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Departamento de Microbiología II

TESIS DOCTORAL

Análisis de elementos genéticos móviles en *Enterococcus faecium*: coste biológico e impacto en la diversificación clonal

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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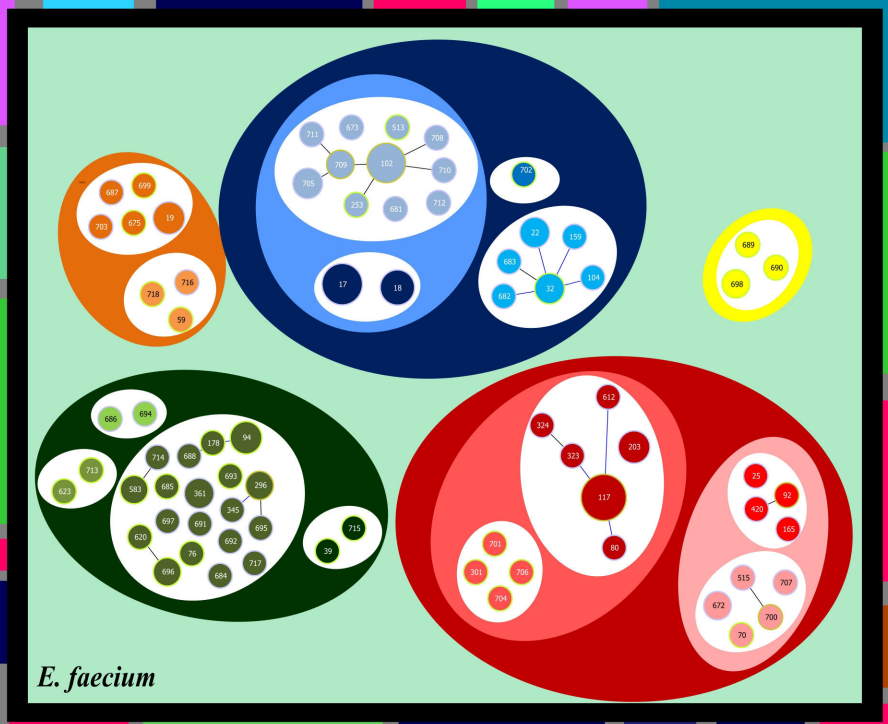

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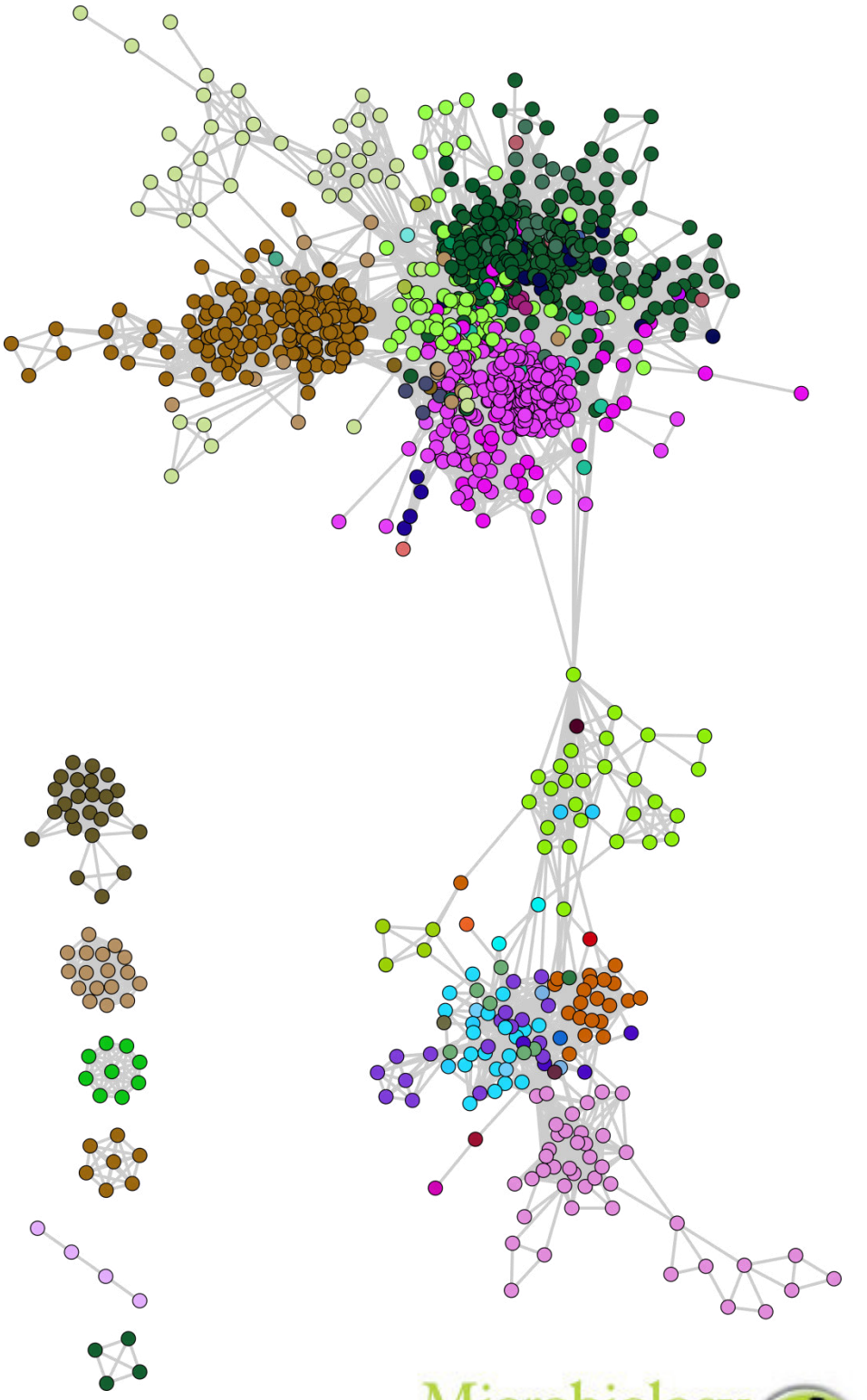

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Tesis doctoral presentada por:

Ana Sofia Santos Tedim Sousa Pedrosa

Para la obtención del grado de doctor

Los directores del trabajo:

Dr. Fernando Baquero Mochales **Dra. Teresa Coque González**

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To my mother and brother for always being close in spite of the distance,

Thank you for your unconditional support!

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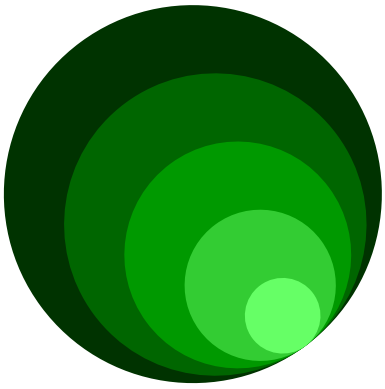
List of Abbreviations

AbR	Antibiotic Resistant
AFLP	Amplified Fragment Length Polymorphism
AmpR	Ampicillin Resistant
AmpS	Ampicillin Susceptible
AREfm	Ampicillin Resistant *Enterococcus faecium*
ASEfm	Ampicillin Susceptible *Enterococcus faecium*
BAPS	Bayesian Analysis of Population Structure
BcR	Biocide Resistant
BHI	Brain Heart Infusion
BSI	Bloodstream Infections
CC	Clonal Complex
CDC	Centers for Disease Control and Prevention
cgMLST	Multilocus Sequence Typing
CLSI	Clinical and Laboratory Standard Institute
CRISPR	Clustered, Regularly Interspaced Short Palindromic Repeats
DNA	Deoxyribonucleic Acid
Efm	*Enterococcus faecium*
Efs	*Enterococcus faecalis*
GAS	Group A Streptococci
GBS	Group B Streptococci
Hfr	High Frequency Recombination
HGT	Horizontal Gene Transfer
HiRCCs	High-risk Clonal Complexes
HRyC	Hospital Universitario Ramón y Cajal
ICE	Integrative Conjugative Element
IDSA	Infectious Disease Society of America
Inc	Incompatibility Groups
IS	Insertion sequence
LAB	Lactic Acid Bacteria
MDR	Multi-drug Resistant
MetR	Metal Resistant
MGE	Mobile Genetic Element
MIC	Minimal Inhibitory Concentrations
MLEE	Multilocus Enzyme Electrophoresis
MLST	Core Genome Multilocus Sequence Typing

MLVA	Multilocus Variable Number Tandem Repeats Analysis
MOB	Mobilization
MRSA	Meticillin Resistant *Staphylococcus aureus*
MS	Mass Spectrometry
nt	Nucleotide
OR	Odd Ratio
PAI	Pathogenicity Island
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PLACNET	Plasmid Constellation Network
RCR	Rolling Circle Replication
REL	Relaxases
Rep	Replication
RFLP	Restriction Fragment Length Polymorphism
RIPs	Replication Initiator Proteins
RM	Restriction-Modification
RNA	Ribonucleic Acid
SLV	Single Locus Variant
ST	Sequence Type
TA	Toxin-Antitoxin
USA	United States of America
UTIs	Urinary Tract Infections
VF	Virulence factors
VRE	Vancomycin Resistant Enterococci
VREfm	Vancomycin Resistant *Enterococcus faecium*
VREfs	Vancomycin Resistant *Enterococcus faecalis*
WGS	Whole genome sequencing

Incredible change happens in your life when you decide to take control of what you do have power over instead of craving control over what you don't.

Steve Maraboli



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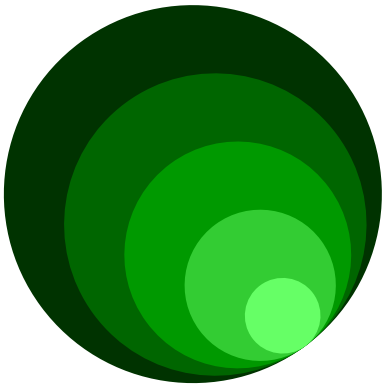
I cannot teach anybody anything. I can only make them think.

Socrates



Abstract

Enterococci were first known to cause human infections in the early 1900s; however, it was not until the last three decades that they emerged as one of the most important common nosocomial pathogens. Enterococci, and particularly *Enterococcus faecium*, are now considered as one of the 21st century medical challenges due to the increasing prevalence of multi-drug resistant (MDR) strains, particularly those resistant to ampicillin and vancomycin. The increasing importance of *E. faecium* as nosocomial pathogen prompted a series of studies regarding its population structure and prevalence of antibiotic resistance particularly in hospitalized patients. Available knowledge regarding the *E. faecium* populations of community-based humans and individuals of different ages is still scarce. Furthermore, little is known about the mobile genetic elements (MGE) associated with antibiotic resistance determinants, particularly vancomycin, which is one of the last therapeutic options to treat infections caused by multidrug resistant isolates of Gram positive species. As such, the main objective of this PhD dissertation is to assess the influence that antibiotic resistance and more specifically, that of the MGEs associated with ampicillin and vancomycin resistance have had in shaping the population structure and evolution of *E. faecium.*

Chapters 1 and 2 describe the comprehensive analysis of MLST data of *E. faecium* isolates from faeces of hospitalized and healthy humans of different ages and *E. faecium* isolates causing bloodstream infections (BSI), via Bayesian Analysis of Population Structure (BAPS) used for analysing the population structure of recombinant bacterial species as *E. faecium.* Such analysis permitted the identification of differences in the content of enterococcal species at different ages, to confirm that ST18, ST17 and ST78 lineages, previously within CC17, have a separate origin, and to validate the suitability of BAPS for analysing the diversity of *E. faecium* populations. The recovery from BSI of all *E. faecium* BAPS groups including those associated with members of the commensal human flora (e.g. BAPS 1 and BAPS 3.3b) pointed out the human gut as the origin of BSI. Changes in the GI tract microbiota of hospitalized patients due to host factors (e.g. age) or different selective pressures in the hospital setting, would have facilitated the selection and the consequent increase in the population size of MDR *E. faecium* clones, leading to a shift in the composition of *E. faecium* populations that increases the chances to be infected by MDR and the chances to transmit MDR strains.

Chapter 3 focuses on a network-based analysis of plasmids that carry resistance to antimicrobials in members of the *Firmicutes* phylum, and it shows the frequent but heterogeneous horizontal gene transfer (HGT) events that occurred among species-specific plasmids. Such results helped to understand the heterogeneous occurrence of resistance to different antibiotics in *E. faecium*. However, to explain the contribution of antibiotic resistance to the increasing recovery of *E. faecium* from hospital infections in the last decades, we further explore the genetic context of resistance to first line therapeutic options, ampicillin and vancomycin, among isolates of predominant in major human lineages of this species.

Chapter 6 showed how ampicillin resistance (*pbp5*) has shaped the population structure of the species through convergent evolution and also horizontal gene transfer, leading to the high prevalence and

further fixation of different clonal lineages of ampicillin resistant *E. faecium* in hospitals, probably associated with the selective pressure exerted by β-lactams in the nosocomial setting since 1940s.

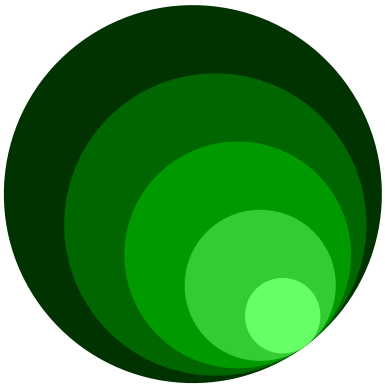
Chapters 4 and 5 describe the diversity of plasmids among vancomycin resistant *E. faecium* from different geographical locations, and we comprehensively characterize those carrying Tn*1546*. These studies reflect the influence of specific narrow- and broad-host range plasmids in the spread of vancomycin resistance in the USA and Europe (pRUM and Inc18, respectively). The diversity of Tn*1546*-*vanA* carrying plasmids, mostly chimeras of narrow and broad host range plasmids, reflects the frequent HGT events between populations of enterococci and other *Firmicutes*. The species specificity of these plasmids besides the significant fitness burden that they confer to the host, and their high stability in the absence of selective pressure, comprehensively described in Chapter 7, would partially explain the confinement of vancomycin resistance to *E. faecium*.

In summary, this PhD dissertation showed the existence of specific populations in both non-hospitalized and hospitalized patients of different ages. The enrichment of such *E. faecium* populations in antibiotic resistance determinants and MGEs appears to be essential for the evolution and adaptation of these lineages to the survival in settings under high selective pressures as hospitals. Even though, the presence of resistant determinants and MGEs might cause a significant burden for the *E. faecium* host, the stability and the frequent HGT events among strains and/or MGEs would justify their maintenance and spread within *E. faecium* populations.

Key Words: *Enterococcus faecium*, mobile genetic elements, plasmids, biological cost, Tn*1546*, *pbp5*, vancomycin, ampicillin.

Prefiero ser aprendiz de todo que maestro de poco.

Guillermo Pérez Villalta



Resumen

La prevalencia de infecciones hospitalarias causadas por especies del género *Enterococcus* ha sido baja desde su descripción como patógenos oportunistas al inicio del siglo XX, hasta finales de los años 70s, coincidiendo con la aparición de las primeras cepas resistentes a antibióticos. La especie *Enterococcus faecium* es actualmente uno de los principales patógenos nosocomiales debido en parte a la alta prevalencia de cepas resistentes a ampicilina y vancomicina. La estructura poblacional de *E. faecium* ha sido analizada mayoritariamente considerando cepas resistentes a antibióticos y de origen hospitalario pero se desconoce su diversidad en individuos no hospitalizados o de diferentes edades. El conocimiento de los elementos genéticos móviles (EGM) que facilitan la transferencia de genes de resistencia a antibióticos entre clones de la misma o diferente especie, principalmente vancomicina (una de las ultimas opciones terapéuticas para el tratamiento de bacterias Gram positivas multi resistentes a los antibióticos), es también muy reducido. El principal objetivo de esta tesis de doctorado es determinar la influencia en la estructura poblacional y evolvabilidad de *E. faecium* de los determinantes de resistencia y de los EGM que facilitan su adquisición, transferencia y persistencia. Los capítulos 1 y 2 consisten en el análisis Bayesiano (BAPS) de los datos de MLST procedentes de heces de pacientes hospitalizados y no-hospitalizados de diferentes edades y de aislados de *E. faecium* causantes de bacteriemias. Este análisis permitió separar los linajes clonales que constituyen el complejo clonal (CC)17 que había sido identificado con la aplicación de metodologías previas en tres líneas clonales de diferente origen, ST18, ST17 y ST78. El aislamiento de cepas de *E. faecium* pertenecientes a grupos BAPS asociados con la flora comensal humana (BAPS 1 y BAPS 3.3b) sugiere la frecuente adquisición endógena a partir de la microbiota intestinal de las bacteriemias causadas por *E. faecium*, bien forma directa o a través de infecciones en territorios cercanos (infección urinaria, infección abdominal). Los cambios ocurridos en la microbiota intestinal de los pacientes hospitalizados debido a factores asociados al hospedador (edad) o diferentes presiones selectivas en el medio hospitalario, habrían facilitado la selección (aumento de la densidad poblacional) y la consecuente expansión de las poblaciones de clones de *E. faecium* resistentes a antibióticos y aumentando por tanto las oportunidades de infección y transmisión de *E. faecium*.

El capítulo 3 describe el análisis de redes de los plásmidos asociados a resistencia a antimicrobianos en las especies del phylum *Firmicutes*, y la resultante demostración de la frecuente pero heterogénea transferencia horizontal de genes (THG) entre plásmidos de muy estrecho rango de hospedador. Estos resultados ayudan a comprender la distribución asimétrica de genes de resistencia a antibióticos en diferentes poblaciones de *E. faecium*. Para poder explicar la contribución de los determinantes de resistencia en el aumento de la prevalencia de *E. faecium* en infecciones nosocomiales en las últimas décadas se estudió detalladamente el contexto genético de los determinantes responsables de resistencia a ampicilina y vancomicina, antibióticos de primera elección para el tratamiento de infecciones graves *E. faecium*.

El capítulo 6 describe la región del cromosoma en la que se localiza el gen responsable de la resistencia a la ampicilina (*pbp5*), sus variaciones y su transferibilidad en poblaciones de *E. faecium*. El análisis

comparativo de genomas de *E. faecium* resistentes y sensibles a ampicilina, indica la influencia de esta región en la estructura poblacional de *E. faecium* a través de procesos de evolución convergente y THG.

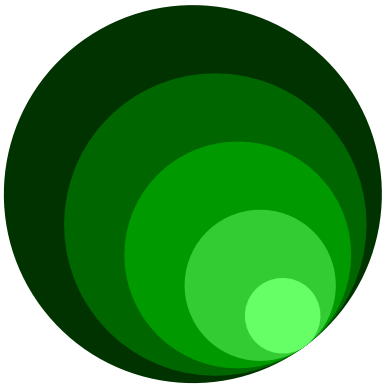
Los capítulos 4 y 5 recogen dos trabajos que analizan la diversidad de los plásmidos portadores de Tn*1546-vanA* en cepas de *E. faecium* resistentes a la vancomicina a nivel local (Portugal) y a nivel global (en diferentes áreas de globo terrestre). Estos estudios demuestran la relevancia de plásmidos particulares de amplio (Inc18) y reducido (pRUM) espectro de hospedador en la diseminación de Tn*1546-vanA* en los Estados Unidos y en Europa. La diversidad de los plásmidos asociados al Tn*1546*, principalmente quimeras de plásmidos de reducido y amplio espectro de hospedador, reflejan la frecuencia de eventos de THG entre diferentes especies de enterococos y otros géneros de *Firmicutes*. El reducido rango de hospedador de los plásmidos Tn*1546,* así como su coste biológico y su estabilidad en ausencia de presión selectiva, descritos en el capítulo 7, podrían contribuir a explicar la casi exclusiva presencia de resistencia a la vancomicina en *E. faecium* dentro del género *Enterococcus*.

En resumen, esta Tesis Doctoral demuestra la existencia de poblaciones especializadas de *E. faecium* asociadas a individuos hospitalizados y no-hospitalizados de diferentes edades. Las poblaciones predominantes en individuos hospitalizados están enriquecidas en determinantes de resistencia a antibióticos y EGM que parecen tener un papel esencial en la adaptación y evolvabilidad de *E. faecium* en condiciones de elevada y variable presión selectiva como el hospital. No obstante, a pesar del significativo coste biológico que los determinantes de resistencia y sus EGM pueden conferir al hospedador, su estabilidad, y a la vez su su variabilidad-adaptabilidad, junto con su capacidad de propagación por eventos de THG, podrían explicar su mantenimiento y diseminación en poblaciones de *E. faecium* a nivel global.

Palabras clave: *Enterococcus faecium*, estructura poblacional, elementos genéticos móviles, plásmidos, ampicilina, vancomicina, coste biológico, *pbp5*, Tn*1546*.

Real knowledge is to know the extent of one's ignorance.

Confucius



Introduction

1. The Genus *Enterococcus* an Historic Perspective

Enterococci emerged as a cause of human infections in the early 1900s and have, in the last three decades, emerged as one of the most important common nosocomial pathogens (1, 2). Enterococci are capable of causing a wide variety of infections as urinary tract infections (UTIs), peritonitis, hepatobiliary sepsis, endocarditis, surgical wound infections, bacteraemia and neonatal sepsis (2, 3).

The term "enterococcus" was firstly used in the 19th century to describe a saprophytic Gram positive diplococcus, of intestinal origin, capable of causing human infection. The term "enterocoque" was proposed in order to emphasize the bacteria morphology and its intestinal origin. This enteric bacteria was found to colonize the GI tract of a patient with diarrhoea and to cause septicaemia afterwards (1, 4). In the same year, MacCallum and Hastings reported a case of acute endocarditis, followed by septicaemia and eventual death, caused by *Micrococcus zymogenes*, latter classified as *Streptococcus faecalis* and now known as *Enterococcus faecalis* (1, 5).

In 1906 *Streptococcus faecalis* was described for the first time, associated with an endocarditis infection (1, 6). In 1919 and 1935 *Streptococcus faecium* and *Streptococcus durans* were firstly described (1, 7, 8). Then, Sherman proposed a classification of *Streptococcus* into four groups: pyogenic, viridans, lactic and "enterococcal group" (1, 9). The creation of an *Enterococcus* taxon, based on cellular arrangement and phenotypic characteristics of the "enterococcal group" was firstly suggested in 1970. However, it was not until 1984 that this taxon was created based on genetic evidences that showed that *S. faecalis* and *S. faecium* were distant members of the *Streptococcus* genus (1, 10, 11).

Nowadays, the genus *Enterococcus* is classified within the phylum Firmicutes, class *Bacilli*, order *Lactobacillales* and family *Enterococcaceae*. This genus is composed of more than 50 species (http://www.bacterio.net/enterococcus.html), of which *E. faecium* and *E. faecalis* are the species most commonly recovered from humans. However, several other species (*E. avium*, *E. durans*, *E. hirae*, *E. casseliflavus*, *E. gallinarum*) are also able to colonize the GI tract of humans and may sporadically cause human infections (1, 2).

1.1. Physiology and Ecology

Enterococci are Gram-positive cocci (spherical cells) frequently arranged in pairs (diplococci). They are non-spore-forming facultative anaerobes that have their optimum growth temperature at 35ºC, but they can grow in a wide range of temperatures (10-45ºC). This bacterial genus can also grow in broth containing 6.5% of NaCl and are able to hydrolyze esculin in the presence of 40% bile salts. Enterococci do not produce a catalase reaction in the presence of hydrogen peroxidase although some species can exhibit a pseudocatalase reaction in blood agar. This behaviour is due to the lack of cytochrome enzymes which also influences the homofermentative metabolism, lactic acid being the end product of

glucose fermentation. In addition, its ability to metabolize a broad range of energy sources (complex carbohydrates, glycerol, lactate, citrate, malate, amino acids such as arginine, and some alpha-keto acids), their capacity to tolerate oxidative stress as well as a wide variety of compounds (heavy metals, azide, detergents, biocides) and prolonged desiccation allows this bacterial genus the survival in diverse habitats (1, 12, 13). Enterococci are also known for being somewhat fastidious bacterial needing a certain number of amino acids and vitamins for maximum growth. This genus reacts with the Lancefield anti-D serum, which allows to differentiate enterococci of other streptococci (1, 13).

Facklam *et al*. proposed a classification based on phenotypic characteristics (hydrolysis of mannitol, sorbose, arginine, arabinose, sorbitol, raffinose and sucrose methyl-α-D-glucopyranoside, motility and pigmentation) that grouped enterococcal species in 5 categories designed by roman numerals (Table 1). Recently Lebreton e*t al* described a new group (VI) that includes *Enterococcus ureilyticus* that did not fit in any of the previously described groups (1, 13). This classification remains useful for diagnostic and ecological purposes, as species from the same groups with similar metabolic characteristics might have similar functional roles. However, it does not reflect any evolutionary relationship between *Enterococcus* species, as accessed by 16S rRNA sequence phylogeny (1). In fact, it has been recently described that some of *Enterococcus* metabolic characteristics might be encoded on plasmids (e. g. raffinose metabolism gene cluster encoded in an *E. faecium* megaplasmid) what might favour the survival in different environments (1, 14). Some species are motile (*E. gallinarum* and *E. casseliflavus*) or shown yellow-pigmentation (*E. sulfureus*, *E. casseliflavus*, and *E. mundtii*) (1, 12, 13).

Table 1 – Facklam *et al* Enterococci classification

Group	Species	MAN	SOR	ARG
I	*E. avium, E. malodoratus, E. raffinosus, E. pseudoavium, E. saccharolyticus, E. pallens, E. gilvus, E. phoeniculicoa, E. devriesei, E. canis*	+	+	-
II	*E. faecalis, E. faecium, E. casseliflavus, E. gallinarum, E. canintestini, E. lactis, E. thailandicus, E. sanguinicola*	+	-	+
III	*E. villorum, E. durans, E. dispar, E. hirae, E. silesiacus, E. rotai**	-	-	+
IV	*E. asini, E. sulfureus, E. cecorum, E. aquamarinus, E. plantarum, E. caccae, E. termitis*	-	-	-
V	*E. columbae, E. rivorum, E. hermaniensis, E. camelliae, E. viikiensis*	+	-	-
VI	*E. ureilyticus*	-	+	-

*some variants of *E. faecium* and *E. faecalis* that do not hydrolyse mannitol can also be found in this group. **some variants of *E. casseliflavus*, *E. gallinarum* and *E. faecalis* that do not hydrolyse arginine can also be included in this group. **Abbreviations:** MAN, mannitol; SOR, sorbose; ARG, Arginine.

Enterococci have been found to colonize a great number of habitats such as the GI tract of humans and other mammals, reptiles and birds, and evironments as: food, water, plants or soil (1, 13, 15).

1.2. Antibiotic Resistance in Enterococci

Enterococcal infections are one of the 21st century medical challenges due to the increasing prevalence of strains that are resistant to therapeutic concentrations of the majority of antibiotics with a

bactericidal activity against enterococci (Section 3, below). In fact, the increase of *E. faecium* infections, described in this section, together with antibiotic resistance led to the inclusion of this species, by the IDSA (Infectious Disease Society of America), in the list of ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter* spp., *Pseudomonas aeruginosa*, and *Enterobacter* spp.), for all of which new therapies are urgently needed. Furthermore, the CDC (Centers for Disease Control and Prevention) has classified Vancomycin Resistant Enterococci (VRE) as a serious threat to human health as VRE is responsible for about 30% of the 66,000 *Enterococcus* healthcare-associated infections registered every year in the USA. (16–18).

Enterococci resistance to antimicrobials (antibiotics, heavy metals, disinfectants and antiseptics) can be classified in intrinsic, acquired and phenotypic tolerance (12, 16). Tolerance can be defined as the ability of a microorganism to resist killing by any antimicrobial well above normal minimal inhibitory concentrations (MIC), and it is caused by a proportion of microbial cells that exist in a transient non-dividing state their bacterial target. Enterococci can exhibit tolerance to antibiotics that target bacteria cell-wall as β-lactams and vancomycin. It can be overcome by using β-lactams besides high concentrations of aminoglycosides. These therapeutic regimens that combine different antibiotic classes (often including a cell wall active agent) are frequently used to treat severe infections as synergy between different classes of drugs provides a bactericidal effect that is not possible to achieve using monotherapy regimens (17).

Intrinsic resistance is defined as an inherent property of a species, making them naturally unsusceptible to particular drugs, and thus chromosomally encoded and present in all species strains. The genus *Enterococcus* is intrinsically resistant to β-lactams, in particular to cephalosporins, low-medium concentrations of aminoglycosides, and trimethoprim-sulfamethoxazole. Some species are also intrinsically resistant to lincosamides and streptogramins A (*E. faecalis*, *E. gallinarum*, *E. avium* and *E. casseliflavus*) or to glycopeptides (*E. gallinarum*, *E. flavescens* and *E. casseliflavus*).

Acquired resistance may result from one or several mutations in existing gene(s) or the acquisition of exogenous DNA (12, 17). Enterococci have acquired resistance to all families of antibiotics (phenicols, tetracyclines, macrolides, aminoglycosides, β-lactams, glycopeptides, quinolones, streptogramins, oxazolidinones, lipopeptides and glycylcyclines) [for comprehensive review see (12, 17)]. We will focus here on the more frequent and clinically relevant (β-lactams and glycopeptides).

1.2.1. β-lactams resistance

β-lactams are able to inhibit cell-wall synthesis by serving as subtracts for penicillin-binding proteins (PBPs, D,D-transpeptidases) that catalyse the union of the peptidoglycan pentapeptide side chains during the synthesis of mature peptidoglycan. Enterococci are intrinsically resistant to β-lactams due to the presence of PBPs with low affinity for these antibiotics, particularly PBP5 in *E. faecium*. However, the level of tolerance to different classes of β-lactams varies, penicillins exhibiting the highest activity

against enterococci (particularly ampicillin, an aminopenicillin), followed by carbapenems and cephalosporins, which barely exhibit activity against enterococci. The level of activity reflects the usefulness of these antibiotics to treat enterococcal infections. While ampicillin is the β-lactam more commonly used to treat enterococcal infections, administration of cephalosporins for the treatment of other bacterial infections constitutes a risk factor for acquiring enterococcal infections (12, 17, 19).

Up to date two acquired mechanisms of resistance to β-lactams have been described, namely production of β-lactamase and mutation of PBP genes. β-lactamase production, firstly described in early 1980s in *E. faecalis,* has scarcely been reported up to date (11 cities and 4 countries including some hospital outbreaks). *E. faecium* β-lactamase producers were firstly described in 1992 in the USA but no further reports were published until recently, when eight β-lactamase producing *E. faecium* strains were reported in Italy (20). β-lactamase producing strains were found to be resistant to penicillins, aminopenicillins, ureido-penicillins and present low level resistance to imipenem. The Enterococcal β-lactamase is inhibited by classical β-lactamase inhibitors (clavulanate, sulbactam and tazobactam) (12, 16, 20–23).

The most common mechanism of acquired resistance to β-lactams occurs through mutations and/or hyperproduction of *pbp* genes. This mechanism of resistance was firstly described in the 1970s and 1980s in American hospitals associated with the *pbp5* gene of *E. faecium* and less frequently, *E. raffinosus* (24–26). The chromosomal region in which *E. faecium pbp5* gene is located has proven to be transferable in independent experiments (27). Recently, other genes that might be responsible for ampicillin resistance were also identified in *E. faecium*, such as, *ddcP* (encoding for D-alanyl-D-alanine carboxypeptidase), ldt_{fm} (encoding for a L,D-transpeptidase), *pgt* predicted to encode a glycosyl transferase group 2 family protein) and *lytG* (predicted to encode an exo-glucosaminidase that might be acting as a peptidoglycan hydrolase) (28). In *E. faecalis,* either the hyperproduction of PBP5 together with deficient binding of penicillin to PBP1 and PBP6 or point mutations in the PBP4, confer resistance to β-lactams (29, 30). The frequency of penicillin resistant *E. faecalis* remains low in most European countries and also in the USA (31–33).

1.2.2. Glycopeptides resistance

Glycopeptides bind specifically to the C-terminus of the pentapeptide precursor of peptidoglycan D-Alanine-D-Alanine (D-Ala-D-Ala); blocking the linkage of the precursors impedes the formation of mature peptidoglycan (17, 34, 35). Enterococci become resistant to glycopeptides by target modification with elimination of the high-affinity peptidoglycan precursors throughout enzymes present in the *van*-operons (34, 35).

Currently eight genotypes coding for acquired glycopeptide resistance have been described in enterococci, which are named by capital letters (*vanA*/*B*/*D*/*E*/*G*/*L*/*M*/*N*). They differ in the ligase gene, the resistance phenotype, gene sequence similarity and synteny (Table 2). The ligase gene encodes

either for a D-Alanine-D-Lactate (D-Ala-D-Lac) or D-Alanine-D-Serine (D-Ala-D-Ser) ligases leading to the synthesis of peptidoglycan precursors with low affinity for glycopeptides. Aside from the ligase genes *van*-operons enclose other 4-6 genes encoding for similar gene functions (17, 34, 35)

E*. gallinarum*, *E. casseliflavus* and *E. flavescens* have intrinsic low level resistance to vancomycin due to the presence of *van* operons in their genomes (designed as *vanC1*, *vanC2* and *vanC3* operons, respectively). VanC elements convert precursors D-Ala-D-Ala into D-Ala-D-Ser. The VanC phenotype, which may be constitutive or inducible, is characterized by the low level of vancomycin and teicoplanin susceptibility (31, 34). Recently, the *vanC* operon has also been identified in *E. faecium* and *E. faecalis* (36, 37).

The first glycopeptide resistant enterococcal strains were two *E. faecium* isolates recovered almost simultaneously in the UK (1986) and France (1987). In the USA, the first VRE was identified as *E. faecalis* in 1988 (34, 38–40).

Table 2 – Enterococci acquired van genotypes characteristics

Phenotype	VanA	VanB	VanD	VanE	vanG	VanL	VanM	VanN
Ligase gene	*vanA*	*vanB*	*vanD*	*vanE*	*vanG*	*vanL*	*vanM*	*vanN*
MIC_{VAN}(mg/L)	16-1000	4-32(-1000)	64-128	8-32	16	8	>256	12-16
MIC_{TEI}(mg/L)	(4)16-512	0.5-1	4-64	0.5	0.5	S	1-96	0.5
Expression	inducible	inducible	constitutive	inducible	inducible	inducible	inducible	constitutive
Location	Pl/Ch	Pl/Ch	Ch	Ch	Ch	Ch?	Pl/Ch	Pl
Transferable by conjugation	+/-	+/-	-	-	+	-	+	+
Distribution among enterococcal species	*E. faecium* *E. faecalis* *E. durans* *E. hirae* *E. gallinarum* *E. casseliflavus* *E. raffinosus* *E. avium* *E. mundtii*	*E. faecium* *E. faecalis* *E. durans* *E gallinarum*	*E. faecium* *E. faecalis* *E. raffinosus*	*E. faecalis*	*E. faecalis*	*E. faecalis*	*E. faecium*	*E. faecium*
MGE	Tn*1546*	Tn*5382* Tn*1547, Tn1549*		Tn*6202*				
Isolation date	1987, 1989	1988	1993	2001	1998	2008	2005	2008

Abbreviations: MIC, Minimal Inhibitory Concentration; MGE, Mobile Genetic Element; VAN, vancomycin; TEI, teicoplanin; Ch, Chromosome; Pl,Plasmid

The most frequent *van* genotypes found in Enterococci are *vanA* and *vanB,* which have also been found in corynebacteria, streptococci and staphylococci (*vanA*) and different *Clostridum* species (*vanB* and *vanG*) (35, 41–44).

The *vanA* genotype is located in Tn*1546* (Tn*3* family transposon) that in turn is located in highly conjugative broad host range plasmids (35, 45, 46). The *vanB* genotype is normally located in a conjugative transposon, Tn*1549* and Tn*5382*, normally chromosomal located although it can also be located in conjugative plasmids (35, 47, 48) (Table 2).

Operons *vanA*, *vanB*, *vanD* and *vanM* harbor several genes: *vanR* (response regulator) and *vanS* (sensor histidine kinase), encoding for a two component regulatory system; *vanH*, encoding for a dehydrogenase that reduces pyruvate to D-Lac (rare in nature and so has to be synthesized) producing

the substrate for the ligases, *vanA*, *vanB*, *vanD* and *vanM,* that catalyze the formation of D-Ala-D-Lac; *vanX* (encoding for a D,D-dipeptidase) and *vanY* (encoding for a D,D-carboxypeptidase) responsible for hydrolysis of D-Ala-D-Ala peptidoglycan precursors that make strains susceptible for glycopeptides. *vanA* and *vanB* operons encode genes of accessory unknown function *vanZ* and *vanW*, respectively (17, 34, 35, 49).

Operons *vanE*, *vanL* and *vanN* have a similar organization to that of *vanC*: i) *vanC*, *vanE*, *vanL* and *vanN* ligases; ii) *vanXY* D,D-dipeptidase-D,D- carboxypeptidase responsible for the hydrolysis of D-Ala-D-Ala peptidoglycan precursors; iii) *vanT* coding for a serine racemase responsible for the production of D-Ser; and iv) *vanR* and *vanS* the two component regulatory system (34, 35, 50, 51). The *vanG* operon is composed of 7 genes that seem to have been recruited from the other *van*-operons. It is composed of 3 regulatory genes, *vanU*, *vanR* and *vanS*, a *vanY*, a *vanW*, a *vanG*, a *vanXY* and *vanT* (34, 35, 52)

1.3. Biological Cost of Antibiotic Resistance Determinants

The acquisition of antibiotic resistance either by point mutations or by HGT leads to a loss of fitness (biological cost) for the bacterial host. The acquisition of mobile genetic elements (MGE), like plasmids, is expected to be associated with a biological cost for the host due to the additional energetic and metabolic burden related with the MGE replication and expression of its genetic content. Furthermore, different resistance mechanisms for the same antibiotic family with different biological cost can be found within the same bacterial population (53–57).

The biological cost of antibiotic resistance has mostly been analysed *in vitro* studies comparing the behaviour of susceptible and resistant strains (competition essays relative growth rates, cost--compensation, and resistance segregation *vs* stability and adaptation) and is highly influenced by the experimental conditions used. The difficulties to simulate *in vitro* the physiological conditions to which a bacterial population is subjected makes it difficult to extrapolate the results obtained in the laboratory to physiological conditions (53, 57). However, the evidence of differences between susceptible and resistant strains in bacteria growing under well defined conditions certainly should *qualitatively* correspond to those expected to occur under natural circumstances. The biological costs of resistance influencing environmental survival, colonization, transmission, or infection rates should be considered to this respect.

Fitness differences between antibiotic susceptible and antibiotic resistant populations due to the lower growth rate and/or lower colonization and inter-host transmission of the antibiotic resistant population should lead to the reduction in frequency and reversion of the resistant phenotype in the absence of selective pressure. However, it has been demonstrated that antibiotic resistant bacteria are able to acquire compensatory mutations that reduce the biological cost of resistance determinants leading to growth rates similar to those of the susceptible strains, making phenotype reversion a rare event (53,

55, 58). Plasmid-host coevolution experiments suggest that compensatory mutations, the presence of post segregational killing (PSK) systems like those in widespread plasmids pRUM (Axe-Txe) or those classified in the Inc18 family (εζ) or coselection by antimicrobials or (antibiotics, heavy metals, biocides) both at high and low concentrations influence the persistence of plasmids. The factors make the loss of MDR plasmids by vegetative segregation unlikely (53, 56, 57, 59).

Biological cost of antibiotic resistance in enterococci has been focused on vancomycin resistant strains and their genetic elements (either transposons or plasmids) due to the "serious microbial threat" that VRE represent (32, 33, 60). The high complexity of vancomycin resistance mechanism (see above) led to the hypothesis of a high fitness cost for this mechanism of antibiotic resistance. In fact, the expression of vancomycin resistance operon (Tn*1546*-*vanA*) in *S. aureus* showed a high fitness loss in this host, which justify the low dissemination of vancomycin resistance in *S. aureus* (54)., Another study focused on the biological cost of plasmids carrying Tn*1546*-*vanA* showed a fitness loss for approximately 4% for the host harbouring these plasmids. Other work showed the vancomycin resistance plasmid pVEF1 was stable on its host after 20 days of serial passages. These preliminary works suggest a low fitness cost and high stability for vancomycin resistance and for vancomycin resistant plasmid (45, 56, 57). In spite these few studies using specific plasmids and laboratory strains many issues about the biological cost of vancomycin resistance in Enterococci remain open.

1.4. Enterococcal Human GI tract colonization

The concentration of bacterial cells vary along the human GI tract from 10^2 to 10^3 bacterial cells in the stomach to 10^9 to 10^{12} bacterial cells in the colon (1, 61, 62). Different studies estimated that more than 1,000 different bacterial species are inhabitants of the human gut microbiota although the intestinal biodiversity, particularly among minority populations, still remains largely unknown (63–66).

Next generation sequencing (NGS) metagenomics analysis, mostly based on 16S rDNA, has provided a great deal of information regarding the full genetic content of the bacteria colonizing the human GI tract (human gut metagenome), as well as the influence of host factors (age, diet, health and immunological status) and also external factors (e.g. antibiotic treatment, access to other hosts', food, water and environmental microbiota) might have on the diversity and dynamics of different bacterial groups (61, 64, 67–72). The studies based on 16S rDNA are limited by the specificity of the techniques that not only overemphasize majority populations, but preclude obtaining information at subspecies level.

In spite of their relevance producing invasive infections, *Enterococcus* constitute a minor part of the GI tract flora (<1% in the ileum and jejunum and about 1% in the colon), *E. faecium* and *E. faecalis* being the species more commonly found in the human GI tract,. Other species as *E. durans*, *E. hirae*, *E. avium*, *E. gallinarum*, *E.*, are also able to colonize the human GI tract (1, 73, 74).

1.4.1. Factors that influence gastrointestinal flora

1.4.1.1. Host's Age.

Establishment of the gut microbiota has been shown to be a complex and dynamic process that, suffers important changes during the first and second years of life, is optimized during infancy and adolescence to reach it optimal composition in adulthood and changes again in elderly (64, 75). Enterococci appear to be one of the first bacteria to colonize the human GI tract, being found within days after birth. In most studies, *E. faecalis* tends to be the most abundant enterococcal species (approximately 50% of all enterococci) followed by *E. faecium* and, to a lesser extent, other *E. durans, E. casseliflavus/E. flavescens, E avium* or *E. raffinosus* (76–78). Only one study, performed in a neonatal ICU, were *E. faecium* (48%) and *E. casseliflavus* (31%) more prevalent than *E. faecalis*. The hospital setting might account for the high prevalence of MDR *E. faecium* colonizing infants gut in this study (79).

Changes in the abundance and diversity of commensal species occur in the intestinal microbiota of elderly, probably related to the immunological alterations and changes in the GI tract physiology (64, 68, 75). They include a shift in the dominant bacterial anaerobic flora (*Bacteroides* and *Bifidofacterium*), an increase in the numbers of fungi and enterobacteria and other facultative anaerobes (lactobacilli, streptococci, staphylococci), as well as, certain *Clostridium* groups (64, 68, 75).

Up to now no studies were performed in order to determine what enterococcal species present in the gut of elderly individuals. In one study enterococci were found in high numbers in antibiotic treated individuals whereas they were absent from the GI tract of elderly healthy individuals (68).

1.4.1.2. Antibiotic treatment

The effect of antibiotics on the GI tract microbiota depends on the antibiotic class. Broad-spectrum antibiotics are usually associated with a decrease in the diversity of gut microbiota (66, 80). Prophylaxis of Gram negative infections with levofloxacin in neutropenic cancer patients' led to a predominance of Gram Positive bacteria that often cause bacteraemia in these patients (81, 82). Narrow host antibiotics, such as vancomycin, seem to lead to expansion of otherwise uncommon Enterobacteriaceae and also to a remarkable decrease of *Bacteroidetes* and *Lactobacillaceae*. Mice models have demonstrated that orally administered antibiotic treatment, particularly those active against Gram-negative anaerobic bacteria, as β-lactam-β-lactamase inhibitors, cefoxitin, clindamycin, and metronidazole favour the colonization by VRE (66, 83, 84).

Antibiotics can greatly modify the gut microbiota of mammals. Even though the overall bacterial numbers may be rapidly recovered, persistent changes in microbiota composition are usually maintained for at least a few months (66, 80, 85, 86). A dominance of Enterobacteriaceae, *Clostridium* and *Enterococcus* occurs during antibiotic treatment which is matter of concern as these prokaryotic groups are frequently involved in hospital-acquired infections, particularly bacteraemia of intestinal origin (32, 33, 61, 66, 87).

1.4.1.3. Bacterial adaptive factors

Different factors enhance the ability of enterococci to colonize, persist, and cause infection in different hosts. They include bacteriocins, microbial surface component recognizing adhesive matrix molecules (MSCRAMM), cell-wall-anchored LPxTG proteins, or those that confer the ability to form biofilms (16).

Bacteriocins are small peptides capable of inhibiting growth of similar or related bacteria. Gram-positive bacteriocins can be classified in two major classes of heat-stable peptides, namely class I or Lantibiotics (which contain amino acids lanthionine or methyllanthionine, dehydroalanine and 2-aminoisobutyric acid) and class II (unmodified non-lantibiotics). Most enterococcal bacteriocins belong to the class II, some of them having been fully characterized (As-48, Bac21, Bac31, Bac41, Bac43, Bac32, Bac51, Entl50a/L50b, EntQ, EntA, EntB and EntP (88). Most of them have been identified in *E. faecalis* and *E. faecium* although can also be found in other species as *E. mundti*, *E. avium*, *E. hirae* and *E. durans* (88). Most enterococcal bacteriocins have activity against *Listeria* spp., *Clostridium* spp. and *S. aureus* (3). Bacteriocin producing strains have been isolated from a wide variety of environments but they appear to be more common in food samples (cheese, meat, fish and vegetable), and human and animals GI tract. Some enterococcal bacteriocin producers have been used for more than 20 years in the food industry as preservatives and more recently as probiotics to prevent the overgrowth of pathogens in the human gut (88, 89).

Cell surface determinants (aggregation substances, MSCRAMMs, among others) contribute to colonize the human GI tract either by direct action or by enabling biofilm formation. Aggregation substance (e.g. *asa1*, *asc10*, *asp1*) are a group of *E. faecalis* proteins, normally encoded in pheromone responsive plasmids (16, 90–92). A pathogenicity island encoding *esp* (enterococcal surface proteins that contribute to biofilm formation) has been described in *E. faecium* and *E. faecalis*. An adhesion molecules MSCRAMMs (Acm in *E. faecium* and Ace and ElrA in *E. faecalis*), contribute to colonization and early stages of infection (16). Some enterococcal proteins with cell-wall anchored LPxTG motifs, such as the loci *ebp* (endocarditis and biofilm-associated pili) and *bee* (biofilm enhancer in enterococci), are attached to pili and play an important role in colonization and infection (16, 93–96).

Secreted proteins as haemolysin-cytolysin (Cyl) or gelatinase (GelE) have been traditionally considered as virulence factors in *E. faecalis*. Cyl is a toxin able to lyse blood cells from humans, cows and horses (97). It is located either on pheromone-responsive plasmids or a pathogenicity island that is present in approximately 30% of the *E. faecalis* isolates making them more virulent than non-Cyl producing isolates in several animal models (16, 98, 99). The protease GelE, seems to be involved in the degradation of the host tissues and modulation of the host immune response as well as in the activation of autolysin (enzyme capable of degrading peptidoglycan) leading to the release of DNA from the bacterial cell and also biofilm formation. This and other *E. faecalis* proteases are regulated by a double component system Fsr, which appears to also influence this bacterial pathogenesis (16).

Megaplasmids (>150kb) containing genes that favour the metabolism of different carbohydrate (e.g. hyl_{Efm}, a glycosyltransferase that allows using complex carbohydrates) are present in the majority of *E. faecium* strains isolated nowadays. Transfer of these plasmids to a plasmid free commensal *E. faecium* strain increased both the colonization and the virulence capabilities of laboratory strains (16, 87, 100).

1.5. Enterococcal infections

Enterococci are opportunistic pathogens able to cause a wide variety of clinical manifestations. Bacteraemia and endocarditis are life-threatening diseases that require bactericidal therapeutic options. Urinary tract infections, intra-abdominal, pelvic, and soft tissue infections caused by enterococci are also frequent and often associated with other microbial species.

The increasing number of enterococcal infections, often caused by antibiotic resistant strains, the high mortality rate of infected patients (from 46% to 75% in individuals with severe underlying diseases) and the ability of enterococci to acquire and transfer antibiotic resistance genes to more pathogenic species as *S. aureus* make enterococci one of the pathogens of concern in the hospital setting (2).

Risks factors to acquire enterococcal infections caused by antibiotic resistant strains include extended hospitalization periods, particularly in surgical or intensive care wards, hospitalization in long-term health care facilities, multiple antibiotic therapy courses, close proximity to colonized or infected patients, transplantation, co-morbidities (e. g. renal failure, diabetes or haemodialysis) and the presence of catheters (e. g. urinary tract, for chemotherapy) (16, 101). Transmission between hospitalized patients, mostly associated with healthcare works hands, has been demonstrated, leading either to directly infection or, more likely, to GI tract colonization (due to reduced colonization resistance) that might, in turn, lead to infection (2).

In the 1970s-80s, *E. faecalis* accounted for 90-95% of the enterococcal clinical isolates. However, the emergence of ampicillin and vancomycin resistant *E. faecium* at the end of the 1980s favoured a change in the occurrence of infections caused by *E. faecium* in hospitals at global level and to a decrease in the ratio of *E. faecalis*:*E. faecium* infections (particularly systemic infections) (102). Nowadays, *E. faecalis* is responsible for approximately 76% of enterococcal infections, while *E. faecium* accounts for most of the remaining infections (approximately 24%) in American hospitals (1, 2). Approximately 21% of the enterococci in USA hospitals were vancomycin resistant the majority (75%) of them being *E. faecium*. Ampicillin resistant *E. faecium* (AREfm) are endemic in USA hospitals with a prevalence close to 90% (1, 2, 16, 33).

This change in the epidemiology of enterococci infections was also observed in Europe. While all European countries reported a high prevalence of AREfm among clinical isolates (ranging from >50% to 100%), vancomycin resistant *E. faecium* (VREfm) occurrence greatly varied among countries (from 0% in

Estonia, Lithuania, Malta and Sweden to 42.7% in Ireland) with an overall prevalence of was 8.9% (Fig. 1 to 4) (16, 31, 32, 103).

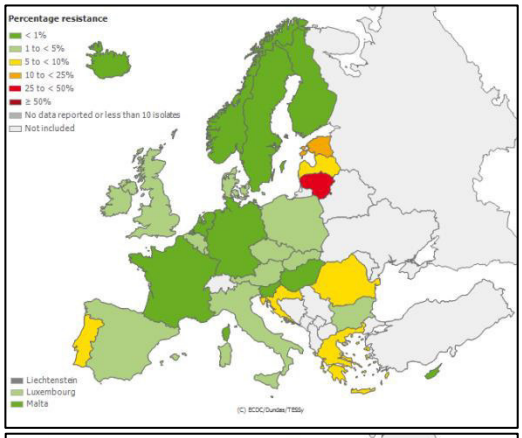


Figure 1. European map showing prevalence of ampicillin resistant *E. faecalis* (source EARSS).

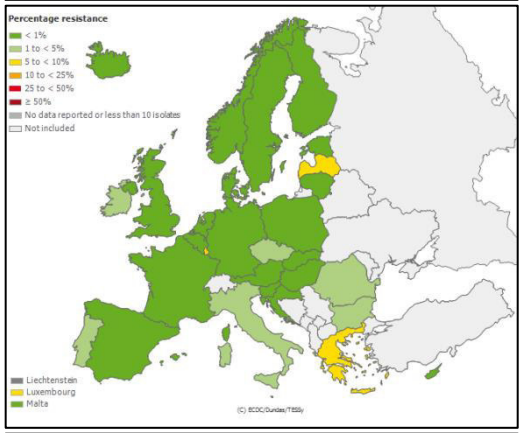


Figure 2. European map showing prevalence of vancomycin resistant *E. faecalis* (source EARSS).

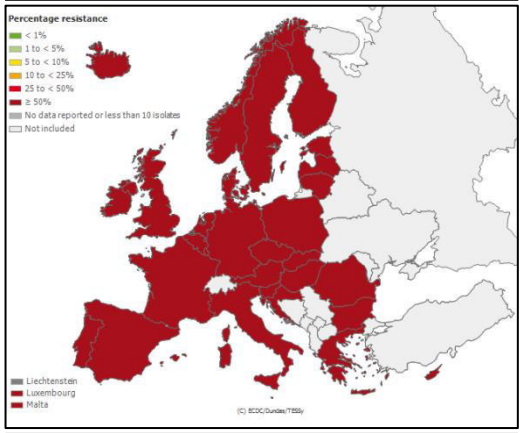


Figure 3. European map showing prevalence of ampicillin resistant *E. faecium* (source EARSS).

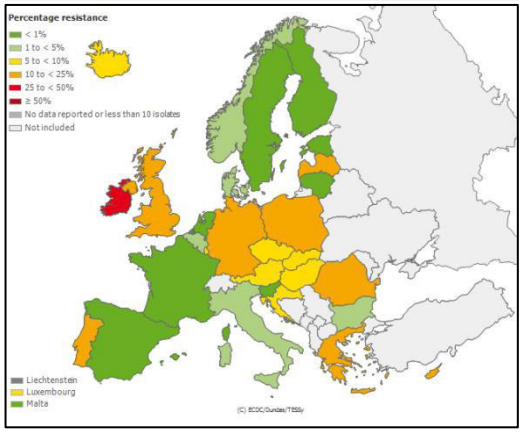


Figure 4. European map showing prevalence of vancomycin resistant *E. faecium* (source EARSS).

Vancomycin resistance is more frequently detected in *E. faecium* than in *E. faecalis* although some areas in different continents (Detroit in the USA and Spain in Europe) consistently describe outbreaks caused by ST6 Vancomycin resistant *E. faecalis* (VREfc) strain throughout years (104). Despite the low occurrence, VREfc is of concern as they can successfully transfer plasmid carrying vancomycin resistance to *S. aureus* (Tn*1546-vanA*) (32, 33, 60).

1.6. *Enterococcus* Population Structure

A great variety of methods have been used to establish the population structure and epidemiological relationship among bacterial strains within a given species. They include AFLP (Amplified Fragment Length Polymorphism), PFGE (Pulsed Field Gel Electrophoresis), MLVA (Multilocus Variable number tandem repeats Analysis), MLEE (Multilocus Enzyme Electrophoresis), MLST (Multilocus Sequence Typing) and whole genome sequencing (WGS). A variety of algorithms are used to analyse MLST data including eBURST, goeBURST and BAPS (Bayesian Analysis of Population Structure). Recently, in order to compare fully sequenced *E. faecium* data from WGS a core genome MLST (cgMLST) was described for this species. Mauve, ARTEMIS, SynView and ssahaSNP (Sequence Search and Alignment by Hashing Algorithm) are used in comparative genomics (105, 106).

1.6.1. E. faecium population structure

MLST using eBURST revealed that clinical and outbreak hospital strains of *E. faecium* grouped in one Clonal Complex (CC), designated CC17 as all STs appeared to descend from the founder ST17, and that these strains were different from those isolated from healthy community humans (gut colonization) and animals (107–111). Strains within CC17 were labelled as hospital-associated *E. faecium* and have been isolated from hospitals worldwide. Most CC17 strains are MDR (resistant to vancomycin, ampicillin, aminoglycosides and fluoroquinolones) and their genomes appears to be enriched in genes coding for putative virulence genes, megaplasmids, and insertion sequences (ISs) (108, 110, 112–115).

Initial studies of *E. faecium* population structure were performed using MLST and eBURST (107). However, this algorithm is not adequate for bacterial species in which recombination, blurring phylogeny, plays an important role in the species evolution (110, 116). Further analysis of concatenated sequences of the 7 MLST genes with neighbour-net tree or Clonalframe indicated that former CC17 was a consequence of large chromosomal recombination events within ST17, ST18 and ST78 lineages of hospital-associated *E. faecium* (109, 110, 117). Later analysis using BAPS (a statistical model based on both clonal ancestry and recombination patterns using concatenated sequences of the 7 MLST genes) revealed the existence of two BAPS groups (2-1 and 3-3) associated with antibiotic resistance (ampicillin and vancomycin) and the hospital setting. Further analysis showed that clinical isolates of these groups are related to ST17 and ST18 lineages (BAPS 3-3), and ST78 lineage (BAPS 2-1). BAPS analysis also showed that strains normally colonized healthy (community) humans belong to BAPS 1 and that these

isolates can be genetic and evolutionary different from hospital associated strains; these hospital strains were more closely related with to animal strains indicating a possible role of animals in the emergence of current hospital associated isolates (110, 115). The recent description of a cgMLST allowed to further look in these lineages and revealed epidemiological links (or lack thereof) in groups off strains where this links were not apparent (106) making it a valuable tool for future epidemiological analysis.

In 2012, the first *E. faecium* closed genome has published (118) and up to date only three more closed *E. faecium* genomes have been released (119–121). Three of these strains are representatives of major lineages of hospital-associated *E. faecium* (ST16, ST17 and ST203) (115, 118–120). The other *E. faecium* genome correspond to ST860, a clonal lineage associated with healthy humans, that has been used for years as a probiotic (121). Comparative genomics of these and other sequenced *E. faecium* strains from clinical and non-clinical environments allows to firstly suggest a core (±1600 genes) and an accessory genome for this species (2272-3318 coding sequences (CDS) that represent 29% to 59% of the *E. faecium* genome (115, 118).

Recent comparative genomic studies using WGS consistently split the *E. faecium* populations in two major groups, Community associated (CA) or Clade B and Hospital associated (HA) or Clade A (also includes animals isolates) (122, 123). Clade A was further divided in A1, including most clinical isolates, and A2, including most animal strains (122). Clade B corresponded to BAPS 1 and clade A1 with BAPS subgroups 2-1 and 3-3 (115, 122). These classifications should be considered as dynamic ones as recombination can occur between strains from the different clades generating new hybrid genomes (122, 124, 125).

The genome size of strains in clade A1 is larger than that of clades A2 and B, consistently with the suggested recent emergence of the former clade. Conversely, clade A2 had a larger pan-genome, which would reflect the diverse origins of the strains that form this clade. Clade A1 is also enriched in MGE as plasmids, ISs, phages and genomic islands compared to clades A2 and B (122). These findings indicates that *E. faecium* has an open pan-genome capable of effectively acquire and incorporate novel DNA into the collective gene pool as most ubiquitous bacteria and opportunistic pathogens (115, 118, 122, 123). The enrichment in genetic determinants seems to be a cumulative process, called “genetic capitalism”, where the acquisition and integration of adaptive elements facilitates the acquisition of additional adaptive elements and afterwards, the transition of *E. faecium* (particularly of clade A1) from commensal to nosocomial pathogen (114, 126).

1.6.2. E. faecalis population structure

The population structure of *E. faecalis* was also initially establish using MLST and eBURST analysis. In this case, an overrepresentation of hospital associated strains within CC2 and CC9 was observed (127). Further studies associated early *E. faecalis* antibiotic resistance isolates with CC2, CC8 and CC9, while a more recent European study revealed that MDR *E. faecalis* isolates were classified within CC16, CC2 and

CC87, the latter two CCs being found almost exclusively in hospitals (128, 129). Even though CC2 is mostly found among hospital strains, it may also be identified in farm animals reflecting the epidemic *E. faecalis* population structure (128, 130, 131). CC21, CC16 and CC40 have been widely isolated from hospitalized and non-hospitalized humans, meat and farm animals. Similarly to that reported in other continents, a decrease in the occurrence of CC9 and an increase in CC2 has been observed in the EU (127, 128).

Analysis of the MLST alleles revealed the *E. faecalis* has a high recombination:mutation rate, gene trees for three individual MLST loci were incongruent and individual MLST alleles are widely distributed among concatenated MLST phylogeny. All these observations indicate that *E. faecalis* has an epidemic population structure that frequently recombines (109, 115, 127). CC2, particularly ST6 β-lactamase-producing Mid-Atlantic clone, are widely disseminated seem to be enriched in MGE and genes coding for virulence and colonization, and antibiotic resistance. However, an epidemiologic study using an historic *E. faecalis* strain collection (1900s-2006) revealed that CC2 was not found prior to the 1980s, suggesting that this CC might have a recent origin (115, 129, 132, 133). Another feature that is absent of CC2 strains, but present in other less successful CCs in the hospital environment, was the CRISPR (clustered regularly interspaced short palindromic repeat) loci. This is a defence mechanism against foreign DNA (phages, transposons and plasmids) so the lack of this system would facilitate the acquisition of new genes (115, 128, 134). The CC87 isolates have a common phenotype that may contribute to colonization and virulence, *cylA*$^{+}$, *asa1*$^{+}$, *esp*$^{+}$ and *gelE*$^{-}$. The absence of *gelE* seems to improve the adherence of bacteria to the host tissue due to the increased production of MSCRAMM Ace (128, 135).

1.6.3. Genome Immunity

Bacteria have multiple mechanisms that can protect against acquisition of foreign DNA such as CRISPR (Clustered, regularly interspaced short palindromic repeats) and restriction modification (RM) systems (125, 136, 137).

Among Enterococci, CRISPR were first identified in *E. faecalis* OG1RF and the presence of CRISPR in this strain was proposed to account for the low number of phages and MGEs (124, 129, 138). CRISPR analysis in several *E. faecalis* strains indicated that CRISPR distribution is variable in different genomes. The identification of spacer regions within the *E. faecalis* CRISPR loci revealed identities with phage and plasmid (as those of pheromone-responsive plasmids and other plasmids found in V583 genome) sequences indicating that these loci confer immunity against these sequences (125, 134, 137). Dissemination of antibiotic resistance genes is frequently associated with plasmids and as these CRISPR loci confer immunity for some of the plasmids more frequently associated with antibiotic resistance it has been hypothesize that CRISPR act as a barrier for the acquisition of these resistance genes. These was further supported by the finding that CRISPR were absent from several *E. faecalis* genomes,

belonging to hospital associated CCs, that had encoded in their genomes both antibiotic resistance and several virulence traits absent in strains were CRISPR had been identified (125, 134).

In *E. faecium* the CRISPR loci have scarcely been studied (8 genomes analysed to date) but the spacers CRISPR loci found so far have spacers that encode sequences from *Clostridium novyi* and *Lactococcus lactis* phages'. Interestingly, CRISPR loci in *E. faecium* have only been found in clade B or community associated or the hybrid strains suggesting that the absence of this immunity system might facilitate the acquisition of genes favouring the emergence of MDR hospital adapted strains (125).

RM systems were firstly described in the 1950s. They inhibit (restrict) the growth of viruses that were able to grow in other strains. This activity is based on the activity of two enzymes: a restriction endonuclease and a methyltransferase. They are highly ubiquitous (approximately 90% of genomes have at least one RM system) and often considered the primitive immune systems in bacteria. (139). Four types of RM have been described (I, II, III and IV), being type II the most common. The presence of RM systems in important for the stabilization of selfish genes or elements (plasmids) and genomes, immigration control, maintenance of species identity and control speciation and recombination and genome rearrangements and therefore genome evolution (139). There is little evidence of the presence of RM systems in enterococci. However, some type II restriction endonucleases have been identified such as SfaGU and SfaNI and M.SfeI and R.SfeI. These enzymes as other possible RM systems identified in enterococci have been identified in MGEs. It remains to be seen if these RM systems can act as barriers to the uptake of additional MGEs (125, 140).

References

1. Lebreton F, Willems RJL, Gilmore MS. 2014. *Enterococcus Diversity, Origins in Nature, and Gut Colonization*. *In* Gilmore, MS, Clewell, DB, Ike, Y, Shankar, N (eds.), *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary, Boston: Massachusetts Eye and Ear Infirmary.

2. Agudelo Higuita N, Huycke MM. 2014. *Enterococcal Disease, Epidemiology, and Implications for Treatment*, *In* Gilmore, MS, Clewell, DB, Ike, Y, Shankar, N (eds.*), Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary, Boston.

3. Fisher K, Phillips C. 2009. *The ecology, epidemiology and virulence of Enterococcus*. Microbiology 155:1749–57.

4. Thiercelin M, Jouhaud L. 1899. *Sur un diplococque saprophyte de l'intestin susceptible de devenir pathogene*. CR French Soc Biol 5:269–271.

5. Maccallum WG, Hastings TW. 1899. *A Case of Acute Endocarditis Caused by Micrococcus Zymogenes (nov. spec.),with a Description of the Microorganism*. J Exp Med 4:521–34.

6. Andrewes F, Horder T. 1906. *A study of the Streptococci pathogenic for man*. Lancet 168:852–855.

7. Orla-Jensen S. 1919. *The lactic acid bacteria. Memoirs of the Academy of the Royal Society of Denmark*. Sect Sci Ser 85:81–197.

8. Sherman J, Wing H. 1935. *An unnoted hemolytic Streptococcus associated with milk products*. J Dairy Sci 18:657–660.

9. Sherman JM. 1937. *The Streptococci*. Bacteriol Rev 1:3–97.

10. Kalina AP. 1970. *The position of enterococci in the system of microorganisms*. Zh Mikrobiol Epidemiol Immunobiol 47:20–1.

11. Schleifer KH, Kilpper-Balz R. 1984. *Transfer of Streptococcus faecalis and Streptococcus faecium to the Genus Enterococcus nom. rev. as Enterococcus faecalis comb. nov. and Enterococcus faecium comb. nov*. Int J Syst Bacteriol 34:31–34.

12. Murray BE. 1990. *The life and times of the Enterococcus*. Clin Microbiol Rev 3:46–65.

13. Facklam R, Carvalho M, Teixeira L. 2002. *History, Taxonomy, Biochemical Characteristics and Antibiotic Susceptibility Testing of Enterococci*, p. 1–54. In Gilmore, M, Clewell, D, Courvalin, P, Dunny, G, Murray, B, Rice, L (eds.), *The Enterococci: Pathogenesis, Molecular Biology and Antibiotic Resistance*. American Society for Microbiology, Washington, DC, USA.

14. Zhang X, Vrijenhoek JEP, Bonten MJM, Willems RJL, van Schaik W. 2011. *A genetic element present on megaplasmids allows Enterococcus faecium to use raffinose as carbon source*. Environ Microbiol 13:518–28.

15. Ramsey M, Hartke A, Huycke M. 2014. *The Physiology and Metabolism of Enterococci*. *In* Gilmore, MS, Clewell, DB, Ike, Y, Shankar, N (eds.), *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary, Boston.

16. Arias CA, Murray BE. 2012. *The rise of the Enterococcus: beyond vancomycin resistance*. Nat Rev Microbiol 10:266–78.

17. Kristich C, Rice L, Arias C. 2014. *Enterococcal Infection—Treatment and Antibiotic Resistance*. *In* Gilmore, MS, Clewell, DB, Ike, Y, Shankar, N (eds.), *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary, Boston.

18. Centers for Disease Control and Prevention. 2013. *Antibiotic Resistance Threats in the United States*.

19. Shepard BD, Gilmore MS. 2002. *Antibiotic-resistant enterococci: the mechanisms and dynamics of drug* introduction and resistance. Microbes Infect 4:215–24.

20. Sarti M, Campanile F, Sabia C, Santagati M, Gargiulo R, Stefani S. 2012. Polyclonal diffusion of beta-*lactamase-producing Enterococcus faecium*. J Clin Microbiol 50:169–72.

21. Coudron PE, Markowitz SM, Wong ES. 1992. *Isolation of a beta-lactamase-producing, aminoglycoside-resistant strain of Enterococcus faecium*. Antimicrob Agents Chemother 36:1125–6.

22. Murray BE, Mederski-Samaroj B. 1983. *Transferable beta-lactamase. A new mechanism for in vitro penicillin resistance in Streptococcus faecalis*. J Clin Invest 72:1168–71.

23. Rhinehart E, Smith NE, Wennersten C, Gorss E, Freeman J, Eliopoulos GM, Moellering RC, Goldmann DA. 1990. *Rapid dissemination of beta-lactamase-producing, aminoglycoside-resistant Enterococcus faecalis among patients and staff on an infant-toddler surgical ward*. N Engl J Med 323:1814–8.

24. al-Obeid S, Gutmann L, Williamson R. 1990. *Modification of penicillin-binding proteins of penicillin-resistant mutants of different species of enterococci*. J Antimicrob Chemother 26:613–8.

25. Fontana R, Aldegheri M, Ligozzi M, Lopez H, Sucari A, Satta G. 1994. *Overproduction of a low-affinity penicillin-binding protein and high-level ampicillin resistance in Enterococcus faecium*. Antimicrob Agents Chemother 38:1980–3.

26. Fontana R, Ligozzi M, Pittaluga F, Satta G. 1996. *Intrinsic penicillin resistance in enterococci*. Microb Drug Resist 2:209–13.

27. Rice LB, Carias LL, Rudin S, Lakticová V, Wood A, Hutton-Thomas R. 2005. *Enterococcus faecium low-affinity pbp5 is a transferable determinant*. Antimicrob Agents Chemother 49:5007–12.

28. Zhang X, Paganelli FL, Bierschenk D, Kuipers A, Bonten MJM, Willems RJL, van Schaik W. 2012. *Genome-wide identification of ampicillin resistance determinants in Enterococcus faecium*. PLoS Genet 8:e1002804.

29. Cercenado E, Vicente MF, Díaz MD, Sánchez-Carrillo C, Sánchez-Rubiales M. 1996. *Characterization of clinical isolates of beta-lactamase-negative, highly ampicillin-resistant Enterococcus faecalis*. Antimicrob Agents Chemother 40:2420–2.

30. Ono S, Muratani T, Matsumoto T. 2005. *Mechanisms of resistance to imipenem and ampicillin in Enterococcus faecalis*. Antimicrob Agents Chemother 49:2954–8.

31. Werner G, Coque TM, Hammerum AM, Hope R, Hryniewicz W, Johnson A, Klare I, Kristinsson KG, Leclercq R, Lester CH, Lillie M, Novais C, Olsson-Liljequist B, Peixe L V, Sadowy E, Simonsen GS, Top J, Vuopio-Varkila J, Willems RJ, Witte W, Woodford N. 2008. *Emergence and spread of vancomycin resistance among enterococci in Europe*. Euro Surveill 13:pii: 19046.

32. European Centre for Disease Prevention and Control. 2014. *Antimicrobial resistance surveillance in Europe 2013*. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm.

33. Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock D a, Fridkin SK. 2008. *NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007*. Infect Control Hosp Epidemiol 29:996–1011.

34. Courvalin P. 2006. *Vancomycin resistance in gram-positive cocci*. Clin Infect Dis 42 Suppl 1:S25–34.

35. Cattoir V, Leclercq R. 2013. *Twenty-five years of shared life with vancomycin-resistant enterococci: is it time to divorce?* J Antimicrob Chemother 68:731–42.

36. Sun M, Wang Y, Chen Z, Zhu X, Tian L, Sun Z. 2014. *The first report of the vanC$_1$ gene in Enterococcus faecium isolated from a human clinical specimen*. Mem Inst Oswaldo Cruz 109:712–5.

37. Nishiyama M, Iguchi A, Suzuki Y. 2015. *Identification of Enterococcus faecium and Enterococcus faecalis as vanC-type Vancomycin-Resistant Enterococci (VRE) from sewage and river water in the provincial city of Miyazaki, Japan*. J Environ Sci Health A Tox Hazard Subst Environ Eng 50:16–25.

38. Leclercq R, Derlot E, Duval J, Courvalin P. 1988. *Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium*. N Engl J Med 319:157–61.

39. Uttley AH, Collins CH, Naidoo J, George RC. 1988. *Vancomycin-resistant enterococci*. Lancet 1:57–8.

40. Frieden TR, Munsiff SS, Low DE, Willey BM, Williams G, Faur Y, Eisner W, Warren S, Kreiswirth B. 1993. *Emergence of vancomycin-resistant enterococci in New York City*. Lancet 342:76–9.

41. Ammam F, Marvaud J-C, Lambert T. 2012. *Distribution of the vanG -like gene cluster in Clostridium difficile clinical isolates*. Can J Microbiol 58:547–551.

42. Berthet N, Périchon B, Mazuet C, Chapetón-Montes D, Bouchier C, Bouvet P, Legeay C, Popoff M-R, Courvalin P. 2015. *A vanG-type locus in Clostridium argentinense*. J Antimicrob Chemother.

43. Marvaud J-C, Mory F, Lambert T. 2011. *Clostridium clostridioforme and Atopobium minutum clinical isolates with vanB-type resistance in France*. J Clin Microbiol 49:3436–8.

44. Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, Flannagan SE, Kolonay JF, Shetty J, Killgore GE, Tenover FC. 2003. *Genetic analysis of a high-level vancomycin-resistant isolate of Staphylococcus aureus*. Science 302:1569–71.

45. Freitas AR, Novais C, Tedim AP, Francia MVMV, Baquero F, Coque TM, Peixe L, Coque TM. 2013. *Microevolutionary Events Involving Narrow Host Plasmid Influences Local Fixation of Vancomycin-Resistance in Enterococcus*. PLoS One e60589.

46. Arthur M, Molinas C, Depardieu F, Courvalin P. 1993. *Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in Enterococcus faecium BM4147*. J Bacteriol 175:117–27.

47. Quintiliani R, Courvalin P. 1994. *Conjugal transfer of the vancomycin resistance determinant vanB between enterococci involves the movement of large genetic elements from chromosome to chromosome*. FEMS Microbiol Lett 119:359–63.

48. Domingo M-C, Huletsky A, Bernal A, Giroux R, Boudreau DK, Picard FJ, Bergeron MG. 2005. *Characterization of a Tn5382-like transposon containing the vanB2 gene cluster in a Clostridium strain isolated from human faeces*. J Antimicrob Chemother 55:466–74.

49. Xu X, Lin D, Yan G, Ye X, Wu S, Guo Y, Zhu D, Hu F, Zhang Y, Wang F, Jacoby GA, Wang M. 2010. *vanM, a new glycopeptide resistance gene cluster found in Enterococcus faecium*. Antimicrob Agents Chemother 54:4643–7.

50. Lebreton F, Depardieu F, Bourdon N, Fines-Guyon M, Berger P, Camiade S, Leclercq R, Courvalin P, Cattoir V. 2011. *D-Ala-d-Ser VanN-type transferable vancomycin resistance in Enterococcus faecium*. Antimicrob Agents Chemother 55:4606–12.

51. Boyd DA, Willey BM, Fawcett D, Gillani N, Mulvey MR. 2008. *Molecular characterization of Enterococcus faecalis N06-0364 with low-level vancomycin resistance harboring a novel D-Ala-D-Ser gene cluster, vanL*. Antimicrob Agents Chemother 52:2667–72.

52. Boyd DA, Du T, Hizon R, Kaplen B, Murphy T, Tyler S, Brown S, Jamieson F, Weiss K, Mulvey MR. 2006. *VanG-type vancomycin-resistant Enterococcus faecalis strains isolated in Canada*. Antimicrob Agents Chemother 50:2217–21.

53. Andersson DI, Levin BR. 1999. *The biological cost of antibiotic resistance*. Curr Opin Microbiol 2:489–93.

54. Foucault M-L, Courvalin P, Grillot-Courvalin C. 2009. *Fitness cost of VanA-type vancomycin resistance in methicillin-resistant Staphylococcus aureus*. Antimicrob Agents Chemother 53:2354–9.

55. Björkman J, Andersson DI. 2000. *The cost of antibiotic resistance from a bacterial perspective*. Drug Resist Updat 3:237–245.

56. Johnsen PJ, Townsend JP, Bøhn T, Simonsen GS, Sundsfjord A, Nielsen KM. 2011. *Retrospective evidence for a biological cost of vancomycin resistance determinants in the absence of glycopeptide selective pressures*. J Antimicrob Chemother 66:608–10.

57. Johnsen PJ, Townsend JP, Bøhn T, Simonsen GS, Sundsfjord A, Nielsen KM. 2009. *Factors affecting the reversal of antimicrobial-drug resistance*. Lancet Infect Dis 9:357–64.

58. Johnsen PJ, Østerhus JI, Sletvold H, Sørum M, Kruse H, Nielsen K, Simonsen GS, Sundsfjord A. 2005. *Persistence of animal and human glycopeptide-resistant enterococci on two Norwegian poultry farms formerly exposed to avoparcin is associated with a widespread plasmid-mediated vanA element within a polyclonal enterococcus faecium population*. Appl Environ Microbiol 71:159–68.

59. Gullberg E, Cao S, Berg OG, Ilbäck C, Sandegren L, Hughes D, Andersson DI. 2011. *Selection of resistant bacteria at very low antibiotic concentrations*. PLoS Pathog 7:e1002158.

60. Flannagan SE, Chow JW, Donabedian SM, Brown WJ, Perri MB, Zervos MJ, Ozawa Y, Clewell DB. 2003. *Plasmid content of a vancomycin-resistant Enterococcus faecalis isolate from a patient also colonized by Staphylococcus aureus with a VanA phenotype*. Antimicrob Agents Chemother 47:3954–9.

61. Vollaard EJ, Clasener HA. 1994. *Colonization resistance*. Antimicrob Agents Chemother 38:409–14.

62. Sartor RB. 2008. *Microbial influences in inflammatory bowel diseases*. Gastroenterology 134:577–94.

63. Sonnenburg JL, Angenent LT, Gordon JI. 2004. *Getting a grip on things: how do communities of bacterial symbionts become established in our intestine?* Nat Immunol 5:569–73.

64. Mariat D, Firmesse O, Levenez F, Guimarães V, Sokol H, Doré J, Corthier G, Furet J-P. 2009. *The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age*. BMC Microbiol 9:123.

65. Hill DA, Hoffmann C, Abt MC, Du Y, Kobuley D, Kirn TJ, Bushman FD, Artis D. 2010. *Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis*. Mucosal Immunol 3:148–58.

66. Ubeda C, Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, Viale A, Socci ND, van den Brink MRM, Kamboj M, Pamer EG. 2010. *Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans*. J Clin Invest 120:4332–41.

67. Woodmansey EJ, Mcmurdo MET, George T, Macfarlane GT. 2004. *Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects*. Appl Environ Microbiol 70: 6113-22.

68. Woodmansey EJ. 2007. *Intestinal bacteria and ageing*. J Appl Microbiol 102:1178–86.

69. Enck P, Zimmermann K, Rusch K, Schwiertz A, Klosterhalfen S, Frick J-S. 2009. *The effects of ageing on the colonic bacterial microflora in adults*. Z Gastroenterol 47:653–8.

70. Tiihonen K, Ouwehand ACA, Rautonen N. 2010. *Human intestinal microbiota and healthy ageing*. Ageing Res Rev 9:107–16.

71. Petersen C, Round JL. 2014. *Defining dysbiosis and its influence on host immunity and disease*. Cell Microbiol 16:1024–33.

72. Firmesse O, Rabot S, Bermúdez-Humarán LG, Corthier G, Furet J-P. 2007. *Consumption of Camembert cheese stimulates commensal enterococci in healthy human intestinal microbiota*. FEMS Microbiol Lett 276:189–92.

73. Layton BA, Walters SP, Lam LH, Boehm AB. 2010. *Enterococcus species distribution among human and animal hosts using multiplex PCR*. J Appl Microbiol 109:539–47.

74. Carvalho M da GS, Shewmaker PL, Steigerwalt AG, Morey RE, Sampson AJ, Joyce K, Barrett TJ, Teixeira LM, Facklam RR. 2006. *Enterococcus caccae sp. nov., isolated from human stools*. Int J Syst Evol Microbiol 56:1505–8.

75. Hopkins MJ, Sharp R, Macfarlane GT. 2001. *Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles*. Gut 48:198–205.

76. Kirtzalidou EI, Mitsou EK, Pramateftaki P, Kyriacou A. 2012. *Screening fecal enterococci from Greek healthy infants for susceptibility to antimicrobial agents*. Microb Drug Resist 18:578–85.

77. Aujoulat F, Roudière L, Picaud J-C, Jacquot A, Filleron A, Neveu D, Baum T-P, Marchandin H, Jumas-Bilak E. 2014. *Temporal dynamics of the very premature infant gut dominant microbiota*. BMC Microbiol 14:2320.

78. Yameen MA, Iram S, Mannan A, Khan SA, Akhtar N. 2013. *Nasal and perirectal colonization of vancomycin sensitive and resistant enterococci in patients of paediatrics ICU (PICU) of tertiary health care facilities*. BMC Infect Dis 13:156.

79. Hufnagel M, Liese C, Loescher C, Kunze M, Proempeler H, Berner R, Krueger M. 2007. *Enterococcal colonization of infants in a neonatal intensive care unit: associated predictors, risk factors and seasonal patterns*. BMC Infect Dis 7:107.

80. Dethlefsen L, Huse S, Sogin ML, Relman DA. 2008. *The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing*. PLoS Biol 6:e280.

81. Sánchez-Díaz AM, Cuartero C, Rodríguez JD, Lozano S, Alonso JM, Rodríguez-Domínguez M, Tedim AP, Campo R del, Cantón R, Ruiz-Garbajosa P. 2016. *The rise of ampicillin-resistant Enterococcus faecium high-risk clones as a frequent intestinal colonizer in oncohaematologic neutropenic patients on levofloxacin prophylaxis: a risk for bacteraemia?* Clin Microbiol Infect. 22:59.e1-8.

82. Holland T, Fowler VG, Shelburne SA. 2014. *Invasive gram-positive bacterial infection in cancer patients*. Clin Infect Dis 59 Suppl 5:S331–4.

83. Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, Fleisher M, Schnabl B, DeMatteo RP, Pamer EG. 2008. *Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits*. Nature 455:804–7.

84. Rice LB. 2013. *The complex dynamics of antimicrobial activity in the human gastrointestinal tract*. Trans Am Clin Climatol Assoc 124:123–32.

85. Croswell A, Amir E, Teggatz P, Barman M, Salzman NH. 2009. *Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric Salmonella infection*. Infect Immun 77:2741–53.

86. Antonopoulos DA, Huse SM, Morrison HG, Schmidt TM, Sogin ML, Young VB. 2009. *Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation*. Infect Immun 77:2367–75.

87. Rice LB, Lakticová V, Carias LL, Rudin S, Hutton R, Marshall SH. 2009. *Transferable capacity for gastrointestinal colonization in Enterococcus faecium in a mouse model*. J Infect Dis 199:342–9.

88. Nes IF, Diep DB, Ike Y. 2014. *Enterococcal Bacteriocins and Antimicrobial Proteins that Contribute to Niche Control*. *In* Gilmore, MS, Clewell, DB, Ike, Y, Shankar, N (eds.*), Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary, Boston.

89. Nes IF, Diep DB, Holo H. 2007. *Bacteriocin diversity in Streptococcus and Enterococcus*. J Bacteriol 189:1189–98.

90. Chuang ON, Schlievert PM, Wells CL, Manias DA, Tripp TJ, Dunny GM. 2009. *Multiple functional domains of Enterococcus faecalis aggregation substance Asc10 contribute to endocarditis virulence*. Infect Immun 77:539–48.

91. Bhatty M, Cruz MR, Frank KL, Laverde Gomez JA, Andrade F, Garsin DA, Dunny GM, Kaplan HB, Christie PJ. 2015. *Enterococcus faecalis pCF10-encoded surface proteins PrgA, PrgB (aggregation substance) and PrgC contribute to plasmid transfer, biofilm formation and virulence*. Mol Microbiol, 95:660-77.

92. Olmsted SB, Dunny GM, Erlandsen SL, Wells CL. 1994. *A plasmid-encoded surface protein on Enterococcus faecalis augments its internalization by cultured intestinal epithelial cells*. J Infect Dis 170:1549–56.

93. Mandlik A, Das A, Ton-That H. 2008*. The molecular switch that activates the cell wall anchoring step of pilus assembly in gram-positive bacteria*. Proc Natl Acad Sci U S A 105:14147–52.

94. Tendolkar PM, Baghdayan AS, Shankar N. 2006. *Putative surface proteins encoded within a novel transferable locus confer a high-biofilm phenotype to Enterococcus faecalis*. J Bacteriol 188:2063–72.

95. Tendolkar PM, Baghdayan AS, Gilmore MS, Shankar N. 2004. *Enterococcal surface protein, Esp, enhances biofilm formation by Enterococcus faecalis*. Infect Immun 72:6032–9.

96. Coburn PS, Baghdayan AS, Craig N, Burroughs A, Tendolkar P, Miller K, Najar FZ, Roe BA, Shankar N. 2010. *A novel conjugative plasmid from Enterococcus faecalis E99 enhances resistance to ultraviolet radiation*. Plasmid 64:18–25.

97. Coque TM, Patterson JE, Steckelberg JM, Murray BE. 1995. *Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons*. J Infect Dis 171:1223–9.

98. Ike Y, Hashimoto H, Clewell DB. 1984. *Hemolysin of Streptococcus faecalis subspecies zymogenes contributes to virulence in mice*. Infect Immun 45:528–30.

99. Booth MC, Bogie CP, Sahl HG, Siezen RJ, Hatter KL, Gilmore MS. 1996. *Structural analysis and proteolytic activation of Enterococcus faecalis cytolysin, a novel lantibiotic*. Mol Microbiol 21:1175–84.

100. Arias CA, Panesso D, Singh K V, Rice LB, Murray BE. 2009. *Cotransfer of antibiotic resistance genes and a hylEfm-containing virulence plasmid in Enterococcus faecium*. Antimicrob Agents Chemother 53:4240–6.

101. Sydnor ERM, Perl TM. 2011. *Hospital epidemiology and infection control in acute-care settings*. Clin Microbiol Rev 24:141–73.

102. Sundsfjord a, Willems R. 2010. *Enterococcus research: recent developments and clinical challenges*. Clin Microbiol Infect 16:525–6.

103. Werner G, Coque TM, Franz CMAP, Grohmann E, Hegstad K, Jensen L, van Schaik W, Weaver K. 2013. *Antibiotic resistant enterococci-tales of a drug resistance gene trafficker*. Int J Med Microbiol 303:360–79.

104. López M, Sáenz Y, Alvarez-Martínez MJ, Marco F, Robredo B, Rojo-Bezares B, Ruiz-Larrea F, Zarazaga M, Torres C. 2010. *Tn1546 structures and multilocus sequence typing of vanA-containing enterococci of animal, human and food origin*. J Antimicrob Chemother 65:1570–5.

105. Guttman DS, Stavrinides J. 2010. *Population Genomics of Bacteria*, p. 121–152. In Robinson, DA, Falush, D, Feil, EJ (eds.), *Bacterial Population Genetics in Infectious Disease*. Wiley-Blackwell, Hoboken, NJ, USA.

106. de Been M, Pinholt M, Top J, Bletz S, Mellmann A, van Schaik W, Brouwer E, Rogers M, Kraat Y, Bonten M, Corander J, Westh H, Harmsen D, Willems RJL. 2015. *Core Genome Multilocus Sequence Typing Scheme for High-Resolution Typing of Enterococcus faecium*. J Clin Microbiol 53:3788–97.

107. Homan WL, Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, Van Embden JDA, Willems RJL. 2002. *Multilocus sequence typing scheme for Enterococcus faecium*. J Clin Microbiol 40:1963–71.

108. Willems RJL, Top J, van Santen M, Robinson DA, Coque TM, Baquero F, Grundmann H, Bonten MJM. 2005. *Global spread of vancomycin-resistant Enterococcus faecium from distinct nosocomial genetic complex*. Emerg Infect Dis 11:821–8.

109. Willems RJL. 2010. *Population Genetics of Enterococcus*, p. 195–216. In Robinson, DA, Falush, D, Feil, EJ (eds.), *Bacterial Population Genetics in Infectious Disease*. Wiley-Blackwell, Hoboken, NJ, USA.

110. Willems RJL, Top J, van Schaik W, Leavis H, Bonten M, Sirén J, Hanage WP, Corander J. 2012. *Restricted gene flow among hospital subpopulations of Enterococcus faecium*. MBio 3:e00151–12.

111. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. 2004. *eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data*. J Bacteriol 186:1518–30.

112. Leavis HL, Willems RJL, Top J, Bonten MJM. 2006. *High-level ciprofloxacin resistance from point mutations in gyrA and parC confined to global hospital-adapted clonal lineage CC17 of Enterococcus faecium*. J Clin Microbiol 44:1059–64.

113. Leavis HL, Willems RJL, van Wamel WJB, Schuren FH, Caspers MPM, Bonten MJM. 2007. *Insertion sequence-driven diversification creates a globally dispersed emerging multiresistant subspecies of E. faecium*. PLoS Pathog 3:e7.

114. Willems RJ, van Schaik W. 2009. *Transition of Enterococcus faecium from commensal organism to nosocomial pathogen*. Future Microbiol 4:1125–35.

115. Palmer KL, van Schaik W, Willems RJL, Gilmore MS. 2014. *Enterococcal Genomics*. *In* Gilmore, MS, Clewell, DB, Ike, Y, Shankar, N (eds.), *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary, Boston.

116. Turner KME, Hanage WP, Fraser C, Connor TR, Spratt BG. 2007. *Assessing the reliability of eBURST using simulated populations with known ancestry*. BMC Microbiol 7:30.

117. Willems RJL, Hanage WP, Bessen DE, Feil EJ. 2011. *Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance*. FEMS Microbiol Rev 35:872–900.

118. Lam MMC, Seemann T, Bulach DM, Gladman SL, Chen H, Haring V, Moore RJ, Ballard S, Grayson ML, Johnson PDR, Howden BP, Stinear TP. 2012. *Comparative analysis of the first complete Enterococcus faecium genome*. J Bacteriol 194:2334–41.

119. Qin X, Galloway-Peña JR, Sillanpaa J, Roh JH, Nallapareddy SR, Chowdhury S, Bourgogne A, Choudhury T, Muzny DM, Buhay CJ, Ding Y, Dugan-Rocha S, Liu W, Kovar C, Sodergren E, Highlander S, Petrosino JF, Worley KC, Gibbs RA, Weinstock GM, Murray BE. 2012. *Complete genome sequence of Enterococcus faecium strain TX16 and comparative genomic analysis of Enterococcus faecium genomes*. BMC Microbiol 12:135.

120. Lam MMC, Seemann T, Tobias NJ, Chen H, Haring V, Moore RJ, Ballard S, Grayson LM, Johnson PDR, Howden BP, Stinear TP. 2013. *Comparative analysis of the complete genome of an epidemic hospital sequence type 203 clone of vancomycin-resistant Enterococcus faecium*. BMC Genomics 14:595.

121. Kopit LM, Kim EB, Siezen RJ, Harris LJ, Marco ML. 2014. *Safety of the surrogate microorganism Enterococcus faecium NRRL B-2354 for use in thermal process validation*. Appl Environ Microbiol 80:1899–909.

122. Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems RJL, Earl AM, Gilmore MS. 2013. *Emergence of epidemic multi-drug-resistant Enterococcus faecium from animal and commensal strains*. MBio 4: e00534-13.

123. Galloway-Peña J, Roh JH, Latorre M, Qin X, Murray BE. 2012. *Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of Enterococcus faecium*. PLoS One 7:e30187.

124. Palmer KL, Godfrey P, Griggs A, Kos VN, Zucker J, Desjardins C, Cerqueira G, Gevers D, Walker S, Wortman J, Feldgarden M, Haas B, Birren B, Gilmore MS. 2012. *Comparative genomics of enterococci: variation in Enterococcus faecalis, clade structure in E. faecium, and defining characteristics of E. gallinarum and E. casseliflavus.* MBio 3:e00318–11.

125. Duerkop BA, Palmer KL, Horsburgh MJ. 2014. *Enterococcal Bacteriophages and Genome Defense*. *In* Gilmore, MS, Clewell, DB, Ike, Y, Shankar, N (eds.), *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary, Boston, MA.

126. Baquero F. 2004. *From pieces to patterns: evolutionary engineering in bacterial pathogens*. Nat Rev Microbiol 2:510–8.

127. Ruiz-Garbajosa P, Bonten MJM, Robinson DA, Top J, Nallapareddy SR, Torres C, Coque TM, Cantón R, Baquero F, Murray BE, del Campo R, Willems RJL. 2006. *Multilocus sequence typing scheme for Enterococcus faecalis reveals hospital-adapted genetic complexes in a background of high rates of recombination*. J Clin Microbiol 44:2220–8.

128. Kuch A, Willems RJL, Werner G, Coque TM, Hammerum AM, Sundsfjord A, Klare I, Ruiz-Garbajosa P, Simonsen GS, van Luit-Asbroek M, Hryniewicz W, Sadowy E. 2012. *Insight into antimicrobial susceptibility and population structure of contemporary human Enterococcus faecalis isolates from Europe*. J Antimicrob Chemother 67:551–8.

129. McBride SM, Fischetti VA, Leblanc DJ, Moellering RC, Gilmore MS. 2007. *Genetic diversity among Enterococcus faecalis*. PLoS One 2:e582.

130. Freitas AR, Coque TM, Novais C, Hammerum AM, Lester CH, Zervos MJ, Donabedian S, Jensen LB, Francia MV, Baquero F, Peixe L. 2011. *Human and swine hosts share vancomycin-resistant Enterococcus faecium CC17 and CC5 and Enterococcus faecalis CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids*. J Clin Microbiol 49:925–31.

131. Novais C, Freitas AR, Silveira E, Antunes P, Silva R, Coque TM, Peixe L. 2013. *Spread of multi-drug-resistant Enterococcus to animals and humans: an underestimated role for the pig farm environment*. J Antimicrob Chemother 68:2746–54.

132. Kawalec M, Pietras Z, Daniłowicz E, Jakubczak A, Gniadkowski M, Hryniewicz W, Willems RJL. 2007. *Clonal structure of Enterococcus faecalis isolated from Polish hospitals: characterization of epidemic clones*. J Clin Microbiol 45:147–53.

133. Solheim M, Brekke MC, Snipen LG, Willems RJL, Nes IF, Brede DA. 2011. *Comparative genomic analysis reveals significant enrichment of mobile genetic elements and genes encoding surface structure-proteins in hospital-associated clonal complex 2 Enterococcus faecalis*. BMC Microbiol 11:3.

134. Palmer KL, Gilmore MS. 2010. *Multi-drug-resistant enterococci lack CRISPR-cas*. MBio 1:e00227–10.

135. Pinkston KL, Gao P, Diaz-Garcia D, Sillanpää J, Nallapareddy SR, Murray BE, Harvey BR. 2011. *The Fsr quorum-sensing system of Enterococcus faecalis modulates surface display of the collagen-binding MSCRAMM Ace through regulation of gelE*. J Bacteriol 193:4317–4325.

136. Labrie SJ, Samson JE, Moineau S. 2010. *Bacteriophage resistance mechanisms*. Nat Rev Microbiol 8:317–27.

137. Palmer KL, Kos VN, Gilmore MS. 2010. *Horizontal gene transfer and the genomics of enterococcal antibiotic resistance*. Curr Opin Microbiol 13:632–9.

138. Bourgogne A, Garsin DA, Qin X, Singh K V, Sillanpaa J, Yerrapragada S, Ding Y, Dugan-Rocha S, Buhay C, Shen H, Chen G, Williams G, Muzny D, Maadani A, Fox KA, Gioia J, Chen L, Shang Y, Arias CA, Nallapareddy SR, Zhao

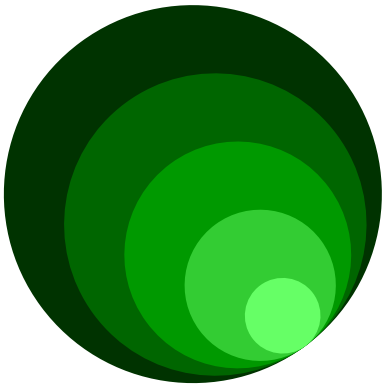
M, Prakash VP, Chowdhury S, Jiang H, Gibbs RA, Murray BE, Highlander SK, Weinstock GM. 2008. *Large scale variation in Enterococcus faecalis illustrated by the genome analysis of strain OG1RF.* Genome Biol 9:R110.

139. Vasu K, Nagaraja V. 2013. *Diverse functions of restriction-modification systems in addition to cellular defense*. Microbiol Mol Biol Rev 77:53–72.

140. Roberts RJ, Vincze T, Posfai J, Macelis D. 2010. *REBASE--a database for DNA restriction and modification: enzymes, genes and genomes*. Nucleic Acids Res 38:D234–6.

The possession of knowledge does not kill the sense of wonder and mystery. There is always more mystery.

Anaïs Nin



Hypothesis and Objectives

Previous studies have demonstrated the influence of age and antibiotic treatment on the bacterial populations (at phyla, genera and/or species level) colonizing the human GI tract. However, the information regarding the influence of age, hospitalizations and other factors on population changes at the subspecies level is still scarce. We are addressing this knowledge gap focusing *Enterococcus faecium* intestinal populations.

Some genomic studies directed to elucidate *E. faecium* population structure have shown differences between ecological groups colonizing the GI tract of healthy volunteers and/or community individuals, which are mostly antibiotic susceptible, and those causing nosocomial infections, which are enriched in adaptive traits (e.g. antibiotic resistance, adhesion, virulence). Nevertheless, longitudinal studies regarding the population structure of *E. faecium* causing nosocomial infections also remain scarce.

Finally, the contribution of MGEs to the adaptation of bacteria to different ecological niches (and microniches), including human pathogenic species, has been traditionally analysed under the dominant "species-centric" view. However, a global, integrated view of the effects of MGEs at population level was needed in order to understand the emergence and dynamics of some relevant traits as antibiotic resistance. This is a necessary requirement for the better understanding of the emergence, spread and persistence of certain ecologic/clonal niche-specialized groups within species.

Our **HYPOTHESIS** postulates that the combination of both human host characteristics (e. g. age), and bacterial factors (e.g. the presence of MGEs) might explain the successful adaptation of particular bacterial populations to well-defined local ecological challenges (e.g. the use of antibiotics in the hospital setting). The biological cost of carrying antibiotic resistance determinants (e. g. *pbp5* and Tn*1546*-*vanA*) and the MGEs in which they are located would play an important role in the possibilities for selection and persistence of specialized populations of *E. faecium* in particular selective environments.

The **MAIN OBJECTIVE** of this dissertation is to assess the influence that MGE and antibiotic resistance have in shaping the population structure and evolution of *E. faecium*, an important nosocomial pathogen.

The more specific **OBJECTIVES (O)** and **ACTIVITIES (A)** are the following:

O1. To comprehensively analyse the diversity of *E. faecium* populations in different age groups and the longitudinal dynamics *E. faecium* in the hospital setting.

A1.1. To analyse the population structure of *E. faecium* faecal isolates from hospitalized and non-hospitalized individuals of different age groups.

A1.2. To assess the population structure of *E. faecium* causing bacteraemia in Hospital Universitario Ramón y Cajal (1995-2015) and its relation with epidemiological patient data in a geographical area where vancomycin resistant *E. faecium* is sporadic.

O2. To characterize the diversity of MGEs responsible for the transmission of antibiotic resistance in *E. faecium*, with emphasis in those conferring resistance to vancomycin.

A2.1. To analyse, using gene exchange networks, based on sequences available in databases, the role of plasmids in the emergence, dissemination and maintenance of antimicrobial resistance determinants (antibiotics, heavy metals and biocides).

A2.2. To comprehensively characterize the plasmids and transposons conferring resistance to glycopeptides since their description in 1986, by analysing documented strains causing outbreaks in different geographical areas of the world, along a 20 years period.

A2.3. To address the dynamics of vancomycin resistance among enterococci by analysing the clonal and plasmid backgrounds influencing the spread and persistence of Tn*1546* in Portugal, one of the developed countries with higher rates of both VREfm (21–23%) and VREfs (1.8–4.1%), and where VanA is prevalent over VanB.

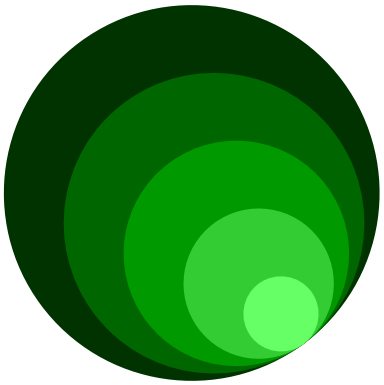
O3. To determine the biological cost of antibiotic resistance determinants and their associated MGEs might have on the fitness of bacterial populations.

A3.1. To assess the genetic context of the *pbp5* gene as well as its transferability and stability in different *E. faecium* clonal backgrounds by analysing a large *E. faecium* collection (obtained from different origins collected in Portugal for more than 20 years) and available *E. faecium* genomes in NCBI database.

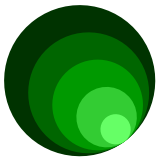
A3.2. To determine the fitness cost and the stability of plasmids carrying different Tn*1546*-*vanA* variants as well as the intrinsic fitness of a well-documented collection of *E. faecium* susceptible and resistant to ampicillin and vancomycin.

I was gratified to be able to answer promptly, and I did. I said I didn't know.

Mark Twain



Chapter 1



Population Biology of Intestinal *Enterococcus* Isolates from Hospitalized and Non-hospitalized Individuals in Different Age Groups

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ABSTRACT

The diversity of enterococcal populations from fecal samples from hospitalized (n=133) and non-hospitalized individuals (n=173) of different age groups (group I, ages 0 to 19 years; group II, ages 20 to 59 years; group III, ages>60 years) was analyzed. Enterococci were recovered at similar rates from hospitalized and non-hospitalized persons (77.44% to 79.77%) of all age groups (75.0% to 82.61%). *Enterococcus faecalis* and *Enterococcus faecium* were predominant, although seven other *Enterococcus* species were identified. *E. faecalis* and *E. faecium* (including ampicillin-resistant *E. faecium*) colonization rates in non-hospitalized persons were age independent. For inpatients, *E. faecalis* colonization rates were age independent, but E. faecium colonization rates (particularly the rates of ampicillin-resistant *E. faecium* colonization) significantly increased with age. The population structure of *E. faecium* and *E. faecalis* was determined by superimposing goeBURST and Bayesian analysis of the population structure (BAPS). Most *E. faecium* sequence types (STs; 150 isolates belonging to 75 STs) were linked to BAPS groups 1 (22.0%), 2 (31.3%), and 3 (36.7%). A positive association between hospital isolates and BAPS subgroups 2.1a and 3.3a (which included major ampicillin-resistant *E. faecium* human lineages) and between community-based ampicillin-resistant *E. faecium* isolates and BAPS subgroups 1.2 and 3.3b was found. Most *E. faecalis* isolates (130 isolates belonging to 58 STs) were grouped into 3 BAPS groups, BAPS groups 1 (36.9%), 2 (40.0%), and 3 (23.1%), with each one comprising widespread lineages. No positive associations with age or hospitalization were established. The diversity and dynamics of enterococcal populations in the fecal microbiota of healthy humans are largely unexplored, with the available knowledge being fragmented and contradictory. The study offers a novel and comprehensive analysis of enterococcal population landscapes and suggests that *E. faecium* populations from hospitalized patients and from community-based individuals differ, with a predominance of certain clonal lineages, often in association with elderly individuals, occurring in the hospital setting.

INTRODUCTION

Enterococci are relatively minor constituents of the human gastrointestinal microbiota (less than 1%) but are able to cause a wide diversity of infections mostly in patients with underlying diseases (1, 2). High-density colonization by antibiotic resistant enterococci increases the risk of bacteremia and transmission, however the population structure and ecological and evolutionary forces influencing population dynamics of gut colonizers remains largely uncomprehended (3–5). Next generation sequencing has provided a wealth of data about the influence of the host (age, diet, health status, and antibiotic treatment) on the diversity and population frequency of different bacterial groups, in which enterococci are included (6–10). However, the information provided by current metagenomic analysis, based on 16S rRNA (11, 12), or by the traditional culture based studies (1, 13, 14) preclude any possible analysis of enterococci at subspecies level. Furthermore, available information about the frequency and diversity of enterococcal species in fecal microbiota with age is fragmented and contradictory (1, 15).

Different methods based on Multilocus Sequence Typing (MLST), comparative genomic hybridization and whole genome sequencing revealed intra-species diversity for *Enterococcus faecalis* and *Enterococcus faecium* which are the predominant enterococcal species colonizing the human gastrointestinal tract (16–25). *E. faecium* has a population structure that has split in two major phylogenomic clusters designated as “clade B”, that includes community-based human isolates, and “clade A” that comprises isolates from humans and animals, with a clade A1 enriched with isolates from hospitalized patients. Specifically, strains belonging to ST17, ST18 and ST78 lineages, within clade A1, are often resistant to antibiotics and the most frequently associated with the hospital environment (21, 26, 27). *E. faecalis*, on the other hand, seems to lack such a clear clade structure probably because this species occupies a higher variety of ecological microniches, having thus access to more heterogeneous spectrum of alleles than *E. faecium* (28). As a result, no clear genotypic differences are observed between hospital and community isolates (25, 28, 29), even though some clonal complexes (CCs) are more prevalent either among hospitalized patients as CC6-ST6, CC9-ST9, CC28-ST87, and CC40-ST40; or among community healthy volunteers, as ST16 and CC58 (30–33). Recombination, as detected previously in enterococci (17, 34, 35), may have a considerable impact on patterns of evolutionary descent as displayed by sequence-based gene trees or even by popular allele-based population snapshots provided by eBURST. This may obscure the genetic relatedness of strains and clones and as such interfere with epidemiological and clinical investigations, in particular when strains are assigned to specific CCs. In addition, knowledge about the population structure of enterococcal species is biased by an overrepresentation of contemporary multi-drug resistant clinical isolates belonging to a few high-risk clonal complexes often associated with nosocomial outbreaks and frequently associated with elderly (36–38). Studies analyzing early isolates document a more diverse enterococcal population able to cause disease, either of nosocomial or community acquisition, and often associated with adults and children. Isolates causing infections or colonizing these populations have less frequently been analyzed at the molecular level (33, 39–41).

The objective of this study was to assess for the first time the population structure of enterococci in the feces of both hospitalized and non-hospitalized individuals within different age groups. In addition, Bayesian Analysis of Population Structure (BAPS), a non-phylogenetic method able to find the best partition of a set of isolates into sub-populations, was applied, broadening former results obtained for *E. faecium*, and providing the first analysis to probabilistically assign *E. faecalis* strains to evolutionary groups.

MATERIAL AND METHODS

Bacterial samples. Three hundred and six faecal samples were collected between April 2009 and April 2011 at Ramón y Cajal University Hospital (HRyC) and its community-care area of influence. HRyC is a tertiary care public hospital with 1,155 beds that provides specialized attention to a population of about 600,000 habitants in the Northern area of Madrid (Spain) which is primarily attended at 20 Primary Health Centers (PHC) of the Madrid Health Service (SERMAS). This study was conducted according to applicable government regulations and approved studies by institutional research policies (e.g., reference CEIC-106/09 [A. M. Sánchez-Díaz, C. Cuartero, J. D. Rodríguez, S. Lozano, J. M. Alonso, M. J. Rodríguez-Domínguez, A. P. Tedim, R. del Campo, J. López, R. Cantón, and P. Ruiz-Garbajosa, unpublished data]).

The samples analysed were recovered from 173 patients with non-severe diseases that attended a PHC or a consult in HRyC (with no hospitalization registered in the 6 months prior to the sample collection) and from 133 hospitalized patients admitted at HRyC. The faecal samples were submitted to HRyC for stool culture, with/without specific request for *Clostridium difficile* or for parasites detection, and were anonymously processed keeping confidential patients' demographic information. Hospitalized patients were mostly located at medical (78.2%), surgical wards (8.3%) and intensive care units (ICU, 9.8%). All but 20 samples from hospitalized patients were collected after more than 48h of hospital admission. However, these 20 patients had history of several recent previous hospitalizations (see Tables S1 and S2 in the supplemental material).

Samples were also classified according with host's age in three age groups designed with roman numerals as group I (young people, 0-19 years old; n=92 [30%]; 57 non-hospitalized persons and 35 hospitalized patients), group II (adults, 20-59 years old; n=108 [35%]; 62 non-hospitalized persons and 46 hospitalized patients) and group III (elderly, ≥60 years old; n=106 [35%]; 54 non-hospitalized persons and 52 hospitalized patients). Only one sample per patient was analysed (see Tables S1 and S2in the supplemental material).

Sample processing. About 0.5 g of each faecal sample was suspended in 1mL of saline solution, plated on m-Enterococcus agar (Difco, Detroit, USA) plain or supplemented either with ampicillin (10 µg/mL) or vancomycin (6 µg/mL), and incubated 48h at 37ºC. For each sample one colony per morphology and plate was selected (28) for further studies. In order to enhance the recovery of minority populations of

ampicillin and vancomycin resistant enterococci (VRE), 0.1 mL of the original suspension of each sample was pre-enriched in Brain-Heart Infusion (BHI) broth (Difco, Detroit, MI, USA) supplemented with 2 µg/mL of ampicillin or 2 µg/mL of vancomycin, incubated 24h at 37ºC and subsequently, plated on m-Enterococcus agar (Difco, Detroit, USA) containing ampicillin (10 µg/mL) or vancomycin (6 µg/mL), respectively.

Identification, antibiotic susceptibility and virulence traits. Bacterial identification was performed by the amplification of species-specific genes: *E. faecalis* D-Alanine-D-Alanine ligase (*ddl*) and *E. faecium aac(6')-Ii* as previously described (42, 43) and by MALDI-TOF MS (Bruker, Daltonics, Bremen, Germany). Susceptibility for ampicillin, vancomycin, teicoplanin, streptomycin, gentamicin, ciprofloxacin, levofloxacin, erythromycin, tetracycline and chloramphenicol (Oxoid, Basingstoke, UK) was determined by disc diffusion according to CLSI guidelines (44).

The presence of putative virulence genes encoding the *E. faecium* enterococcal surface protein (*esp*), glycosyl hydrolase (hyl_{Efm}) and collagen-binding adhesin (*acm*), and the *E. faecalis* enterococcal surface protein (*esp*), hyaluronidase (hyl_{Efc}), cytolysin/haemolysin (*cyl*A), gelatinase (*gel*E) and aggregation substance (*asa*1) were investigated by PCR and sequencing as described before (45, 46).

Clonal Relatedness. Clonal relationship among isolates of each enterococcal species were established by Pulsed Field Gel Electrophoresis (PFGE) and MLST as previously described (16, 47) and it is detailed in Tables S1 and S2 in the supplemental material. Clusters of related STs for *E. faecalis* (differing in no more than two of the seven MLST loci) were considered as belonging to the same clonal complex (CC) using the goeBURST algorithm (48, 49). CCs were defined based of goeBURST analysis of the 524 STs present in the *E. faecalis* MLST database (http://efaecalis.mlst.net/).

Analysis of population structure. A BAPS software was used to probabilistically assign *E. faecalis* and *E. faecium* STs to non-overlapping evolutionary groups (27, 50). BAPS clustering was performed with the second-order Markov model and the standard MLST data input option in a hierarchical manner. For *E. faecium*, the major clusters identified at the first stage were re-analysed after excluding the remaining data. The rationale for this approach is to increase statistical power to detect more fine-scale genetic structure of a population when analyzing particular lineages separately from the remaining population. In all BAPS analyses, 10 runs of the estimation algorithm were performed using a priori upper bounds (10-30 for the major groups analysis and 2-10 for subgroups analysis) for the number of clusters over the interval and in each case the runs converged to a nearly identical partition of the data in question, indicating a high level of peakedness of the posterior distribution (estimated p=1.000).

The accuracy of BAPS for establishing *E. faecium* population structure was determined using different sample sizes and discarding the inclusion of *E. faecalis* as outgroup (see Fig. S1 to S3 in supplemental material) (27). Correlation analysis was performed for each of the comparisons mentioned above using Microsoft Excel 2010. This study constitutes the first application of BAPS to investigate *E. faecalis*

population and evolutionary genetics, following the same approach that was previously used for *E. faecium* (27).

Statistical analysis. Statistical significance of the results was calculated by the Chi-square test; *P* values <0.05 were considered as being statically significant.

The STATA Generalized Estimating Equations (GEE) model (takes into account clone related data) (51) was used for calculating odd ratios (ORs) and 95% confidence intervals (CIs) related to the colonization isolates. They were done in comparison with major BAPS 3.3a for *E. faecium,* and relative to BAPS 1 for *E. faecalis*.

For the analysis of all isolates available at MLST databases, ORs were calculated between BAPS groups and different sources (hospitalized patients, non-hospitalized persons and animals). Environmental, food and other sources were also considered but due to the low number of isolates in these categories ORs analysis was not performed.

RESULTS

Prevalence and diversity of enterococcal species in human faecal samples. Enterococci were recovered by culture from 78.8% of the individuals analysed (n=241/306), at similar rates among hospitalized and non-hospitalized individuals (77.4% vs 79.8%) and among all age groups (75.0-82.6%). They corresponded to three of the five groups of enterococci previously described by Facklam *et al* on the basis of phenotypic and genotypic characteristics which used to be designed by roman numerals (1, 52). The rate of individuals colonized by different species varied in each age group, with *E. faecalis* and *E. faecium* being the predominant species identified (Fig. 1 and 2). Among non-hospitalized persons, *E. faecalis* and *E. faecium* colonization rates were age-independent (*E. faecalis*/*E. faecium* ratios 1.14, 0.71, 1.12 for age groups I, II and III, respectively). *E. faecalis* colonization rate was also age-independent among hospitalized patients but *E. faecium*, and particularly ampicillin resistant *E. faecium* colonization rate, significantly (*P*<0.01) increased with age (*E. faecalis*/*E. faecium* ratios 1.90, 0.71, 0.65, for age groups I, II and III, respectively) (Fig. 1 and 3). Besides *E. faecium* and *E. faecalis*, both classified within the enterococcal Facklam´s group II, other species within enterococcal groups I (20 *E. avium*, 7 *E. raffinosus*, 2 *E. malodoratus*); II (4 *E. casseliflavus*, 3 *E. gallinarum*, 1 *E. thailandicus*) and group III (8 *E. hirae*) were identified (Fig. 2).

Ampicillin resistance (22.2%, *n*=68/306) was detected among *E. faecium* (94.1%, n=64/68) and *E. raffinosus* (5.9%, n=4/68) isolates. Ampicillin-resistant *E. faecium* were significantly associated with hospitalized patients (44.7%, n=46/103), when compared with non-hospitalized individuals (13.0%, n=18/138). A low number of individuals colonized with VRE, all identified as *E. faecalis,* was also detected (1.6%, n=5/306, consisting of 2 non-hospitalized and 3 hospitalized individuals of different ages) (see Table S2 in the supplemental material).

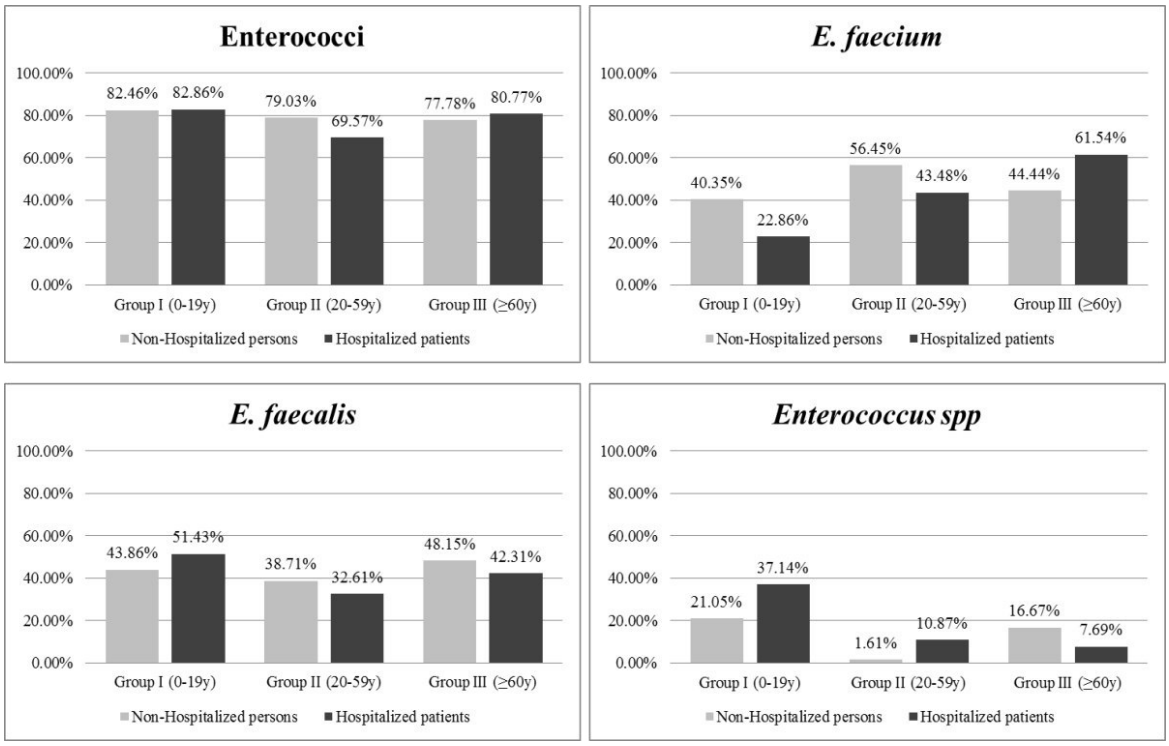

Figure 1. Proportion (%) of Non-hospitalized and Hospitalized individuals colonized by Enterococci by age group.

E. faecalis/*E. faecium* colonization ratios in non-hospitalized persons per age group were: group I (0-19 years old)=1.08, group II (20-59 years old)=0.68, and group III (≥60 years old)=1.08. *E. faecalis*/*E. faecium* colonization ratios in hospitalized patients were: group I (0-19 years old)= 2.25, group II (20-59 years old)= 0.75, and group III (≥60 years old)= 0.68.

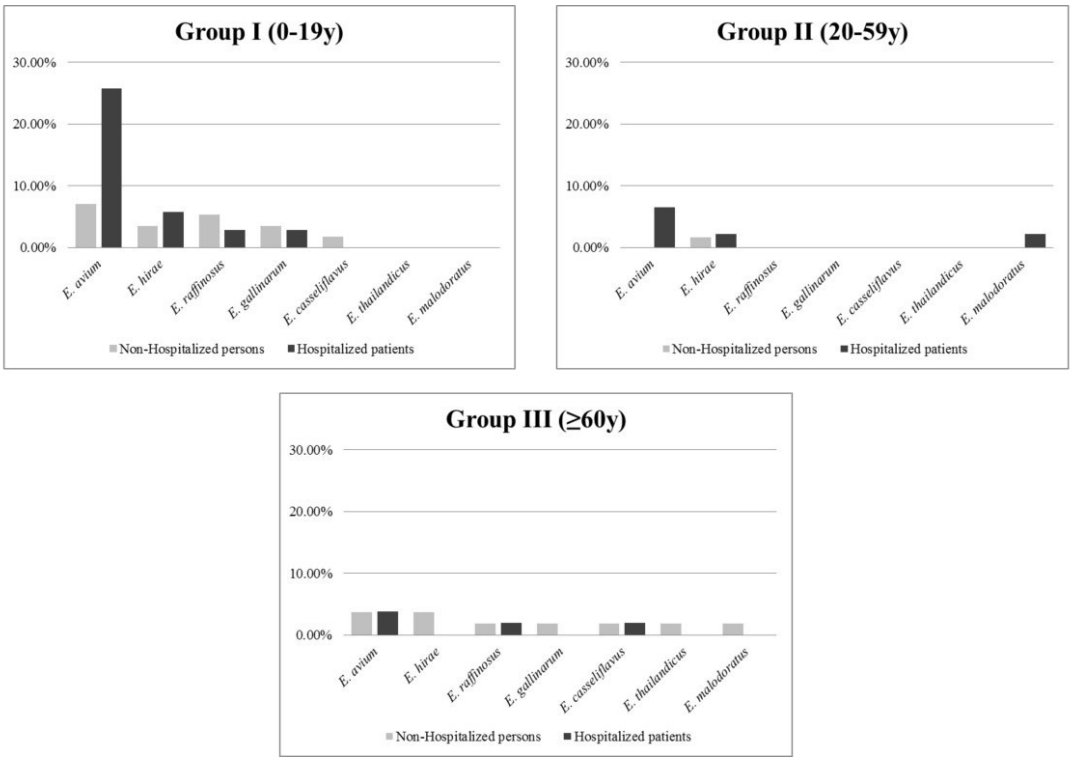

Figure 2. Proportion (%) of Non-hospitalized and Hospitalized individuals colonized by different *Enterococcus* spp by age group.

The population structure of the *E. faecium* and *E. faecalis* isolates is detailed below. For other enterococci, isolates of the same species exhibited different PFGE-types with the exception of some *E. avium* isolates (data not shown). All these species were resistant to quinupristin-dalfopristin, often resistant to erythromycin (*E. avium, E. hirae, E. raffinosus, E. gallinarum, E. casseliflavus*) and tetracycline (*E. raffinosus*) and eventually to levofloxacin (*E. raffinosus, E. gallinarum*), and high concentrations of streptomycin (*E. avium, E. raffinosus, E. gallinarum*) and gentamicin (*E. avium, E. raffinosus*).

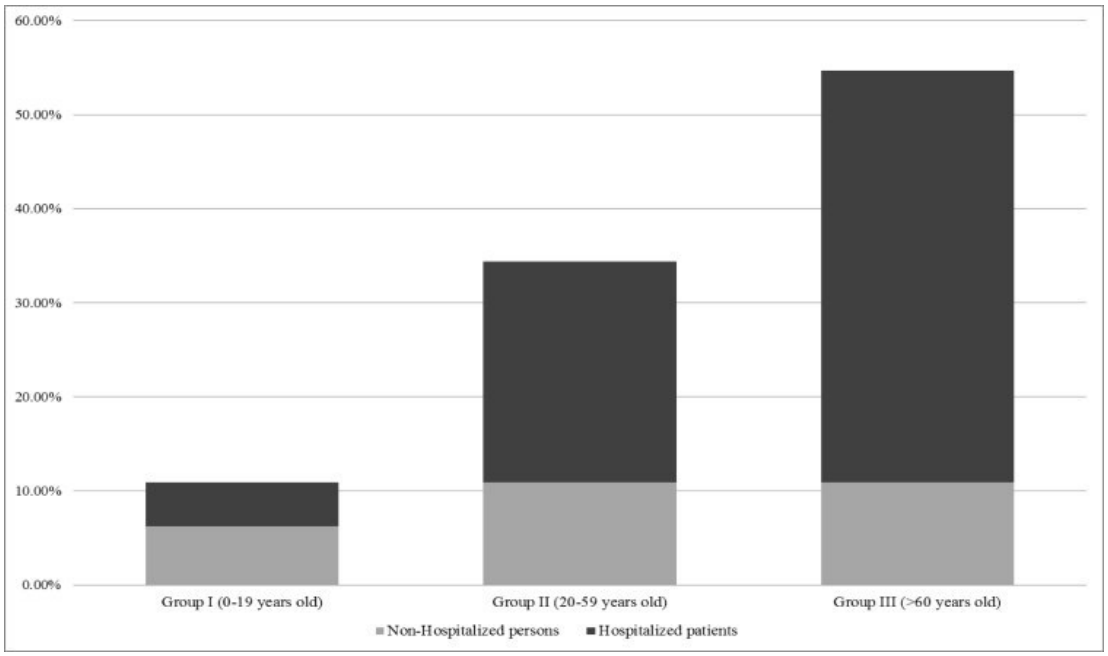

Figure 3. Proportion (%) of Non-hospitalized and Hospitalized individuals colonized by Ampicillin-resistant *E. faecium* by age group.

The proportion of Hospital/Non-hospital Ampicillin-resistant *E. faecium* for the different age groups was: Group I (0-19 years old)=0.75; Group II (20-59 years old)=2.14 and Group III (≥60 years old)=4.

BAPS analysis of *E. faecium* population structure. A BAPS analysis was used to infer the population structure of *E. faecium* according with previous findings that demonstrated that eBURST is not sufficient to reliably delineate the patterns of recent evolutionary descent of *E. faecium* (27, 53). The analysis was repeated taking in account the significant enlargement of the MLST database (http://efaecium.mlst.net/) in which the number of STs increased from 492 to 837 in the 2 years since the time of publication of the original 2012 study (27).

A hierarchical BAPS clustering analysis of the currently available 837 *E. faecium* STs representing 2,402 isolates of different origins yield 8 BAPS groups. The majority of STs grouped in BAPS 1, BAPS 2, BAPS 3 and BAPS 7 (15.1%, 39.7%, 31.5%, and 8.5%, respectively) while BAPS 4, BAPS 5, BAPS 6 and BAPS 8 were much more infrequently detected (1.9%, 1.3%, 0.8% and 1.2%, respectively). BAPS nested analysis subdivided BAPS 1 in six subgroups (BAPSs 1.1-1.6) and BAPS 2 (BAPSs 2.1a, 2.1b, 2.3a, 2.3b), BAPS 3 (BAPSs 3.1, 3.2, 3.3a, 3.3b) and BAPS 7 (BAPSs 7.1-7.4) in four subgroups each (Table 1). The original BAPS subgroups 2.1, 2.3, and 3.3 described by Willems *et al.* (27), were now split in two subgroups each,

arbitrarily designed here as BAPS 2.1a and 2.1b, BAPS 2.3a and 2.3b, and BAPS 3.3a and 3.3b, for backwards compatibility (see Fig. S3 in the supplemental material).

Table 1. *E. faecium* BAPS analysis data.

BAPS Group	BAPS Subgroup	No STs	% STs	No isolates
BAPS 1	**1.1**	9	1.08%	11
	1.2	61	7.29%	100
	1.3	12	1.43%	16
	1.4	2	0.24%	2
	1.5	36	4.30%	41
	1.6	6	0.72%	6
	Subtotal	**126**	**15.05%**	**176**
BAPS 2	**2.1a**	88	10.51%	577
	2.1b	133	15.89%	321
	2.3a	78	9.32%	135
	2.3b	33	3.94%	49
	Subtotal	**332**	**39.67%**	**1082**
BAPS 3	**3.1**	72	8.60%	122
	3.2	28	3.35%	59
	3.3a	107	12.78%	679
	3.3b	57	6.81%	92
	Subtotal	**264**	**31.54%**	**952**
BAPS 4		**11**	**1.31%**	**11**
BAPS 5		**16**	**1.91%**	**19**
BAPS 6		**7**	**0.84%**	**9**
BAPS 7	**7.1**	54	6.45%	120
	7.2	6	0.72%	6
	7.3	10	1.19%	14
	7.4	1	0.12%	2
	Subtotal	**71**	**8.48%**	**142**
BAPS 8		10	1.19%	11
TOTAL		**837**		**2402**

Next, we analyzed the congruence between the BAPS grouping of 492 STs using the BAPS assignment as described previously by Willems *et al* (27), and the BAPS grouping from this study. A correlation coefficient of 0.5958 indicates some discrepancies between the partitioning of the 492 STs. These discrepancies, probably related with the presence of an *E. faecalis* outgroup in Willems *et al* (27) BAPS analysis, are mostly due to STs that moved from the BAPS 2 (15 STs), BAPS 3 (25 STs), and BAPS 5 (1 ST) in the 2012 study to BAPS 7 in our study (see Fig. S1 in the supplemental material). Subsequently, the 492 *E. faecium* STs included in the work of Willems *et al* (27) were compared to BAPS grouping of the same 492 STs using the extended *E. faecium* MLST database of 837 STs in order to infer the influence of the sample size on BAPS assignment. The correlation coefficients analysis revealed almost perfect

correlations for classification of BAPS groups (0.9996) and BAPS subgroups (0.9988) based on 492 and 837 STs (see Fig. S2 and S3 in the supplemental material) and that only a small number of changes occurred (44/837 STs, 5.2%) in BAPS assignment, either at a group or subgroup level, when the number of STs analysed was significantly increased. This further indicates that, for *E. faecium*, BAPS analysis is both reproducible and robust and may accurately describe the *E. faecium* population structure.

Since the extended dataset of 837 STs slightly changed BAPS grouping of STs, we decided to recalculate ORs to assess significance between BAPS groups and the origin of isolates (see Table S3 in the supplemental material). This revealed that isolates from hospitalized individuals were positively associated with BAPS 2.1a and 3.3a and negatively associated with all other BAPS groups. Conversely, isolates of all BAPS groups from non-hospitalized individuals were negatively associated with BAPSs 2.1a and 3.3a but positively associated with BAPS 1.2 and BAPS 3.3b (Fig. 4).

Isolates of animal origin were negatively associated with BAPS 3.3a and BAPS 1.2 but showed a positive association with BAPSs 1.5, 2.1a, 2.1b, 2.3a, 2.3b, 3.1, 3.2, and 7.1 (Fig. 4; see also Table S3 in the supplemental material).

Genotypic relatedness of *E. faecium* colonizing different age groups. The 150 *E. faecium* isolates, obtained from 142 samples in this study, corresponded to 75 distinct STs. Forty-seven STs, representing 62.7% of the studied isolates, were STs firstly reported here (see Table S1 in the supplemental material). The remaining ones corresponded to globally spread STs like ST78, (n=34, 7 STs), ST17 (n=14, 1 ST), and ST18 (n=6, 1 ST), and also ST102 (n=20, 7 STs), ST22 (n=13, 9 STs), ST94 (n=12, 7 STs), ST9 (2, 2 STs) and ST5 (1 ST), which were previously detected among community based isolates (see Table S1 in the supplemental material). The 75 STs were partitioned into BAPS 1 (24 STs, 22.0% of isolates), BAPS 2 (19 STs, 31.3% of isolates), BAPS 3 (20 STs, 36.7% of isolates), BAPS 7 (8 STs, 7.3% of isolates), and BAPS 8 (3 STs, 2.7% of isolates) (Fig. 5).

STs classified as BAPS 1 mainly corresponded to subgroup 1.2 (n=27 [81.2%], 19 STs). The proportion of isolates with STs that group in BAPS 1 steadily decreased with age (Fig. 5 and 6), but isolates of this group were still prevalent among the adults of group II (15/27). All strains within BAPS 1 were ampicillin susceptible, mainly recovered from non-hospitalized persons (23/27, $P<0.01$).

Within BAPS 2, the subgroup 2.1a was predominant (70.2%, 33/47) and increasingly detected with age, constituting the leading group in elderly patients (Fig. 5 and 6). Most isolates were recovered in hospitals, exhibited ampicillin-resistance and harboured genes encoding adhesive surface protein (Esp) and collagen-adhesin (Acm) (27/33 and 31/33, respectively) that are associated with colonization and pathogenicity (see Table S1 in the supplemental material).

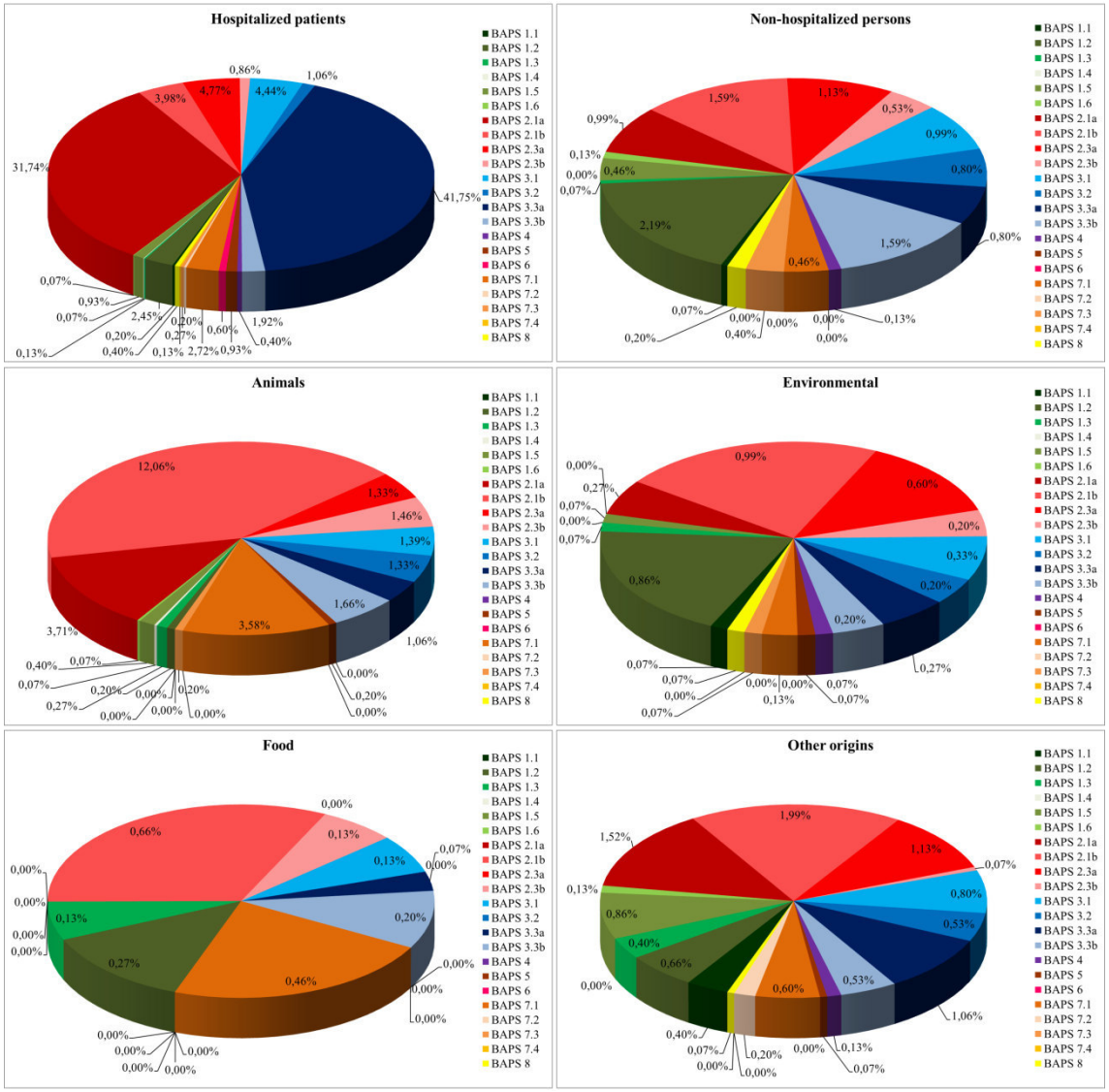

Figure 4. *E. faecium* BAPS group distribution by origin.

This distribution by origin included all isolates present in the *E. faecium* MLST database (http://efaecium.mlst.net/) on August 2013.

STs contained within BAPS 2.1a were ST117 (n=25), ST203 (n=4), ST80 (n=1), ST323 (n=1), ST324 (n=1) and ST612 (n=1). *E. faecium* ST117 (CEfm1) is, apart from being predominantly a colonizing clone, also frequently associated with severe infections in our institution (54). The other BAPS 2 subgroups, namely BAPS 2.3b (n=6 [12.8%], 5 STs), BAPS 2.3a (n=4 [8.5%], 4 STs) and BAPS 2.1b (n=4 [8.5%], 4 STs) were detected among both hospitalized and non-hospitalized individuals (Fig. 5 and 6).

BAPS 3 was represented by subgroups 3.1, 3.2, 3.3a and 3.3b. Most isolates in BAPS 3.1 (6 STs, 18.2% of isolates) were ampicillin-susceptible *E. faecium* (9/10) from non-hospitalized individuals (7/10, $P<0.01$) of age groups I and II (Fig. 5 and 6; see also Table S1 in the supplemental material). The subgroups BAPS 3.3a and BAPS 3.3b, previously described as BAPS 3.3 differed in the susceptibility to ampicillin. BAPS 3.3a (n=20 [36.4%] 14 ST17 and 6 ST18) comprised ampicillin-resistant *E. faecium* isolates (19/20, $P<0.01$) containing hyl_{Efm} (16/20) and predominantly recovered from hospitalized patients (16/20, $P<0.01$). Conversely, the BAPS 3.3b subgroup (11 STs 43.6% of isolates) was significantly associated with

ampicillin-susceptible *E. faecium* isolates (21/24 *P*<0.01), mostly from non-hospitalized persons (18/24, *P*<0.01).

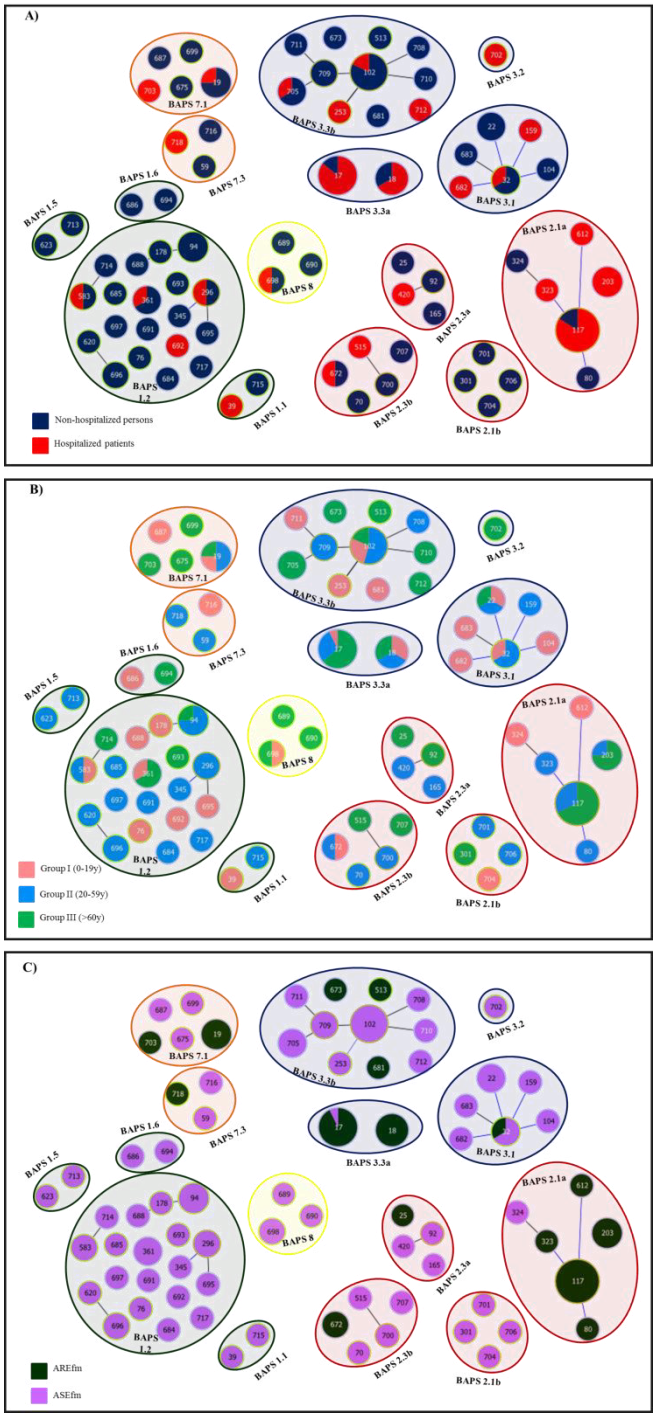

Figure 5. *E. faecium* colonization population structure.
A) by origin; B) by age group; C) by susceptibility to Ampicillin.

BAPS subgroup 7.1 is most predominant within BAPS 7 comprised of 5 STs, representing 8 isolates. Finally, 3 STs comprising BAPS 8 and representing four isolates (2 ST698, 1 ST689 and 1 ST690) were all ampicillin-susceptible *E. faecium* (of which 3 where *esp*$^{+}$) and were recovered from non-hospitalized persons (see Table S1 in the supplemental material).

Differences in the recovery rate of ampicillin resistant enterococci were noticed when samples were cultured without or with selective enrichment (56.3%, 36/64) but not for those resistant to vancomycin (100%, 5/5). Ampicillin-resistant *E. faecium* isolates that were only cultured after enrichment mostly belonged to BAPS 2.1a (n=14, 9 ST117, 3 ST203, 1 ST80 and 1 ST323) and BAPS 3.3a (n=8, 6 ST17 and 2 ST18), the majority of these isolates were recovered from hospitalized patients. Isolates of other BAPS groups were also found and are described in Table S1 in the supplemental material.

BAPS analysis of *E. faecalis* population structure. Previous studies based on MLST have suggested that recombination may play an important role in the diversification of *E. faecalis* (17, 20, 22). As methods to infer evolutionary descent are highly influenced by recombination, we analyzed the *E. faecalis* population structure using Bayesian-based population genetic modeling implemented in BAPS software in addition to goeBURST. The sample included 1,310 isolates corresponding to 523 STs available at public database (http://efaecalis.mlst.net/).

A maximum likelihood based phylogenetic reconstruction of STs using concatenated MLST gene sequences, placed ST80 far apart from all other STs. When this ST80 (amounting to only 1 isolate from the MLST database) was excluded from the analysis to better observe differences among tree features, practically all clades showed low bootstrap support, which supports previous analyses indicating that recombination may obscure the phylogenetic signal in nucleotide-based phylogenetic reconstructions in *E. faecalis*. A hierarchical BAPS clustering analysis subdivided the *E. faecalis* population into 5 BAPS groups (Fig. 7). Most of STs and isolates were distributed among BAPSs 1, 2 and 3 (44.7%, 27.5%, and 20.6%, respectively), while BAPSs 4 (1.0%) and 5 (6.1%) only represented a small fraction of the STs analyzed (see Table S4 in supplemental material).

ORs calculations revealed that isolates from hospitalized patients were not significantly associated with any of the BAPS groups, while BAPS 2 was positively associated with isolates from non-hospitalized persons (ORs=1.8507, $P<0.01$) and negatively associated with animal isolates (ORs=0.4659, $p<0.01$) (Fig. 7; see also Table S5 in supplemental material). Although signals of microevolutionary hospital specialization within the different BAPS groups were not found, some STs were enriched in isolates from hospitalized patients as ST6 (107/123), ST64 (12/18), ST9 (22/25), ST28 (16/17), ST87 (15/16), ST49 (4/4), ST88 (4/4) and ST159 (4/4). Furthermore, ST58 (8/8), ST82 (25/27) and ST174 (11/11) were frequently found in isolates from animals. We also analysed traces of significant admixture in the *E. faecalis* population as recombination is the driving force of admixture dynamics and it might influence the evolvability of specific amplified lineages. Admixture was significantly present in some STs from animal and community-based hosts. However, additional analyses revealed that admixture was not significantly found in STs that are unique or shared between hosts, STs from hospital or non-hospital origin, STs from human and non-human origin or STs that represent antibiotic resistant isolates (data not shown). The combination of these results suggests that the majority of *E. faecalis* seems to belong to one single recombining population that exchanges alleles regardless of the genetic background (BAPS

groups), ecological origin (isolation source; hospital or non-hospital; human or non-human) or antibiotic-resistance phenotype.

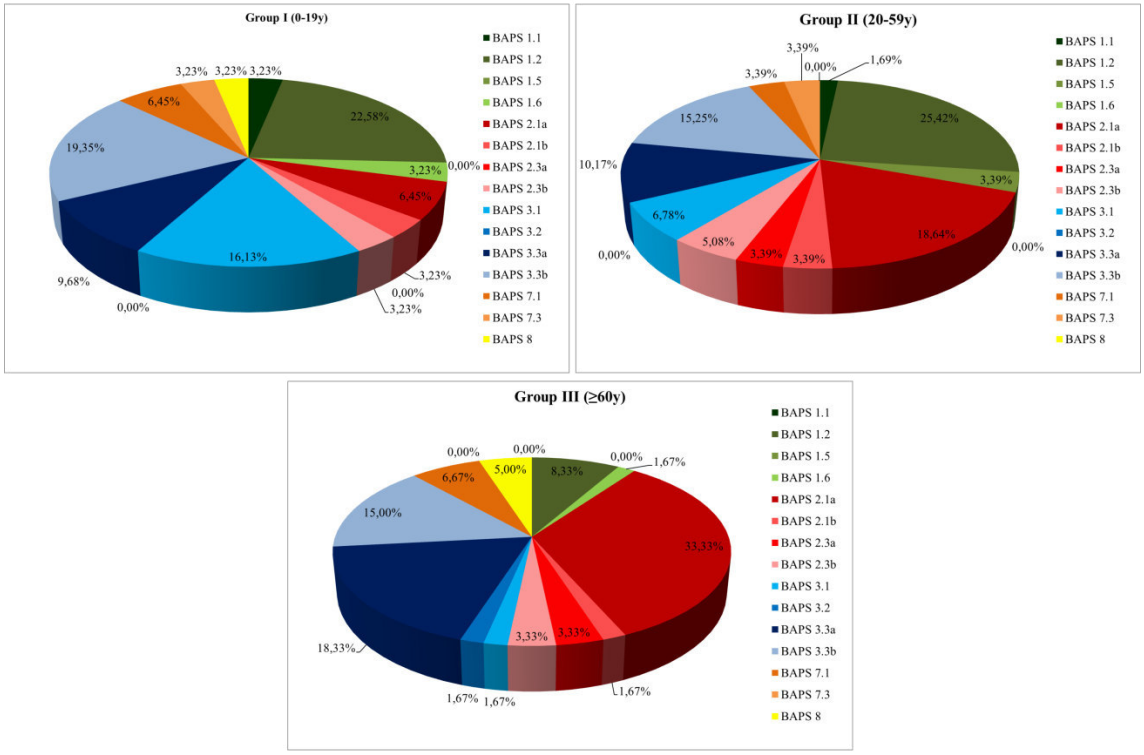

Figure 6. *E. faecium* BAPS distribution by age group in human colonization.

The influence of the sample size (and therefore the underlying diversity) in the accuracy of BAPS for establishing the *E. faecalis* population structure was assessed using two datasets (see Fig. S4 in supplemental material). The first dataset consisted of 433 STs available at MLST database (http://efaecalis.mlst.net/) before including the new *E. faecalis* STs found in this study. The second dataset included 523 STs available in the MLST database (end 2013). In both analyses ST80 was excluded. The negative correlation coefficient of 0.6439 obtained when comparing ST assignments to BAPS groups of the 433 ST and 523 ST set, is due to the split of BAPS groups 1 and 2 and the existence of three more BAPS groups when using the second larger dataset (see Fig. S4 in supplemental material). These results indicate that in *E. faecalis,* BAPS analysis is highly influenced by the sample size, as larger samples contain a representation of a higher diversity of strains of different spatial-temporal origins.

Genotypic relatedness of *E. faecalis* colonizing different age groups. The 130 *E. faecalis* isolates identified in this study represented 58 STs (see Table S2 in supplemental material) that were partitioned into *E. faecalis* groups BAPS 1 (36.9%), BAPS 2 (40.0%) and BAPS 3 (23.1%). ORs calculations revealed that none of the three BAPS groups were significantly associated with a particular source or age group as all the BAPS groups contained isolates from both hospitalized and non-hospitalized individuals of all ages in more or less equal numbers (Fig. 8).

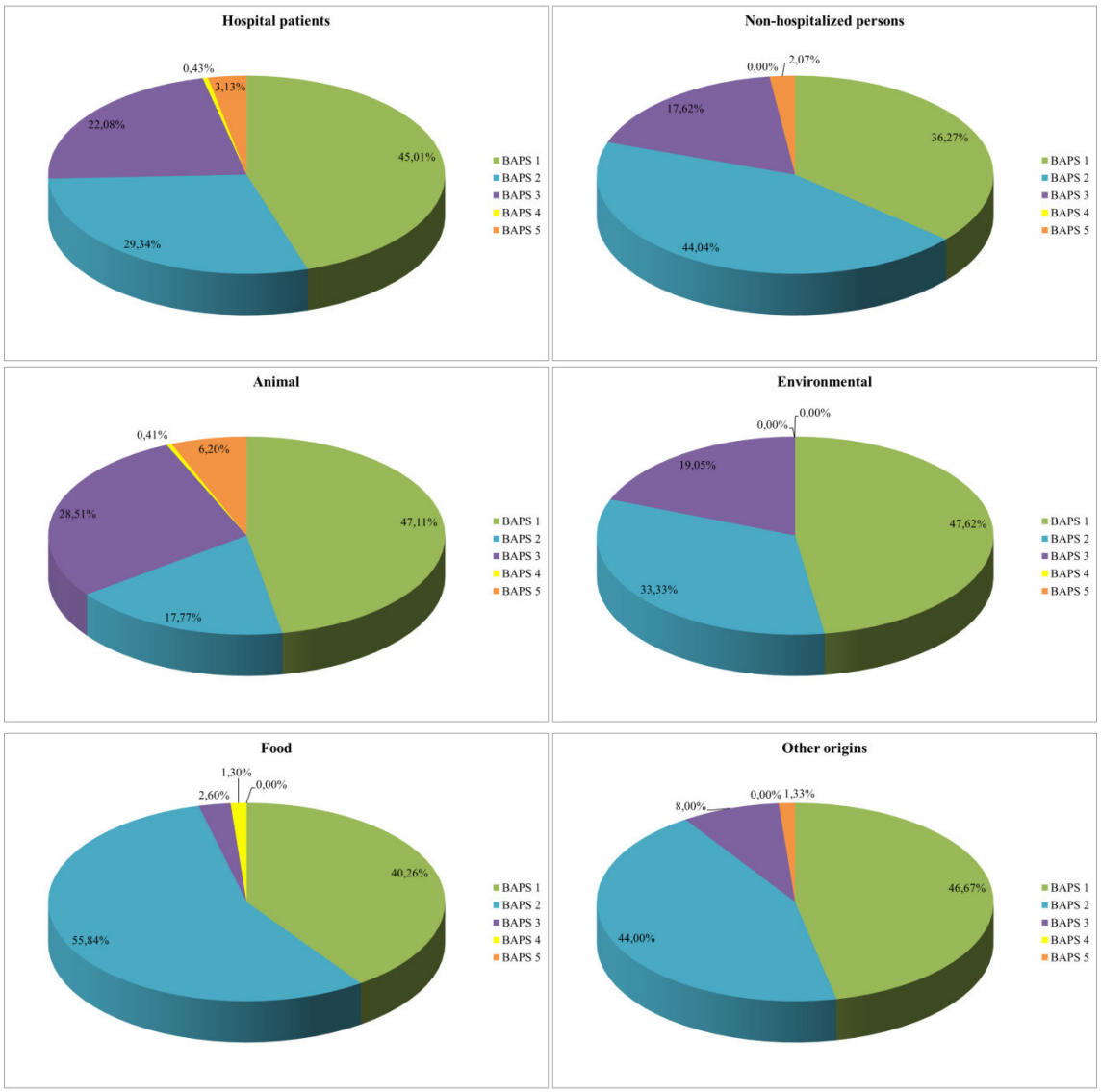

Figure 7. *E. faecalis* BAPS group distribution by origin.

This distribution by origin included all isolates present in the *E. faecalis* MLST database (http://efaecalis.mlst.net/) on August 2013.

Within BAPS 1 (n=48/130 [36.9%], 20 STs), ST6 (n=16) was predominant and mainly comprised isolates from hospitalized patients (13/16) and elderly (11/13) (Fig. 8; see also Table S2 in supplemental material). All were multi-drug resistant (MDR) showing resistance to high levels of gentamicin or streptomycin, and also to erythromycin (100% of isolates), tetracycline (93.8%, 15/16) and levofloxacin (87.5%, 14/16) and exhibiting a highly similar PFGE-profile (ST6-H10 profile) identical to the widespread international Mid-Atlantic clone, which also causes bacteraemia infections in our hospital (55). The 5 VREfc isolates (*vanA*, data not shown) found in this study are also ST6-H10. Putative virulence factors *asa1* (100%) and *gelE* (81.3%) were identified in most ST6 isolates while *cylA* (56.3%) and *esp* (37.5%) were less frequent. Other STs were represented by a very few number of isolates, usually susceptible to antibiotics and with a highly variable presence of virulence factors.

Within BAPS 2 (n=52/130 [40.0%], 26 STs), ST40 isolates (n=15) were predominant. These isolates were recovered from both non-hospitalized and hospitalized individuals of different ages that often

harboured *gelE* (88.2%) and less frequently *asa1* (41.2%) and *esp* (47.1%) and were resistant to tetracycline (70.6%) and erythromycin (47.1%). Similarly to BAPS 1, other STs were represented by single or very few isolates that often contain *esp* (Table S2). Among them were STs that were identified over several decades as ST55, ST30 or ST19 (31, 33).

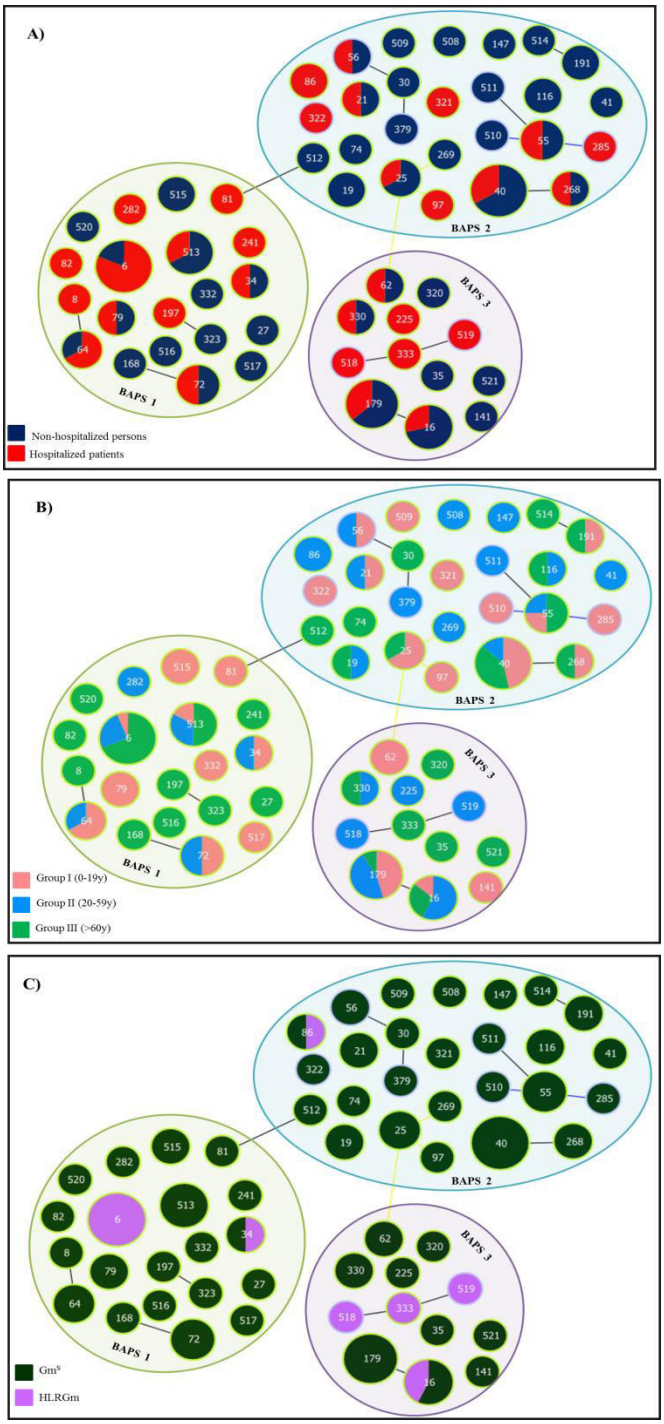

Figure 8. *E. faecalis* colonization population structure: A) by origin; B) by age group; C) by susceptibility to gentamicin.

Finally, BAPS 3 (n=30/130, 12 STs) was predominantly comprised of ST16 and ST179, previously classified as CC16 by goeBURST (7 ST16 and 11 ST179). These STs also included isolates from both non-

hospitalized and hospitalized individuals of different ages that often harboured *asa1* (99.4%), *esp* (77.8%) or *gelE* (61.1%), often resistant to different antibiotics (see Table S2 in supplemental material). Other STs classified before as CC28 by goeBURST (1 ST333, 1 ST518 and 1 ST519), were recovered from adults or elderly hospitalized patients were also enriched in putative virulence factors (all harboured *asa1, gelE*, *esp* and *cylA*) and were also MDR (all showing high level of resistance to gentamicin and streptomycin, tetracycline and erythromycin and levofloxacin) (see Table S2 in supplemental material).

DISCUSSION

This study describes a consistent high recovery rate of enterococci in human faeces, both in hospitalized and non-hospitalized individuals and different age groups, similar to that reported in other studies, that ranges from 71% to 80% (1, 56, 57). These equilibrated constant rates of colonization indicate a major resiliency for the genus *Enterococcus* along heterogeneous conditions imposed by age, changing environments, and highly variable host niches. Previous studies (6, 58–60) have described changes in the recovery rates of the genus *Enterococcus* in faecal microbiota with ageing, which was not confirmed in our work, and a consistent predominance of the species *E. faecalis* in the fecal flora of young individuals and elderly, which is essentially consistent with our findings, with the important exception of the growing predominance of *E. faecium* in elderly particularly in hospitalized patients. Other studies yielded contradictory information about the frequency and diversity of *E. faecium* and other enterococcal species in faecal microbiota (1, 15). Shifts in the prevalence of *Enterococcus* populations might result from fluctuating changes in the environmental conditions over time as diet (10), health status or antibiotic treatment (1, 5, 61–64), all of them delineating particular selective landscapes in hospitals (58, 61). Aging interacts with these conditions, and age dependent enterococcal colonization dynamics has also been demonstrated for chicken and calves (1, 65), probably in interaction with antibiotic consumption (1, 66, 67).

Considering the currently available diversity of known genotypes, the superimposing of goeBURST analysis of clonal relationship among multiple isolates with BAPS allowed the detection of a low number of presumptive evolutionary and functionally heterogeneous clades for the *E. faecium* species (21, 25–27). BAPS 1 *E. faecium*, associated with the “clade B” phylogenetic lineage (a clade with pathways of complex carbohydrate utilization linked to host diet and with a majority of ASEfm strains) was highly represented in the different age groups although its incidence was slightly reduced in the elderly (26, 27). Conversely, BAPS 2.1a and BAPS 3.3a subgroups (containing most of the AREfm strains), associated with the “clade A1” (26, 27) and mostly found in elderly hospitalized patients, represent *E. faecium* strains that are spreading in hospitals and causing clinical infections. The rates of these populations in the nosocomial and the community settings might be underestimated, as we have demonstrated here that if you do not pre-enrich the sample some of the even more widespread clones might escape screening, probably due to low colonization densities. The observed population structure of *E. faecium* indicates a certain specialization of subpopulations in colonization of particular age-groups which are usually associated with several other host associated factors, and also differences in harboured

selectable characters, as antibiotic resistance genes. Interestingly, some groups evolve independently from the acquisition of ampicillin resistance, suggesting a certain genetic isolation as seems to be the case of different lineages within BAPS 3.3b, BAPS 1 and BAPS 2. These results further confirm a population structure comprised of ecotypes representing specialization in different hosts (16, 68).

E. faecalis populations showed a considerable level of genetic diversity. Because of that, and in contrast with *E. faecium*, no BAPS groups were significantly associated with ageing, hospital exposure, or host species and, with the exception of BAPS 2, showed positive association with non-hospitalized individuals. The wide recovery of certain STs (e.g. ST6, ST16, ST40 or ST55) able to colonize hospitalized and non-hospitalized humans (this study) and also animals (30, 31, 69), may be related with the more generalist lifestyle of this enterococcal species that weakens the possibility of recognition of ecotypes associated with a particular environment, at least, by using the same approach that was so useful with *E. faecium*. However, despite possible limitations in the available methods analysing the *E. faecalis* population structure, it is now clear that certain multihost *E. faecalis* subpopulations as ST6 or ST16 have developed different strategies of adaptation to harsh and fluctuating habitats (31, 33). Among them, the lack of CRISPR loci (Cluster Regulatory Interspaced Short Palindromic Repeats, a bacterial defense system against foreign DNA that facilitates the acquisition of foreign DNA as antibiotic resistance and virulence genes) (70), and the frequent acquisition of phages (71).

Other enterococcal species have been largely recognized as part of human faecal microbiota's and this is confirmed in our study (1). The inverse parallel trends in the population frequencies of these species and that of *E. faecium* is of particular interest. Dynamics of colonization by these might reflect differences in the functional requirements of the host with the age and deserves further analysis.

This study provides a novel, integrated and comprehensive image of the landscape of *Enterococcus* populations in a balanced amount of non-hospital and hospital-based individuals of different ages, and suggests that a number of enterococcal lineages might be predominant in certain age groups and/or hospital environment. However, a number of clones are spread in different types of individuals, and its prevalence is reduced in others, in a kind of source-sink dynamics (72–74), with frequent cases of coexistence, and preservation of rare clonal populations. That suggests a frequency-dependent evolution of enterococcal populations, which prevents the extinction of different genotypes playing not equivalent ecological roles (75–78).

The work also illustrates a high plasticity of *E. faecium* and *E. faecalis* genomes reflected by admixture analysis (27; this study), with variable intra-clonal PFGE patterns (31, 69) (see Tables S1 and S2 in the supplemental material), and recombination of large fragments of the chromosome (79–83, our unpublished results). The consequences of such large variability has been scarcely explored on a population-based perspective. However, it can be expected that genome plasticity would contribute to variation and selection of genes from a common genetic intra-species pool, needed for the adaptation to environments imposing different stress conditions. Future progresses in understanding enterococcal

population biology requires global analysis combining many ecological features, population dynamics, and population genetics (78, 84, 85).

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REFERENCES

1. Lebreton F, Willems RJL, Gilmore MS, Lebreton, Francois; Willems RJL. 2014. *Enterococcus Diversity, Origins in Nature, and Gut Colonization*. *In* Gilmore, MS, Clewell, DB, Ike, Y, Shankar, N (eds.), *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary, Boston: Massachusetts Eye and Ear Infirmary.

2. Arias CA, Murray BE. 2012. *The rise of the Enterococcus: beyond vancomycin resistance*. Nat. Rev. Microbiol. 10:266–78.

3. Taur Y, Xavier JB, Lipuma L, Ubeda C, Goldberg J, Gobourne A, Lee YJ, Dubin KA, Socci ND, Viale A, Perales M-A, Jenq RR, van den Brink MRM, Pamer EG. 2012. *Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation*. Clin. Infect. Dis. 55:905–14.

4. Bonten MJ, Willems R, Weinstein RA. 2001. *Vancomycin-resistant enterococci: why are they here, and where do they come from?* Lancet Infect. Dis. 1:314–25.

5. Donskey CJ. 2004. *The role of the intestinal tract as a reservoir and source for transmission of nosocomial pathogens*. Clin. Infect. Dis. 39:219–26.

6. Woodmansey EJ, McMurdo MET, Macfarlane GT, Macfarlane S. 2004. *Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects*. Appl. Environ. Microbiol. 70:6113–22.

7. Enck P, Zimmermann K, Rusch K, Schwiertz A, Klosterhalfen S, Frick J-S. 2009. *The effects of ageing on the colonic bacterial microflora in adults*. Z. Gastroenterol. 47:653–8.

8. Tiihonen K, Ouwehand AC, Rautonen N. 2010. *Human intestinal microbiota and healthy ageing*. Ageing Res. Rev. 9:107–16.

9. Petersen C, Round JL. 2014. *Defining dysbiosis and its influence on host immunity and disease*. Cell. Microbiol. 16:1024–33.

10. Firmesse O, Rabot S, Bermúdez-Humarán LG, Corthier G, Furet J-P. 2007. *Consumption of Camembert cheese stimulates commensal enterococci in healthy human intestinal microbiota*. FEMS Microbiol. Lett. 276:189–92.

11. Medini D, Donati C, Tettelin H, Masignani V, Rappuoli R. 2005. *The microbial pan-genome*. Curr. Opin. Genet. Dev. 15:589–94.

12. Dobrindt U, Hacker J. 2001. *Whole genome plasticity in pathogenic bacteria*. Curr. Opin. Microbiol. 4:550–7.

13. Finegold SM, Sutter VL, Methisen GE. 1983. *Normal Indegenous Intestinal flora*, p. 3–32. *In* Hentges, D (ed.), *Human intestinal microflora in health and disease*. Academic Press, New York.

14. Tannock G.W. and Cook G. 2002. *Enterococci as members of the intestinal microflora of humans*, p. 101–132. *In* Gilmore, MS (ed.), *The Enterococci: Pathogenesis, Molecular Biology and Antibiotic Resistance*. ASM Press, Whashington DC.

15. Silva N, Igrejas G, Gonçalves A, Poeta P. 2011. *Commensal gut bacteria: distribution of Enterococcus species and prevalence of Escherichia coli phylogenetic groups in animals and humans in Portugal*. Ann. Microbiol. 62:449–459.

16. Homan WL, Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, Van Embden JDA, Willems RJL. 2002. *Multilocus sequence typing scheme for Enterococcus faecium*. J. Clin. Microbiol. 40:1963–71.

17. Ruiz-Garbajosa P, Bonten MJM, Robinson DA, Top J, Nallapareddy SR, Torres C, Coque TM, Cantón R, Baquero F, Murray BE, del Campo R, Willems RJL. 2006. *Multilocus sequence typing scheme for Enterococcus faecalis reveals hospital-adapted genetic complexes in a background of high rates of recombination*. J. Clin. Microbiol. 44:2220–8.

18. Leavis HL, Willems RJL, van Wamel WJB, Schuren FH, Caspers MPM, Bonten MJM. 2007. *Insertion sequence-driven diversification creates a globally dispersed emerging multiresistant subspecies of E. faecium*. PLoS Pathog. 3:e7.

19. Van Schaik W, Willems RJL. 2010. *Genome-based insights into the evolution of enterococci*. Clin. Microbiol. Infect. 16:527–32.

20. Nallapareddy SR, Duh R-W, Singh K V, Murray BE. 2002. *Molecular typing of selected Enterococcus faecalis isolates: pilot study using multilocus sequence typing and pulsed-field gel electrophoresis*. J. Clin. Microbiol. 40:868–76.

21. Galloway-Peña J, Roh JH, Latorre M, Qin X, Murray BE. 2012. *Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of Enterococcus faecium*. PLoS One 7:e30187.

22. Nallapareddy SR, Wenxiang H, Weinstock GM, Murray BE. 2005. *Molecular characterization of a widespread, pathogenic, and antibiotic resistance-receptive Enterococcus faecalis lineage and dissemination of its putative pathogenicity island*. J. Bacteriol. 187:5709–18.

23. Qin X, Galloway-Peña JR, Sillanpaa J, Roh JH, Nallapareddy SR, Chowdhury S, Bourgogne A, Choudhury T, Muzny DM, Buhay CJ, Ding Y, Dugan-Rocha S, Liu W, Kovar C, Sodergren E, Highlander S, Petrosino JF, Worley KC, Gibbs RA, Weinstock GM, Murray BE. 2012. *Complete genome sequence of Enterococcus faecium strain TX16 and comparative genomic analysis of Enterococcus faecium genomes*. BMC Microbiol. 12:135.

24. Palmer KL, Godfrey P, Griggs A, Kos VN, Zucker J, Desjardins C, Cerqueira G, Gevers D, Walker S, Wortman J, Feldgarden M, Haas B, Birren B, Gilmore MS. 2012. *Comparative genomics of enterococci: variation in Enterococcus faecalis, clade structure in E. faecium, and defining characteristics of E. gallinarum and E. casseliflavus.* MBio 3:e00318–11.

25. Howden BP, Holt KE, Lam MMC, Seemann T, Ballard S, Coombs GW, Tong SYC, Grayson ML, Johnson PDR, Stinear TP. 2013. *Genomic insights to control the emergence of vancomycin-resistant enterococci*. MBio 4: e00412-13.

26. Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems RJL, Earl AM, Gilmore MS. 2013. *Emergence of epidemic multi-drug-resistant Enterococcus faecium from animal and commensal strains*. MBio 4: e00534-13.

27. Willems RJL, Top J, van Schaik W, Leavis H, Bonten M, Sirén J, Hanage WP, Corander J. 2012. *Restricted gene flow among hospital subpopulations of Enterococcus faecium*. MBio 3:e00151–12.

28. Ruiz-Garbajosa P, Cantón R, Pintado V, Coque TM, Willems R, Baquero F, del Campo R. 2006. *Genetic and phenotypic differences among Enterococcus faecalis clones from intestinal colonisation and invasive disease*. Clin. Microbiol. Infect. 12:1193–8.

29. Waar K, Muscholl-Silberhorn AB, Willems RJL, Slooff MJH, Harmsen HJM, Degener JE. 2002. *Genogrouping and incidence of virulence factors of Enterococcus faecalis in liver transplant patients differ from blood culture and fecal isolates*. J. Infect. Dis. 185:1121–7.

30. Kuch A, Willems RJL, Werner G, Coque TM, Hammerum AM, Sundsfjord A, Klare I, Ruiz-Garbajosa P, Simonsen GS, van Luit-Asbroek M, Hryniewicz W, Sadowy E. 2012. *Insight into antimicrobial susceptibility and population structure of contemporary human Enterococcus faecalis isolates from Europe*. J. Antimicrob. Chemother. 67:551–8.

31. Freitas AR, Novais C, Ruiz-Garbajosa P, Coque TM, Peixe L. 2009. *Clonal expansion within clonal complex 2 and spread of vancomycin-resistant plasmids among different genetic lineages of Enterococcus faecalis from Portugal*. J. Antimicrob. Chemother. 63:1104–11.

32. Kawalec M, Pietras Z, Daniłowicz E, Jakubczak A, Gniadkowski M, Hryniewicz W, Willems RJL. 2007. *Clonal structure of Enterococcus faecalis isolated from Polish hospitals: characterization of epidemic clones*. J. Clin. Microbiol. 45:147–53.

33. McBride SM, Fischetti VA, Leblanc DJ, Moellering RC, Gilmore MS. 2007. *Genetic diversity among Enterococcus faecalis*. PLoS One 2:e582.

34. Willems RJL, Top J, van Santen M, Robinson DA, Coque TM, Baquero F, Grundmann H, Bonten MJM. 2005. *Global spread of vancomycin-resistant Enterococcus faecium from distinct nosocomial genetic complex*. Emerg. Infect. Dis. 11:821–8.

35. De Been M, van Schaik W, Cheng L, Corander J, Willems RJ. 2013. *Recent recombination events in the core genome are associated with adaptive evolution in Enterococcus faecium*. Genome Biol. Evol. 5:1524–35.

36. Safdar N, Maki DG. 2002. *The commonality of risk factors for nosocomial colonization and infection with antimicrobial-resistant Staphylococcus aureus, enterococcus, gram-negative bacilli, Clostridium difficile, and Candida*. Ann. Intern. Med. 136:834–44.

37. Willems RJL, Hanage WP, Bessen DE, Feil EJ. 2011. *Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance*. FEMS Microbiol. Rev. 35:872–900.

38. Leavis HL, Bonten MJM, Willems RJL. 2006. *Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance*. Curr. Opin. Microbiol. 9:454–60.

39. Chowdhury SA, Nallapareddy SR, Arias CA, Murray BE. 2014. *The majority of a collection of U.S. endocarditis Enterococcus faecalis isolates obtained from 1974 to 2004 lack capsular genes and belong to diverse, non-hospital-associated lineages*. J. Clin. Microbiol. 52:549–56.

40. Galloway-Peña JR, Nallapareddy SR, Arias CA, Eliopoulos GM, Murray BE. 2009. *Analysis of clonality and antibiotic resistance among early clinical isolates of Enterococcus faecium in the United States*. J. Infect. Dis. 200:1566–73.

41. Butler KM. 2006*. Enterococcal infection in children*. Semin. Pediatr. Infect. Dis. 17:128–39.

42. Coque TM, Murray BE. 1995*. Identification of Enterococcus faecalis strains by DNA hybridization and pulsed-field gel electrophoresis*. J. Clin. Microbiol. 33:3368–9.

43. Depardieu F, Perichon B, Courvalin P. 2004. *Detection of the van alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR*. J. Clin. Microbiol. 42:5857–60.

44. CLSI. 2013. *Performance Standards for Antimicrobial Susceptibility Testing*; Twenty-Second Informational Supplement. Clin. Lab. Stand. Inst. 32:1–184.

45. Nallapareddy SR, Singh K V, Okhuysen PC, Murray BE. 2008. *A functional collagen adhesin gene, acm, in clinical isolates of Enterococcus faecium correlates with the recent success of this emerging nosocomial pathogen*. Infect. Immun. 76:4110–9.

46. Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, Jabes D, Goossens H. 2004. *Development of a multiplex PCR for the detection of asa1, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among European hospital isolates of Enterococcus faecium*. J. Clin. Microbiol. 42:4473–9.

47. Coque TM, Willems RJL, Fortún J, Top J, Diz S, Loza E, Cantón R, Baquero F. 2005. *Population structure of Enterococcus faecium causing bacteremia in a Spanish university hospital: setting the scene for a future increase in vancomycin resistance*? Antimicrob. Agents Chemother. 49:2693–700.

48. Francisco AP, Vaz C, Monteiro PT, Melo-Cristino J, Ramirez M, Carriço JA. 2012. *PHYLOViZ: phylogenetic inference and data visualization for sequence based typing methods*. BMC Bioinformatics 13:87.

49. Francisco AP, Bugalho M, Ramirez M, Carriço JA. 2009. *Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach*. BMC Bioinformatics 10:152.

50. Corander J, Marttinen P, Sirén J, Tang J. 2008. *Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations*. BMC Bioinformatics 9:539.

51. Hardin JW, Hilbe JH. 2002. *Generalized Estimating Equations*. Chapman and Hall/CRC, London.

52. Facklam RR, Carvalho MG, Teixeira LM. 2002. *Enterococcus*, p. 1–54. *In* Gilmore, MS, Clewell, DB, Courvalin, P, Dunny, GM, Rice, LB (eds.*), The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance*. ASM Press, Washington, DC.

53. Turner KME, Hanage WP, Fraser C, Connor TR, Spratt BG. 2007. *Assessing the reliability of eBURST using simulated populations with known ancestry*. BMC Microbiol. 7:30.

54. Tedim AP, Ruiz-Garbajosa P, Rodriguez CM, Derdoy L, Cardenas G, Loza E, Cantón R, Baquero F, Coque TM. 2014. *Evolution of healthcare bacteraemic episodes of Enterococcus faecium (1995-2012) reflects changes in phylogenomic groups with internal clonal epidemic waves24th European Congress of Clinical Microbiology and Infectious Diseases*. European Society of Clinical Microbiology and Infectious Diseases, Barcelona.

55. Tedim AP, Miranda AC, Ruiz-Garbajosa P, Freitas AR, Francia MV, Baquero F, Coque TM. 2010. *Plasmid Diversity among Enterococcus faecalis clinical isolates from Spain (2001-2009)3rd International ASM Conference on Enterococci*. American Society for Microbiology, Portland, Oregon.

56. Mundt JO. 1963. *Occurrence of enterococci in animals in a wild environment*. Appl. Microbiol. 11:136–40.

57. Coque TM, Arduino RC, Murray BE. 1995. *High-level resistance to aminoglycosides: comparison of community and nosocomial fecal isolates of enterococci*. Clin. Infect. Dis. 20:1048–51.

58. Woodmansey EJ. 2007. *Intestinal bacteria and ageing*. J. Appl. Microbiol. 102:1178–86.

59. Stewart CJ, Marrs ECL, Magorrian S, Nelson A, Lanyon C, Perry JD, Embleton ND, Cummings SP, Berrington JE. 2012. *The preterm gut microbiota: changes associated with necrotizing enterocolitis and infection*. Acta Paediatr. 101:1121–7.

60. Kirtzalidou EI, Mitsou EK, Pramateftaki P, Kyriacou A. 2012. *Screening fecal enterococci from Greek healthy infants for susceptibility to antimicrobial agent*s. Microb. Drug Resist. 18:578–85.

61. Ubeda C, Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, Viale A, Socci ND, van den Brink MRM, Kamboj M, Pamer EG. 2010. *Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans*. J. Clin. Invest. 120:4332–41.

62. Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, Fleisher M, Schnabl B, DeMatteo RP, Pamer EG. 2008. *Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits*. Nature 455:804–7.

63. Berg RD. 1996. The indigenous gastrointestinal microflora. Trends Microbiol. 4:430–5.

64. O'Sullivan O, Coakley M, Lakshminarayanan B, Conde S, Claesson MJ, Cusack S, Fitzgerald AP, O'Toole PW, Stanton C, Ross RP. 2013. *Alterations in intestinal microbiota of elderly Irish subjects post-antibiotic therapy*. J. Antimicrob. Chemother. 68:214–21.

65. Devriese LA, Laurier L, De Herdt P, Haesebrouck F. 1992. *Enterococcal and streptococcal species isolated from faeces of calves, young cattle and dairy cows*. J. Appl. Bacteriol. 72:29–31.

66. Aarestrup FM, Butaye P, Witte W. 2002. *Non-human reservoirs of enterococci, p. 55–99. In Enterococci : Pathogenesis, Molecular Biology, and Antibiotic Resistance*. American Society for Microbiology, Washington, DC, USA.

67. Devriese LA, Pot B, Collins MD. 1993. *Phenotypic identification of the genus Enterococcus and differentiation of phylogenetically distinct enterococcal species and species groups*. J. Appl. Bacteriol. 75:399–408.

68. Willems RJ, Top J, van Den Braak N, van Belkum A, Endtz H, Mevius D, Stobberingh E, van Den Bogaard A, van Embden JD. 2000. *Host specificity of vancomycin-resistant Enterococcus faecium*. J. Infect. Dis. 182:816–23.

69. Freitas AR, Coque TM, Novais C, Hammerum AM, Lester CH, Zervos MJ, Donabedian S, Jensen LB, Francia MV, Baquero F, Peixe L. 2011. *Human and swine hosts share vancomycin-resistant Enterococcus faecium CC17 and CC5 and Enterococcus faecalis CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids*. J. Clin. Microbiol. 49:925–31.

70. Palmer KL, Gilmore MS. 2010. *Multi-drug-resistant enterococci lack CRISPR-cas*. MBio 1: e00227-10.

71. Duerkop BA, Clements C V, Rollins D, Rodrigues JLM, Hooper L V. 2012. *A composite bacteriophage alters colonization by an intestinal commensal bacterium*. Proc. Natl. Acad. Sci. U. S. A. 109:17621–6.

72. Pulliam H. 1988. *Sources, sinks, and population regulation*. Am. Nat. 132:652–611.

73. Perron GG, Gonzalez A, Buckling A. 2007. *Source-sink dynamics shape the evolution of antibiotic resistance and its pleiotropic fitness cost*. Proc. Biol. Sci. 274:2351–6.

74. Hermsen R, Hwa T. 2010. *Sources and sinks: a stochastic model of evolution in heterogeneous environments*. Phys. Rev. Lett. 105:248104.

75. Levin BR. 1988. *Frequency-dependent selection in bacterial populations*. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 319:459–72.

76. Dugatkin LA, Perlin M, Lucas JS, Atlas R. 2005. *Group-beneficial traits, frequency-dependent selection and genotypic diversity: an antibiotic resistance paradigm*. Proc. Biol. Sci. 272:79–83.

77. Gonzalez A, Ronce O, Ferriere R, Hochberg ME. 2013. *Evolutionary rescue: an emerging focus at the intersection between ecology and evolution*. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 368:20120404.

78. Cordero OX, Polz MF. 2014. *Explaining microbial genomic diversity in light of evolutionary ecology*. Nat. Rev. Microbiol. 12:263–73.

79. Quintiliani R, Courvalin P. 1994. *Conjugal transfer of the vancomycin resistance determinant vanB between enterococci involves the movement of large genetic elements from chromosome to chromosome*. FEMS Microbiol. Lett. 119:359–63.

80. Laverde Gomez J a, Hendrickx AP a, Willems RJ, Top J, Sava I, Huebner J, Witte W, Werner G. 2011. *Intra- and interspecies genomic transfer of the Enterococcus faecalis pathogenicity island*. PLoS One 6:e16720.

81. Manson JM, Hancock LE, Gilmore MS. 2010. *Mechanism of chromosomal transfer of Enterococcus faecalis pathogenicity island, capsule, antimicrobial resistance, and other traits*. Proc. Natl. Acad. Sci. U. S. A. 107:12269–74.

82. De Regt MJA, van Schaik W, van Luit-Asbroek M, Dekker HAT, van Duijkeren E, Koning CJM, Bonten MJM, Willems RJL. 2012. *Hospital and community ampicillin-resistant Enterococcus faecium are evolutionarily closely linked but have diversified through niche adaptation*. PLoS One 7:e30319.

83. Novais C, Escada R, Freitas AR, Silveira E, Coque TM, Peixe L. 2011. *High incidence of horizontal transfer of ampicillin resistance (pbp5) among CC17 Enterococcus faecium*, p. S129. In 21st European Congress of Clinical Microbiology and Infectious Diseases. Milan, Italy.

84. Luck GW, Daily GC, Ehrlich PR. 2003. *Population diversity and ecosystem services*. Trends Ecol. Evol. 18:331–336.

85. Urban MC, Skelly DK. 2006. *Evolving metacommunities: toward an evolutionary perspective on metacommunities*. Ecology 87:1616–26.

Table S1. *E. faecium* isolates: epidemiological data.

BAPS		ST		PFGE-type (No)	m	Year	Age Group (No)			Ward (No)		Origin	VF (No isolates)			Anbiotic resistance (No isolates)							
Group	Subgroup (No)	ST (No)*	No PFGE		(No)		I	II	III	Name	Type	(No)	*esp*	hyl_{Efm}	*acm*	ERY	VAN	TEI	CIP	LEV[l]	STR[l]	GEN	TET	CHL
BAPS 1	1.1 (2)	**39** (1)	1	ASEfm57	-	2009	1			P	M	H	+	-	+	R	S	S	R	ND	ND	S	S	S
		715 (1)	1	ASEfm46	-	2009		1		ER	O	NH	-	-	-	R	S	S	R	ND	ND	S	S	S
	1.2 (27)	**94** (4)	4	ASEfm11-14	-	2009-2010		3	1	PHC	O	NH	+ (2)	-	+ (3)	R	S	S	S	ND	ND	S	R (1)	S
		361 (3)	2[a]	ASEfm30(2) - 31	-	2009	1		2	ER; ID; PHC	O (2); M	NH (2); H	-	-	+	R	S	S	S	ND	ND	S	R (1)	S
		178 (1)	1	ASEfm23	-	2009	1			PHC	O	NH	+	-	+	S	S	S	S	ND	ND	S	S	S
		688 (1)	1	ASEfm15	-	2009	1			PHC	O	NH	-	-	+	R	S	S	S	ND	ND	S	S	S
		691 (1)	1	ASEfm19	-	2009		1		PHC	O	NH	+	-	+	R	S	S	S	ND	ND	S	S	S
		296 (2)	2[b]	ASEfm 21, ASEfm63	-	2009		2		NE; PHC	O; M	NH; H	+ (1)	-	+ (1)	R	S	S	S	ND	ND	S	S	S
		345 (1)	1	ASEfm29	-	2009		1		PHC	O	NH	+	-	+	R	S	S	S	ND	ND	S	S	S
		695 (1)	1	ASEfm22	-	2009	1			PHC	O	NH	-	-	+	R	S	S	S	ND	ND	S	R	S
		583 (2)	2[c]	ASEfm44; ASEfm64	-	2009-2011	1	1		PHC	O	NH; H	-	-	+ (1)	R	S	S	S	ND	ND	S	S	S
		714 (1)	1[c]	ASEfm44	-	2009			1	ER	O	NH	-	-	+	R	S	S	S	ND	ND	S	S	S
		696 (2)	1[d]	ASEfm26	-	2009		2		PHC	O	NH	+ (1)	-	+	R	S	S	S	ND	ND	S	S	S
		620 (1)	1	ASEfm17	-	2009		1		PHC	O	NH	-	-	+	R	S	S	S	ND	ND	S	S	S
		76 (1)	1	ASEfm10	-	2010	1			P	O	NH	+	-	+	S	S	S	S	ND	ND	S	S	S
		684 (1)	1	ASEfm52	-	2009		1		PHC	O	NH	+	-	+	R	S	S	S	ND	ND	S	S	S
		685 (1)	1	ASEfm9	-	2009		1		PHC	O	NH	-	-	-	R	S	S	S	ND	ND	S	S	S
		692 (1)	1	ASEfm20	-	2011	1			P	M	H	-	-	+	R	S	S	S	ND	ND	S	R	S
		693 (1)	1	ASEfm65	-	2010			1	PHC	O	NH	+	-	+	R	S	S	S	ND	ND	S	S	S
		697 (1)	1	ASEfm25	-	2010		1		E	O	NH	-	-	+	R	S	S	S	ND	ND	S	S	S
		717 (1)	1	ASEfm45	-	2010		1		GDS	O	NH	+	-	+	R	S	S	S	ND	ND	S	S	S
	1.5 (2)	**623** (1)	1	ASEfm24	-	2009		1		PHC	O	NH	+	-	+	R	S	S	S	ND	ND	S	S	S
		713 (1)	1	ASEfm62	-	2009		1		PHC	O	NH	-	-	+	S	S	S	S	ND	ND	S	S	S
	1.6 (2)	**686** (1)	NT	NT	-	2009	1			PHC	O	NH	+	+	+	R	S	S	S	ND	ND	S	S	S
		694 (1)	1[b]	ASEfm63.1	-	2009			1	ER	O	NH	-	-	+	R	S	S	R	ND	ND	S	S	S
BAPS 2	2.1a (33)	**117** (25/9)	1[e]	CEfm1	+	2009-2010		8	17	ID (3); IM (3); GDS (3); NP (3); T (3); G (2); UR (2); C; CV; ER; GI; NM; PHC	M (14); I (4); O (4); S (3)	H (21); NH (4)	+ (22)	+ (2)	+	R (23)	S	S	R (24)	R (23)	R (6)	S	S	R (1)
		203 (4/3)	2[k]	CEfm7 (3); CEfm27	+	2009-2010		1	3	IM (2); NM; PS	M (3); S	H	+ (3)	-	+	R (3)	S	S	R (3)	R (3)	R (3)	S	S	R (1)
		80 (1/1)	1	CEfm39	+	2009		1		ID	O	NH	-	-	+	R	S	S	R	R	R	R	ND	ND
		323 (1/1)	1	CEfm18	+	2009		1		G	M	H	+	+	+	R	S	S	R	R	S	S	S	S
		612 (1)	1	CEfm37	+	2011	1			P	M	H	+	+	-	R	S	S	R	R	R	S	ND	ND
		324 (1)	1	ASEfm51	-	2009	1			PHC	O	NH	-	-	+	S	S	S	S	S	S	S	R	S
	2.1b (4)	**704** (1)	1	ASEfm42	-	2009	1			PHC	O	NH	-	-	+	S	S	S	S	ND	ND	S	S	S
		301 (1)	1	ASEfm56	-	2009			1	ER	O	NH	-	-	+	S	S	S	S	ND	ND	S	S	S
		701 (1)	1	ASEfm38	-	2009		1		PHC	O	NH	+	-	-	R	S	S	R	ND	ND	S	R	S
		706 (1)	1	ASEfm54	-	2009		1		PHC	O	NH	-	-	-	S	S	S	S	ND	ND	S	S	S

Table S1. *E. faecium* isolates: epidemiological data (continuation).

BAPS		ST		PFGE-type (No)	m	Year	Age Group (No)			Ward (No)		Origin	VF (No isolates)			Anbiotic resistance (No isolates)							
Group	Subgroup (No)	ST (No)*	No PFGE		(No)		I	II	III	Name	Type	(No)	*esp*	*hyl*$_{Efm}$	*acm*	ERY	VAN	TEI	CIP	LEV[j]	STR[l]	GEN	TET	CHL
	2.3a (4)	**92** (1)	1	ASEfm6	-	2009			1	PHC	O	NH	+	-	+	R	S	S	S	ND	ND	S	S	S
		420 (1)	1	CEfm24	-	2009		1		G	M	H	-	-	+	R	S	S	R	ND	ND	S	R	S
		25 (1)	1	CEfm23	+	2009			1	PHC	O	NH	-	-	+	R	S	S	R	S	R	S	R	R
		165 (1)	1	ASEfm34	-	2009		1		G	O	NH	-	-	-	R	S	S	R	S	S	S	S	S
	2.3b (6)	**672** (2/1)	1	Cefm20	+	2009-2011	1	1		P; PHC	M; O	NH; H	+ (1)	+ (1)	+ (1)	R	S	S	R	R (1)	R (1)	S	R (1)	S
		515 (1)	1	ASEfm43	-	2009			1	NE; PHC	M	H	-	-	+	R	S	S	R	ND	ND	S	R	S
		700 (1)	1	ASEfm37	-	2009		1		PHC	O	NH	+	-	+	R	S	S	R	ND	ND	S	S	S
		70 (1)	1	ASEfm40	-	2009		1		ER	O	NH	-	-	+	R	S	S	R	ND	ND	S	S	S
		707 (1)	1	ASEfm49	-	2009			1	ER	O	NH	+	-	-	S	S	S	S	ND	ND	S	S	S
BAPS 3	3.1 (10)	**32** (3/1)	3	ASEfm3; ASEfm59; CEfm32	+ (1)	2009-2010	1	2		PHC	O (2); M	NH (2); H (1)	-	-	+ (2)	R (1)	S	S	R	ND	ND	S	R (1)	S
		22 (3)	3	ASEfm1-2; ASEfm9	-	2009-2011	1	1	1	PHC (2); ER	O	NH	-	-	+	R (2)	S	S	R (1)	ND	ND	S	R (2)	S
		104 (1)	1	ASEfm32	-	2009	1			PHC	O	NH	+	+	+	R	S	S	R	ND	ND	S	S	S
		159 (1)	1	ASEfm33	-	2009		1		P	M	H	-	-	+	R	S	S	R	ND	ND	S	R	S
		682 (1)	1	ASEfm4	-	2009	1			CP	I	H	-	-	+	S	S	S	R	ND	ND	S	R	S
		683 (1)	1	ASEfm5	-	2009	1			P	O	NH	+	-	+	R	S	S	R	ND	ND	S	R	S
	3.2 (1)	**702** (1)	1	ASEfm53	-	2009			1	T	S	H	-	-	+	S	S	S	R	ND	ND	S	R	S
	3.3a (20)	**17** (14/6)	13[m]	CEfm3 (2); CEfm6; CEfm11; CEfm13; CEfm14 (2); CEfm16; CEfm25; CEfm30(3); CEfm34; ASEfm50	+ (13)	2009-2010	1	4	9	IM (5); PHC (3); CV (2); GDS; HE; P; UR	M (9); O (2); I (2); S	H (12); NH (2)	+ (10)	+ (13)	+ (12)	R (13)	S	S	R (12)	R (12)	R (3)	R (3)	R (4)	R (2)
		18 (6/2)	6	CEfm8; CEfm10; CEfm15; CEfm28; CEfm35-36	+	2009-2011	2	2	2	ID (2); PHC (2); HE; NE	M (4); O (2)	H (4); NH (2)	-	+ (3)	+	R	S	S	R (3)	R (4)	R (2)	S	R (3)	R (1)
	3.3b (24)	**102**[n] (11)	1[d]	ASEfm7	-	2009-2010	3	6	2	PHC (5); ID (2); ER; G; NE; P	O (9); M (2)	NH (9); H (2)	+ (6)	+ (1)	-	R (6)	S	S	R (9)	ND	ND	S	S	S
		705 (3)	1[g]	ASEfm39	-	2009			1	ER (2); T	O (2); S	NH (2); H	+ (1)	+ (1)	-	R (2)	S	S	R (1)	ND	ND	S	S	S
		709[n] (2)	1[h]	ASEfm7.6	-	2009		2		ER; PHC	O	NH	+ (1)	-	-	R	S	S	S	ND	ND	S	S	S
		708[n] (1)	1[i]	ASEfm7.3'	-	2010		1		PHC	O	NH	-	-	-	S	S	S	S	ND	ND	S	S	S
		710[n] (1)	1[j]	ASEfm7.1	-	2010			1	PHC	O	NH	-	-	-	R	S	S	S	ND	ND	S	S	S
		711 (1)	1	ASEfm8	-	2009	1			PHC	O	NH	-	-	-	S	S	S	R	ND	ND	S	S	S
		513 (1)	1	CEfm41	+	2010			1	PHC	O	NH	-	-	+	R	S	S	R	R	S	S	R	S
		681 (1/1)	1	CEfm33	+	2009	1			P	O	NH	+	-	+	R	S	S	R	ND	ND	S	S	S
		673 (1/1)	1	CEfm31	+	2010			1	PHC	O	NH	-	-	+	R	S	S	S	S	S	S	S	S
		253 (1)	1	ASEfm60	-	2011	1			PHC	O	H	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
		712 (1)	1	ASEfm58	-	2009			1	IM	M	H	-	-	-	R	S	S	R	ND	ND	S	R	S
BAPS 7	7.1 (8)	**19** (1)	1[k]	CEfm5	+	2009	1	2	1	PHC (2); C; ER	O (3); M	NH (3); H	-	-	+	R	S	S	R	R (2)	R (2)	S	R	R (1)
		687 (1)	1	ASEfm16	-	2011	1			PHC	O	NH	-	-	+	R	S	S	S	ND	ND	S	S	S
		703 (1/1)	1	CEfm38	+	2009			1	GDS	S	H	-	-	-	ND	S	S	ND	ND	ND	ND	ND	ND
		675 (1)	1	ASEfm55	-	2009			1	PHC	O	NH	-	-	-	S	S	S	S	ND	ND	S	S	S
		699 (1)	1	ASEfm35	-	2009			1	PHC	O	NH	-	-	-	S	S	S	S	ND	ND	S	S	S
	7.3 (3)	**718** (1/1)	1	CEfm2	+	2009		1		R	M	H	-	-	-	R	S	S	R	S	S	S	R	S
		716 (1)	1[f]	ASEfm7.5	-	2011	1			PHC	O	NH	+	-	-	S	S	S	S	ND	ND	S	S	S
		59 (1)	1	ASEfm36	-	2009		1		PHC	O	NH	-	-	-	R	S	S	R	ND	ND	S	S	S
BAPS 8	(4)	**698** (2)	2	ASEfm27-28	-	2009	1		1	PHC; T	O; S	NH; H	+	-	+	R (1)	S	S	S	ND	ND	S	S	S
		689 (1)	1	ASEfm61	-	2009			1	PHC	O	NH	-	-	+	S	S	S	S	ND	ND	S	S	S
		690 (1)	1	ASEfm18	-	2009			1	PHC	O	NH	+	-	+	S	S	S	S	ND	ND	S	S	S

*the numbers in after the bar (/) corresponds to the number of Ampicillin-resistant (AREfm) isolates that were only cultured after culture enrichment; PFGE clones were named as CEfm plus the number of the clone, or eventually ASEfm and AREfm to highlight the ampicillin susceptibility or resistance phenotype; [a]two strains have a PFGE pattern that has 3 different bands; [b]PFGE patterns with 1 band of difference; [c]although strains have different STs they have the same PFGE-type; [d]PFGE pattern that has up to 5 bands of difference; [e]3 of the 25 strains that belong to this PFGE-type have 2 bands of difference; [f]PFGE-type with 5 bands of difference regarding ST102-PFGE-type; [g]PFGE-type with 1 band of difference; [h]PFGE-type with 6 bands of difference regarding ST102-PFGE-type; [i]PFGE-type with 3 bands of difference regarding ST102-PFGE-type; [j]PFGE-type with 1 band of difference regarding ST102-PFGE-type; [k]PFGE pattern that has up to 2 bands difference; [l]High level resistance; [m]It is of note that these ST17 strains were among those more frequently detected from samples of patients with bacteremia in our hospital admitted to different wards and locations; [n]Isolates classified as ST102 and its SLVs (ST709, ST708, ST710 and ST711) showed very similar PFGE types (ASEfm7, up to 5 bands difference). **Abbreviations:** BAPS, Bayesian Analysis of Population Structure; ST, Sequence Type; AREfm, Ampicillin resistance *E. faecium*; VF, Virulence factors; *esp*, Enterococcal Surface Protein; hyl_{Efm}, Glycosyl Hydrolase; *acm*, Collagen-binding Adhesin Gene ; NH, Non-Hospital; H, Hospital; O, Outpatient; M, Medical; S, Surgical; I, ICU; ND, Not determined; Group I, 0-19 years old; Group II, 20-59 years old; Group III, ≥60 years old; C, Cardiology; CV, Cardiovascular; CP, Cardiopediatrics; E, Endocrinology; ER, Emergency room; G, Gastroenterology; GDS, General and Digestive Surgery; GI, Gynecology; HE, Hematology; ID, Infectious Diseases; IM, Internal Medicine; NE, Neurology; NM, Pneumology; NP, Nephrology; OC, Oncology; OT, Otorhinolaryngology; P, Pediatrics; PS, Plastic Surgery; PHC, Primary health center; R, Rheumatology; T, Traumatology; UR, Urology; AMP, Ampicillin; ERY, Erythromycin; VAN, Vancomycin; TEI, Teicoplanin; CIP, Ciprofloxacin; LEV, Levofloxacin; STR, Streptomycin; GEN, Gentamicin; TET, Tetracycline; CHL, Chloramphenicol.

Table S2. *E. faecalis* isolates: epidemiological data.

BAPS	ST			PFGE-type (No)	Year	Age Group (No)			Ward (No)		Origin	VF (No)					Anbiotic resistance (No)								
(No)	ST No)	No PFGE	CC (No)			I	II	III	Name	Type	(No)	*asa1*	*gelE*	*esp*	hyl_{Efc}	*cylA*	AMP	VAN	TEI	STR^n	GEN^n	LEV	ERI	TET	CHO
BAPS 1 (48)	6 (16)	1[c]	6 (16)	H10	2009-2010	1	4	11	IM (6); NE (3); PHC (2); T (2); CV; ER; P	M (12); O (3); I	H (13); NH (3)	+	+ (13)	+ (6)	-	+ (9)	S	R (5)	R (5)	R	R	R (14)	R	R (15)	R (1)
	513 (6)	1	242 (6)	CEfc35	2009-2010	1	2	3	PHC (4); GDS; ID	O (4); M; I	NH (4); H (2)	+ (2)	+ (1)	+ (1)	-	+ (3)	S	S	ND	R (2)	S	R (1)	R (2)	R (3)	S
	72 (5)	4	72 (5)	CEfc30-33	2009-2011	2	2		ER; P; PHC; R	O (2); M (2)	NH (2); H (2)	+ (2)	+ (3)	-	-	-	S	S	S	S	S	S	S	S	S
	168 (1)	1		CEfc34	2009			1	PHC	O	NH	+	+	-	-	-	S	S	ND	S	S	S	S	R	S
	64 (3)	3	8 (4)	CEfc2-4	2009	2	1		HE; P; PHC	M (2); O (1)	H (2); NH (1)	+	+	-	-	-	S	S	S	S	S	S	R (2)	R (2)	S
	8 (1)	1		CEfc1	2009			1	HE	M	H	+	+	+	-	-	S	S	ND	S	S	S	S	R	S
	34 (2)	1[m]	34 (2)	CEfc21	2009	1	1		G; PHC	O; M	NH; H	+ (1)	+	+ (1)	-	-	S	S	S	S	R (1)	S	R (1)	R (1)	ND
	79 (2)	2	79 (2)	CEfc59-60	2009	2			P; PHC	O; M	NH; H	-	+	-	-	-	S	S	ND	S	S	S	S	S	ND
	81 (1)	1	81 (2)	CEfc55	2010	1			CP	I	H	+	+	-	-	-	S	S	ND	S	S	S	S	S	S
	332 (1)	1	272 (2)	CEfc47	2009	1			PHC	O	NH	+	+	-	-	-	S	S	S	S	S	S	S	S	ND
	517 (1)	1		CEfc48	2009	1			PHC	O	NH	+	+	-	-	-	S	S	ND	S	S	S	S	S	ND
	27 (1)	1	27 (1)	CEfc57	2009			1	PHC	O	NH	+	+	-	-	-	S	S	S	S	S	S	S	S	ND
	82 (1)	1	82 (1)	CEfc69	2009			1	IM	M	H	-	+	-	-	-	S	S	S	S	S	S	S	S	ND
	282 (1)	1	121 (1)	CEfc37	2009		1		G	M	H	-	+	-	-	-	S	S	S	S	S	S	R	S	ND
	241 (1)	1	241 (1)	CEfc43	2009			1	GDS	I	H	-	+	+	-	+	S	S	S	S	S	S	S	S	S
	197 (1)	1	D (1)	CEfc42	2009			1	PHC	O	H	-	-	-	-	-	S	S	S	S	S	S	S	S	ND
	323 (1)	1	D (1)	CEfc56	2009			1	ER	O	NH	-	-	-	-	-	S	S	S	S	S	S	S	S	ND
	515 (2)	2	S (2)	CEfc44-45	2009	2			PHC	O	NH	+	+ (1)	-	-	+ (1)	S	S	ND	S	S	S	S	S	S
	516 (1)	1	S (1)	CEfc46	2009			1	ER	O	NH	-	+	-	-	-	S	S	ND	S	S	S	I	S	S
	520 (1)	1	S (1)	CEfc67	2009			1	PHC	O	NH	-	+	-	-	-	S	S	S	S	S	S	S	S	ND
BAPS 2 (52)	40 (15)	9[c]	40 (17)	CEfc23; CEfc24 (2); CEfc25 (3); CEfc26 (5); CEfc27; CEfc28.5; CEfc52	2009-2011	7	2	6	PHC (7); ER (2); P (2); ID; NP; T; UR	O (10); M (5)	NH (10); H (5)	+ (6)	+ (13)	+ (6)	-	+ (1)	S	S	S	R (1)	S	S	R (7)	R (10)	S
	268 (2)	1[d]		CEfc28	2009-2011	1		1	P; PHC	O; M	NH; H	+ (1)	+	+	-	-	S	S	S	S	S	S	R (1)	R	S
	25 (3)	2	25 (7)	CEfc11; CEfc12 (2)	2009	2		1	GDS; P; PHC	O (2); M	NH (2); H	+ (2)	+	+	-	+ (1)	S	S	S	S	S	S	R (1)	R (1)	S
	97 (1)	1			2009	1			P	M	H	+	+	-	-	-	S	S	ND	S	S	S	S	S	S
	269 (1)	1		CEfc15	2009		1		PHC	O	NH	-	+	+	-	-	S	S	S	S	S	S	S	S	ND
	55 (4)	1[g]	55 (7)	CEfc29	2009-2011	1	1	2	PHC (2); CP; UR	O (2); M; I	NH (2); H (2)	+	+ (1)	+ (3)	-	-	S	S	ND	R (1)	S	S	R (3)	R	S
	285 (1)	1[h]		CEfc29	2011	1			P	M	H	-	-	+	-	-	S	S	ND	S	S	S	R	R	R
	510 (1)	1[i]		CEfc29.1'	2009	1			PHC	O	NH	-	+	+	-	-	S	S	ND	S	S	S	R	R	R
	511 (1)	1[h]		CEfc29	2009		1		ER	O	NH	-	+	+	-	-	S	S	ND	S	S	S	S	R	R
	56 (2)	2	30 (4)	CEfc18-19	2009	1	1		G; PHC	O; M	NH; H	+ (1)	+ (1)	-	-	+	S	S	ND	S	S	S	R (1)	R	S
	30 (1)	1		CEfc17	2009			1	PHC	O	NH	+	+	+	-	-	S	S	ND	S	S	S	S	R	ND
	379 (1)	1		CEfc20	2009		1		ER	O	NH	-	+	+	-	-	S	S	S	R	S	S	R	S	ND
	191 (2)	2	191 (3)	CEfc39-40	2009-2010	1		1	PHC	O	NH	+ (1)	+	+	-	-	S	S	ND	S	S	R (1)	R (1)	R	ND
	514 (1)	1		CEfc41	2009			1	PHC	O	NH	+	+	+	-	-	S	S	ND	S	S	S	S	S	ND
	19 (2)	1[l]	19 (2)	CEfc7	2009		1	1	PHC	O	NH	+ (1)	+ (1)	+	-	-	S	S	S	S	S	S	S	R	S
	21 (2)	2	21 (2)	CEfc8-9	2009-2010	1	1		HE; PHC	O; M	NH; H	+ (1)	+	+	-	-	S	S	ND	S	S	S	S	R (1)	S
	512 (1)	1		CEfc22	2009			1	PHC	O	NH	+	+	-	-	-	S	S	ND	S	S	S	R	S	ND

Table S2. *E. faecalis* isolates: epidemiological data (continuation).

BAPS (No)	ST			PFGE-type (No)	Year	Age Group (No)			Ward (No)		Origin	VF (No)					Anbiotic resistance (No)								
	ST No)	No PFGE	CC (No)			I	II	III	Name	Type	(No)	*asa1*	*gelE*	*esp*	hyl_{Efc}	*cylA*	AMP	VAN	TEI	STR[n]	GEN[n]	LEV	ERI	TET	CHO
	116 (2)	1[g]	116 (2)	CEfc36	2009		1	1	ER; PHC	O	NH	-	+	-	-	-	S	S	S	R (1)	S	S	R	R (1)	ND
	509 (1)	1	26 (1)	CEfc16	2009	1			PHC	O	NH	-	+	+	-	-	S	S	ND	S	S	S	S	R	ND
	41 (1)	1	41 (1)	CEf21	2009		1		PHC	O	NH	-	+	+	-	-	S	S	ND	R	S	S	S	R	ND
	74 (1)	1	73 (1)	CEfc58	2010			1	PHC	O	NH	-	-	-	-	-	S	S	ND	S	S	S	S	S	S
	508 (1)	1	206 (1)	CEfc51	2009		1		PHC	O	NH	+	+	-	-	+	S	S	ND	S	S	S	S	R	S
	86 (2)	1	S (2)	CEfc61	2009		2		G; NP	M	H	+	+	+	-	+	S	S	S	S	R (1)	R (1)	R	R	ND
	147 (1)	1	S (1)	CEfc62	2009		1		PHC	O	NH	-	-	-	-	-	S	S	S	S	S	S	S	S	ND
	321 (1)	1	S (1)	CEfc64	2009	1			P	M	H	+	+	+	-	+	S	S	S	S	S	S	R	S	ND
	322 (1)	1	S (1)	CEfc65	2009	1			P	M	H	+	-	+	-	-	S	S	S	R	S	S	S	S	ND
BAPS 3 (30)	179 (11)	1[a]	16 (18)	EFC11	2009-2011	5	5	1	PHC (3); P (3); ER (2); G; IM; OT	O (7); M (3); I	NH (7); H (4)	+	+ (8)	+ (9)	-	+ (4)	S	S	S	S	S	S	R (4)	R (8)	S
	16 (7)	4[b]		CEfc5 (4); CEfc6; CEfc50; Degraded	2009-2011	1	4	2	PHC (4); ER; OC; P	O (5); M (2)	NH (5); H (2)	+ (6)	+ (3)	+ (5)	-	+ (6)	S	S	S	R (3)	R (3)	R (2)	R (6)	R (6)	R (4)
	62 (2)	2[f]		CEfc12.5; CEfc13	2009	2			P; PHC	O; M	NH; H	+ (1)	+	+	-	-	S	S	ND	S	S	S	S	R	S
	333 (1)	1[j]	28 (3)	CEfc49.4	2009			1	IM	M	H	+	+	+	-	+	S	S	S	R	R	R	R	R	ND
	518 (1)	1		CEfc49	2009		1		CV	I	H	+	+	+	-	+	S	S	ND	R	R	S	R	R	ND
	519 (1)	1[k]		CEfc49	2009		1		CV	I	H	+	+	+	-	+	S	S	ND	R	R	R	R	R	S
	35 (1)	1	35 (1)	CEfc22	2010			1	PHC	O	NH	-	+	-	-	-	S	S	ND	S	S	R	R	R	S
	320 (1)	1	58 (1)	CEfc53	2009			1	PHC	O	NH	-	+	-	-	-	S	S	S	S	S	S	S	S	ND
	141 (1)	1	141 (1)	CEfc38	2009	1			PHC	O	NH	-	+	-	-	-	S	S	S	S	S	S	S	S	ND
	225 (1)	1	S (1)	CEfc63	2009		1		ID	M	H	-	+	-	-	-	S	S	ND	S	S	S	R	S	ND
	330 (1)	1	S (1)	CEfc66	2009		1	1	IM; ID	O; M	NH; H	+ (1)	+	+	-	-	S	S	S	R	S	R	R	R (1)	ND
	521 (1)	1	S (1)	CEfc68	2009			1	ER	O	NH	-	-	-	-	-	S	S	ND	S	S	S	S	S	S

[a]CEfc5 has up to 5 bands of difference; [b]up to 3 bands of difference; [c]Several PFGE-types here prevalent. Among this PFGE-types there are up to 5 bands of difference; [d]5 bands of difference compared with ST40-CEfc28; [e]up to 6 bands of difference when comparing to V583; [f]5 bands of difference to ST25-CEfc12; [g]up to 2 bands of difference; [h]1 band of difference regarding ST50-CEfc29; [i]the same PFGE type as ST50-CEfc29; [j]the same PFGE as ST518; [k]4 bands of difference regarding ST518; [l]up to 6 bands of difference; [m]up to 1 bands of difference; [n]High level resistance. **Abbreviations:** BAPS, Bayesian Analysis of Population Structure; MLST, Multilocus Sequence Typing; CC, Clonal Complex; ST, Sequence Type; VR, Virulence factors; *esp*, Enterococcal Surface Protein; hyl_{Efc}, Glycosyl Hydrolase; *cylA*, cytolysin/haemolysin; *gelE*, gelatinase; *asa1*, aggregation substance; NH, Non-Hospital; H, Hospital; O, Outpatient; M, Medical; S, Surgical; I, ICU; ND, Not determined; Group I, 0-19 years old; Group II, 20-59 years old; Group III, ≥60 years old; CV, Cardiovascular; CP, Cardiopediatrics; ER, Emergency room; G, Gastroenterology; GDS, General and Digestive Surgery; HE, Hematology; ID, Infectious Diseases; IM, Internal Medicine; NE, Neurology; NP, Nephrology; OC, Oncology; OT, Otorhinolaryngology; P, Pediatrics; PHC, Primary health center; R, Rheumatology; T, Traumatology; UR, Urology; AMP, Ampicillin; ERY, Erythromycin; VAN, Vancomycin; TEI, Teicoplanin; CIP, Ciprofloxacin; LEV, Levofloxacin; STR, Streptomycin; GEN, Gentamicin; TET, Tetracycline; CHL, Chloramphenicol.

Table S3. ORs analysis of *E. faecium* BAPS groups/subgroups regarding the origin of isolates.

BAPS		Hospitalized patients						Non-hospitalized patients						Animal						Environmental		Food		Others	
Group	Subgroup	No	%	OR[a]	p	95% CI		No	%	OR[a]	p	95% CI		No	%	OR[a]	p	95% CI		No	%	No	%	No	%
BAPS 1	**1.1**	3	0.20%	ND				1	0.07%	ND				0	0.00%	ND				1	0.07%	0	0.00%	6	0.40%
	1.2	37	2.45%	0.0386	<0.01	0.0232	0.0640	33	2.19%	35.1080	<0.01	17.0839	72.1480	3	0.20%	1.2816	0.698	0.3667	4.4793	13	0.86%	4	0.27%	10	0.66%
	1.3	2	0.13%	0.0109	<0.01	0.0024	0.0492	1	0.07%	ND				4	0.27%	ND				1	0.07%	2	0.13%	6	0.40%
	1.4	1	0.07%	ND				0	0.00%	ND				1	0.07%	ND				0	0.00%	0	0.00%	0	0.00%
	1.5	14	0.93%	0.0394	<0.01	0.0194	0.0802	7	0.46%	ND				6	0.40%	7.1036	<0.01	2.6186	19.2698	1	0.07%	0	0.00%	13	0.86%
	1.6	1	0.07%	ND				2	0.13%	ND				1	0.07%	ND				0	0.00%	0	0.00%	2	0.13%
BAPS 2	**2.1a**	479	31.74%	0.3672	<0.01	0.2551	0.5289	15	0.99%	1.7320	0.165	0.7978	3.7625	56	3.71%	4.4539	<0.01	2.5256	7.8547	4	0.27%	0	0.00%	23	1.52%
	2.1b	60	3.98%	0.0174	<0.01	0.0116	0.0262	24	1.59%	4.9073	<0.01	2.3729	10.1485	182	12.06%	54.2563	<0.01	31.5295	93.3649	15	0.99%	10	0.66%	30	1.99%
	2.3a	72	4.77%	0.0844	<0.01	0.0539	0 .1320	17	1.13%	8.5132	<0.01	4.3025	20.2869	20	1.33%	7.2065	<0.01	3.6271	14.3184	9	0.60%	0	0.00%	17	1.13%
	2.3b	13	0.86%	0.0275	<0.01	0.0136	0 .0552	8	0.53%	ND				22	1.46%	33.7639	<0.01	15.9460	71.4912	3	0.20%	2	0.13%	1	0.07%
BAPS 3	**3.1**	67	4.44%	0.0927	<0.01	0.0584	0.1471	15	0.99%	8.5132	<0.01	3.8086	19.0289	21	1.39%	8.6157	<0.01	4.3507	17.0619	5	0.33%	2	0.13%	12	0.80%
	3.2	16	1.06%	0.0283	<0.01	0.0148	0.0539	12	0.80%	ND				20	1.33%	21.2500	<0.01	10.2157	44.2028	3	0.20%	0	0.00%	8	0.53%
	3.3a	630	41.75%	#	#	#	#	12	0.80%	#	#	#	#	16	1.06%	#	#	#		4	0.27%	1	0.07%	16	1.06%
	3.3b	29	1.92%	0.0333	<0.01	0.0195	0.0567	24	1.59%	22.6594	<0.01	10.6789	48.0809	25	1.66%	15.4618	<0.01	7.8651	30.3956	3	0.20%	3	0.20%	8	0.53%
BAPS 4		6	0.40%	ND				2	0.13%	ND				0	0.00%	ND				1	0.07%	0	0.00%	2	0.13%
BAPS 5		14	0.93%	0.2130	<0.01	0.0736	0.6163	0	0.00%	ND				3	0.20%	ND				1	0.07%	0	0.00%	1	0.07%
BAPS 6		9	0.60%	ND				0	0.00%	ND				0	0.00%	ND				0	0.00%	0	0.00%	0	0.00%
BAPS 7	**7.1**	41	2.72%	0.0395	<0.01	0.0245	0.0637	7	0.46%	3.7619	<0.01	1.4284	9.9075	54	3.58%	33.9034	<0.01	18.3744	62.5597	2	0.13%	7	0.46%	9	0.60%
	7.2	3	0.20%	ND				0	0.00%	ND				0	0.00%	ND				0	0.00%	0	0.00%	3	0.20%
	7.3	4	0.27%	ND				6	0.40%	ND				3	0.20%	ND				1	0.07%	0	0.00%	0	0.00%
	7.4	2	0.13%	ND				0	0.00%	ND				0	0.00%	ND				0	0.00%	0	0.00%	0	0.00%
BAPS 8		6	0.40%	ND				3	0.20%	ND				0	0.00%	ND				1	0.07%	0	0.00%	1	0.07%
TOTAL		**1509**						**189**						**437**						**68**		**31**		**168**	

[a]using BAPS 3.3a as a reference group.

Table S4. *E. faecalis* BAPS analysis data.

	BAPS		
Group	**No STs**	**% STs**	**No isolates**
BAPS 1	234	44.74%	576
BAPS 2	144	27.53%	417
BAPS 3	108	20.65%	270
BAPS 4	5	0.96%	5
BAPS 5	32	6.12%	42
TOTAL	**523**		**1310**

Table S5. ORs analysis of *E. faecalis* BAPS group regarding the origin of isolates.

BAPS	Hospitalized patients						Non-hospitalized patients						Animal						Environmental		Food		Others	
	No	%	OR[a]	p	95% CI		No	%	OR[a]	p	95% CI		No	%	OR[a]	p	95% CI		No	%	No	%	No	%
BAPS 1	316	45,01%	#	#	#	#	70	36,27%	#	#	#	#	114	47,11%	#	#	#	#	10	47,62%	31	40,26%	35	46,67%
BAPS 2	206	29,34%	0,8033	0,089	0,6240	1,0341	85	44,04%	1,8507	<0,01	1,3103	2,6139	43	17,77%	0,4659	<0,01	0,3198	0,6788	7	33,33%	43	55,84%	33	44,00%
BAPS 3	155	22,08%	1,1090	0,487	0,8284	1,4847	34	17,62%	1,0414	0,856	0,6721	1,6136	69	28,51%	1,3912	0,058	0,9885	1,9580	4	19,05%	2	2,60%	6	8,00%
BAPS 4	3	0,43%	ND				0	0,00%	ND				1	0,41%	ND				0	0,00%	1	1,30%	0	0,00%
BAPS 5	22	3,13%	0,9051	0,755	0,4833	1,6949	4	2,07%	ND				15	6,20%	ND				0	0,00%	0	0,00%	1	1,33%
TOTAL	**702**						**193**						**242**						**21**		**77**		**75**	

[a]using BAPS 1 as a reference group.

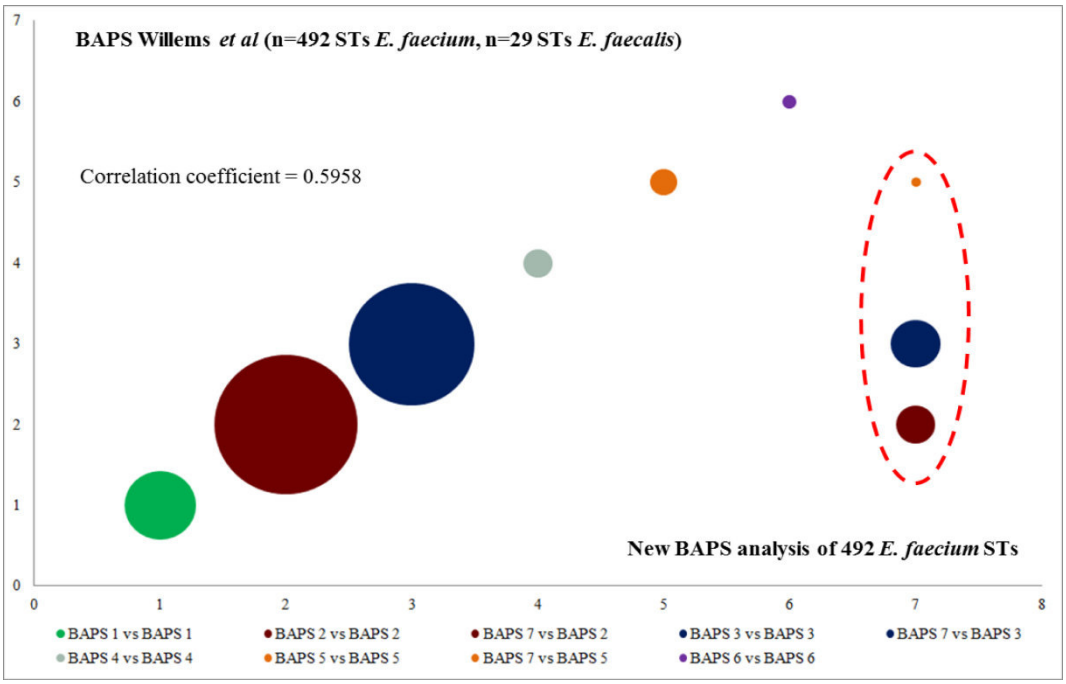


Figure S1. Correlation between the *E. faecium* BAPS groups obtained by Willems *et al* (27) using a MLST dataset of 492 *E. faecium* STs plus 29 *E. faecalis* (Y) and those obtained by us when using the same 492 *E. faecium* STs but excluding *E. faecalis* STs (X).

The size of each circle represents the population size included in each group. Dotted red line, groups with no correlation.

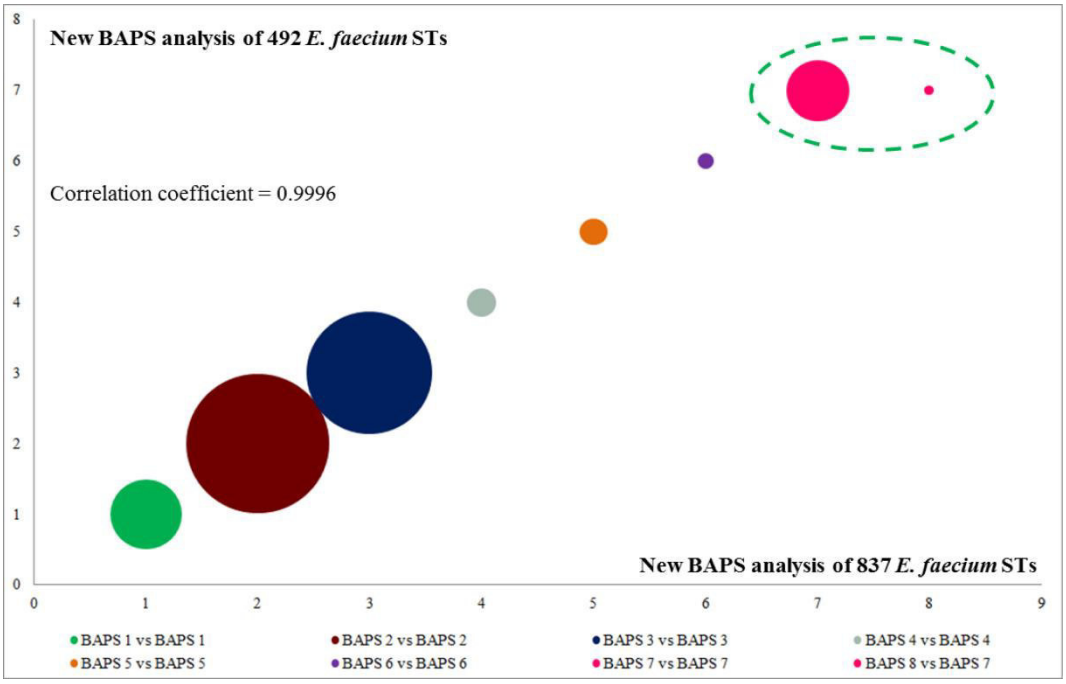
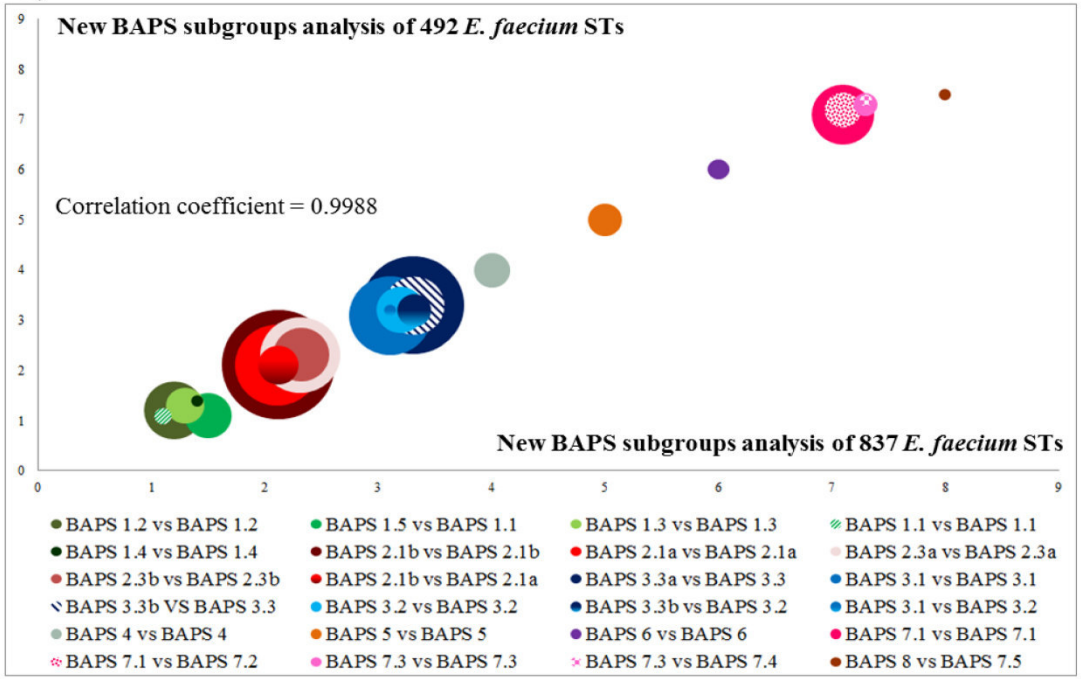


Figure S2. Correlation between the *E. faecium* BAPS groups obtained using the *E. faecium* dataset (492 STs) (Y) or using an updated database (837 STs) (X).

The size of each circle represents the population size included in each group. Green line, groups that change with the is increase in size of the population analyzed (27).

A)



B)

BAPS Willems *et al*				New BAPS			
Group	**Subgroup**	**No STs**	**%**	**Group**	**Subgroup**	**No STs**	**%**
BAPS 1	-	53	10.77%	**BAPS 1**	**1.1**	2	0.41%
					1.2	25	5.08%
					1.3	10	2.03%
					1.4	1	0.20%
					1.5	15	3.05%
					1.6	0	0.00%
					Total	**53**	**10.77%**
BAPS 2	**2.1**	149	30.28%	**BAPS 2**	**2.1a**	49	9.96%
					2.1b	99	20.12%
					2.3b	1	0.20%
	2.3	63	12.80%		**2.3a**	43	8.74%
					2.3b	20	4.07%
	2.4	15	3.05%	**BAPS 7**	**7.1**	15	3.05%
	2.2	1	0.20%	**BAPS 8**		1	0.20%
	Total	**228**	**46.34%**		**Total**	**228**	**46.34%**
BAPS 3	**3.1**	45	9.15%	**BAPS 3**	**3.1**	45	9.15%
	3.2	17	3.46%		**3.2**	16	3.25%
					3.3b	1	0.20%
	3.3	103	20.93%		**3.3a**	71	14.43%
					3.3b	32	6.50%
	3.4	4	0.81%	**BAPS 7**	**7.3**	4	0.81%
	3.5	21	4.27%		**7.1**	21	4.27%
	Total	**190**	**38.62%**		**Total**	**190**	**38.62%**
BAPS 4		9	1.83%	**BAPS 4**		9	1.83%
BAPS 5		9	1.83%	**BAPS 5**		8	1.63%
				BAPS 7	**7.3**	1	0.20%
					Total	**9**	**1.83%**
BAPS 6		3	0.61%	**BAPS 6**		3	0.61%
	TOTAL	**492**				**492**	

Figure S3. A) Correlation between the *E. faecium* BAPS subgroups obtained using the original *E. faecium* dataset analyzed in previous publications (492 STs) (Y) or using an updated database (837 STs) (X). B) Table showing correspondence between BAPS groups/subgroups described in Willems *et al* (27) and those described in this study.

The size of each circle represents the population size included in each group. The size of each circle represents the population size included in each group.

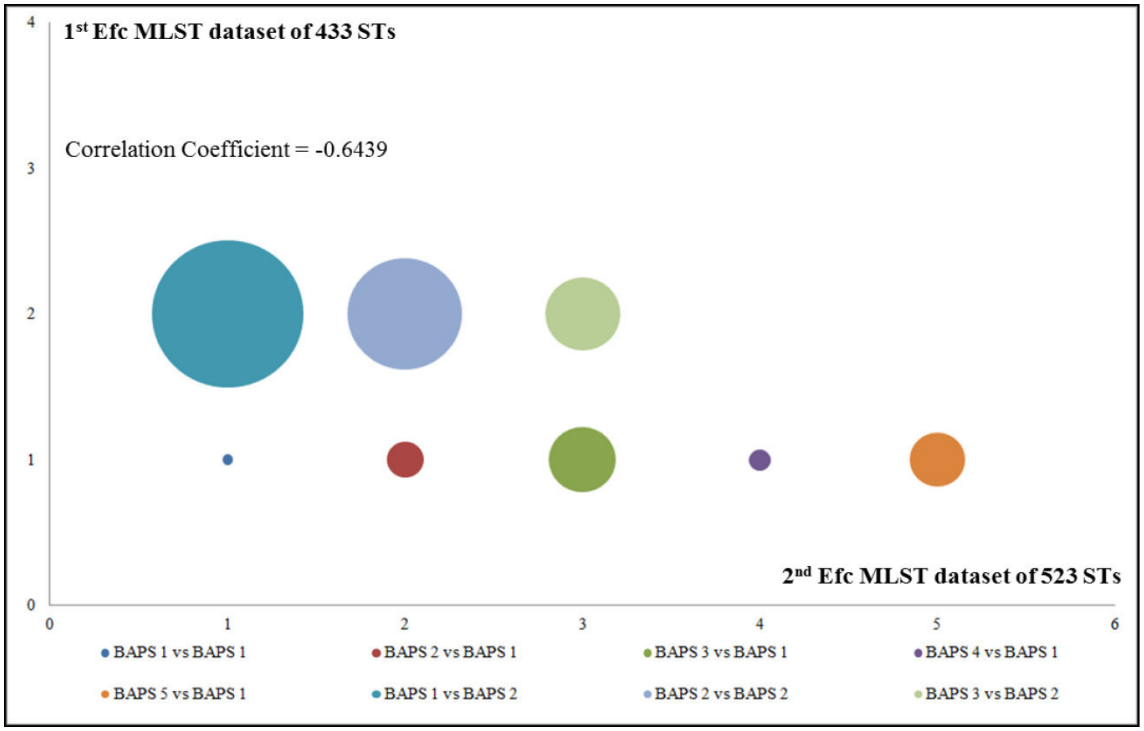
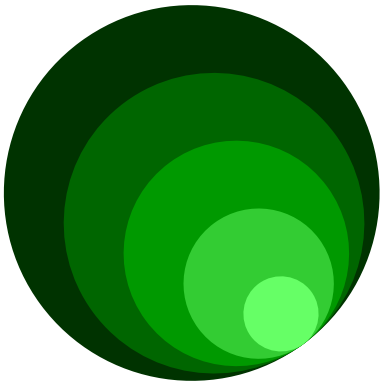


Figure S4. Correlation between the *E. faecalis* BAPS subgroups obtained using the first dataset of 433 STs (Y) and the second dataset of 523 STs (X).

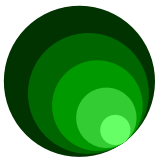
The size of each circle represents the population size included in each group.

To acquire knowledge, one must study; but to acquire wisdom, one must observe.

Marilyn vos Savant



Chapter 2



Long-Term Clonal Dynamics of *Enterococcus faecium* Strains causing Bloodstream Infections (1995-2015) in an area with low occurrence of vancomycin resistance.

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ABSTRACT

Objectives. Emergence of multi-drug resistant (MDR) *Enterococcus faecium* in hospitals is associated with lineages 17, 18, and 78. The lack of unbiased long-term longitudinal studies of *E. faecium* causing infections limits our knowledge about the emergence and dynamics of high risk clonal complexes. The population structure of *E. faecium* causing bloodstream infections (BSI) in a tertiary Spanish hospital with low glycopeptide resistance is provided.

Methods. All available BSI (n=413) registered in our hospital (1995-2015) were analysed for antibiotic susceptibility (CLSI), putative virulence traits (PCR) and clonal relationship (*Sma*I-PFGE, MLST evaluated by goeBURST and BAPS).

Results. BSI incidence decreased during the study (39‰ patients in 1996 to 30‰ patients in 2014). The increased in incidence in enterococcal BSI (2.3‰ patients in 1996 to 3.0‰ patients in 2014) appeared to be related with *E. faecium* BSI "blooming" (0.33‰ patients in 1996 to 1.3‰ patients in 2014; *E. faecalis:E. faecium* BSI ratio changed from 5:1 in 1996 to 1:1 in 2012) paralleling *E. faecium* BSI episodes in cancer patients (10.9% in 1995-2005 and 37.1% in 2006-2015). Ampicillin-susceptible (ASEfm, different STs/BAPS) and ampicillin-resistant (AREfm, ST18-BAPS3.3a1) isolates were recovered throughout the study. Successive waves of BAPS2.1a-AREfm (ST117, ST203 and ST80) partially replaced ASEfm and ST18-AREfm since 2006.

Conclusions. A high diversity of *E. faecium* populations can cause BSI. The increase of major *E. faecium* hospital associated lineages, in areas with different rates of MDR-*E. faecium*, suggests the relevance of common sociodemographic factors responsible for the emergence of *E. faecium* as nosocomial pathogen at global level.

INTRODUCTION

Enterococci, especially *Enterococcus faecalis* and *Enterococcus faecium*, have long been recognized as important causes of major nosocomial infections.[1,2] These species currently represent the second or third leading cause of healthcare-associated bacteraemia in American and European hospitals[1,3], in contrast with studies from the 1980s that ranked enterococci as the sixth most common hospital opportunistic pathogens.[4]

Most enterococcal nosocomial infections were caused by *E. faecalis* up to the early 1990s when a steady increase in the number of cases of multi-drug resistant (MDR) *E. faecium,* started to be detected.[2,4–6] Some of these studies also reported a change in the ratio of *E. faecalis:E. faecium* causing disease from 10:1 before 1990 to 3:1 in the late 1990s[5,7], suggesting a coming challenge in the management of the enterococcal infections.

With a few exceptions, most of the available information regarding *E. faecium* population structure is based on cross-sectional targeted studies, often focused on MDR or vancomycin resistant enterococcal (VRE) isolates.[8–11] The majority of MDR *E. faecium* causing nosocomial infections belong to the major hospital lineages 17, 18 and 78 (originated from ST17, ST18 and ST78, respectively) that have progressively acquired traits coding for antibiotic resistance and traits that provide colonization advantages.[11] Although isolates of these lineages are now predominant in hospitals worldwide, temporal and spatial differences have been observed within and between different geographical areas.[10–13] Thus, long-term population genetics studies seem to be needed in order to understand the transmission, evolutionary biology, and the resulting changes in the dynamics of this species in the hospital setting.

Available information is mostly based on eBURST analysis of data obtained by multilocus sequence typing (MLST) [14,15] data but this approach does not reliably establish evolutionary descent patterns for recombinant species as *E. faecium*. The recent application of Bayesian Analysis of Population Structure (BAPS) to *E. faecium* seems to accurately establish relatedness among populations within this species.[11,12] Such approaches seem necessary to analyse the bacterial shifts in pathogenicity and antibiotic resistance in response to changes in patient demographics and medical strategies, within the paradigm of the “Hamiltonian medicine” (based on the application of social-evolution theory in order to understand kinship interactions between the human host and core medicine issues as bacterial opportunistic pathogens) and the increasing demand for evolutionary biology studies to understand medical problems.[16] The objective of this work was to comprehensively analyse the population structure of *E. faecium* strains recovered from BSI and its relation with epidemiological patient data in a geographical area where the occurrence of vancomycin resistant enterococci is still very low.

MATERIAL AND METHODS

Epidemiological data and Bacterial samples

A total 21,695 positive blood cultures were detected in Hospital Universitario Ramón y Cajal (HRyC) between January of 1996 and May of 2015. HRyC is a tertiary care public hospital with 1,155 beds that provides specialized attention to a population size of about 600,000 habitants in the Northern area of Madrid (Spain). Isolates up to 2002 were previously analysed elsewhere.[3,17] Of the total number of microorganisms isolated from positive blood cultures, 1,816 were identified as enterococci (8.4%), of which 531 were *E. faecium* (2.4%). To avoid duplications, only one positive sample per Bloodstream infection (BSI) was considered. *E. faecium* isolates obtained from the same patient at different hospitalizations more than one month apart (n=6 patients) were also included. Using this criterion we identified 403 cases of *E. faecium* BSI between 1996 and 2015 corresponding to 395 patients. Due to the changes in the software used in HRyC's Microbiology Department in 1996, data from 1995 was not available for epidemiological analysis. We were, however, able to recover 10 *E. faecium* BSI isolates from 1995 making the total number of *E. faecium* isolates analyzed 413 (405 patients). The study was approved by the local ethics committee.

E. faecium BSI were classified as community-acquired if the sample was obtained within the first 48h after hospital admission and the patients had no history of previous hospitalization or antibiotic therapy in the 6 months previous to the positive blood culture. Otherwise, we considered that the BSIs were acquired in the hospital setting.[12]

Bacterial identification and antibiotic susceptibility

Bacterial identification was performed using WIDER, MICROSCAN (Francisco Soria Melguizo, Madrid, Spain) and MALDI-TOF MS (Bruker, Bellerica, Massachusetts, USA). Susceptibility to twelve antibiotics was determined using WIDER and MICROSCAN standard panels and also by the agar dilution method (Oxoid, Basingstoke, UK) according to CLSI guidelines.[18]

Clonal Relationship

Clonal relationship was established by Pulsed Field Gel Electrophoresis (PFGE) and MLST as previously described.[14,17] *E. faecium* population structure was further characterized using the BAPS scheme previously described.[11,12] updated for this paper in order to include new STs described in this work (a total sample of 1115 STs; MLST database updated in February 2016, see supplementary data).

Presence of putative virulence factors

The presence of known *E. faecium* putative virulence genes, *esp* (Enterococci surface protein) and hyl_{Efm} (glycosyl hydrolase) were investigated by PCR and further sequencing. The presence of IS*16*, a marker of hospital-associated infections, was carried out as previously described.[19,20]

Statistical analysis

Statistical significance was calculated using Chi-square test using RStudio; p values <0.05 were considered as statically significant.[21]

RESULTS

The rising trend of enterococcal BSI is due to the increase in the number of MDR *E. faecium.*

Figure 1 represents the number of BSI cases (total BSI cases, incidence of BSI, BSI caused by *Enterococcus* spp, *E. faecium* and *E. faecalis*) in our institution. Despite some fluctuations, the incidence of BSI in our hospital showed a decreasing trend (p<0.001) throughout the study period (39 cases per 1000 patients in 1996 to 30 cases per 1000 patients in 2014) (Figure 1 and Figure S1). A decrease in the number of positive blood cultures was recorded in and 2009. The last one was coincidental with a better adherence to the rule that each culture flask should be extracted from different arms in order to reduce false positives due to skin microbiota contamination.

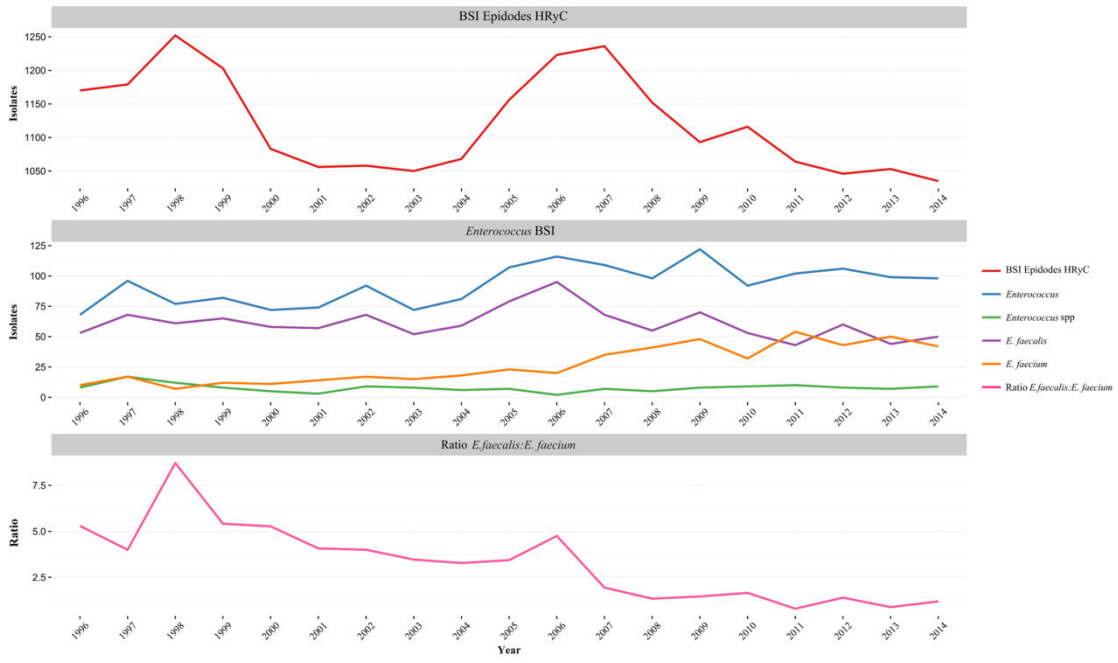

Figure 1. Trend of BSI in Hospital Ramón y Cajal, 1996 to 2014.

The incidence of *Enterococcus* spp BSI increase from 2.3‰ patients (n=68, 5.8% of the total BSI) in 1996 to a 3.0‰ patients (n=98, 9.5% of the total BSI) in 2014. Such increase in incidence was paralleled that of *E. faecium* BSI that changed from 0.33‰ patients (n=10, 0.85% of total BSI) in 1996 to 1.3‰ patients (n=42, 4.6% of the total BSI) in 2014. The incidence of *E. faecalis* BSI was steadily maintained during the study (1.8‰ patients (n=53) in 1996 and 1.6‰ patients (n=50) in 2014), thus the ratio of *E. faecalis*:*E. faecium* BSI decreased throughout the study from 5:1 in 1996 to 1:1 in 2014. The increase in *E. faecium* BSI is probably due to the increase in the number of *E. faecium* strains resistant to different antibiotics, mainly ampicillin, levofloxacin and high-level resistance (HLR) to streptomycin (Figure 1 and 2).

The number of *E. faecium* BSI increased with age (average, 62.7±20.0 years; range, 0-95 years old) and was mostly associated with the elderly (≥60y, 257/405, p<0.0001) compared with adults (20-59y, 133/405) and young people (0-19y, 15/405). *E. faecium* BSI was also more frequent among males (59.8%, p<0.0001) and hospitalized patients (85.9%, p<0.0001) (Figure 3).

Inpatients were located at medical wards (56.8%, mainly in Gastroenterology, Haematology, Internal Medicine and Oncology; 28.3% 19.1%, 16.1% and 15.2%, respectively), surgical areas (17.0%) and ICUs (12.1%). An increase in *E. faecium* BSI at Oncology, Haematology and Internal Medicine (from 10.9% to 37.1% in the periods of 1995-2005 and 2006-2015, respectively) was observed (Figure S2 and S3 supplementary data).

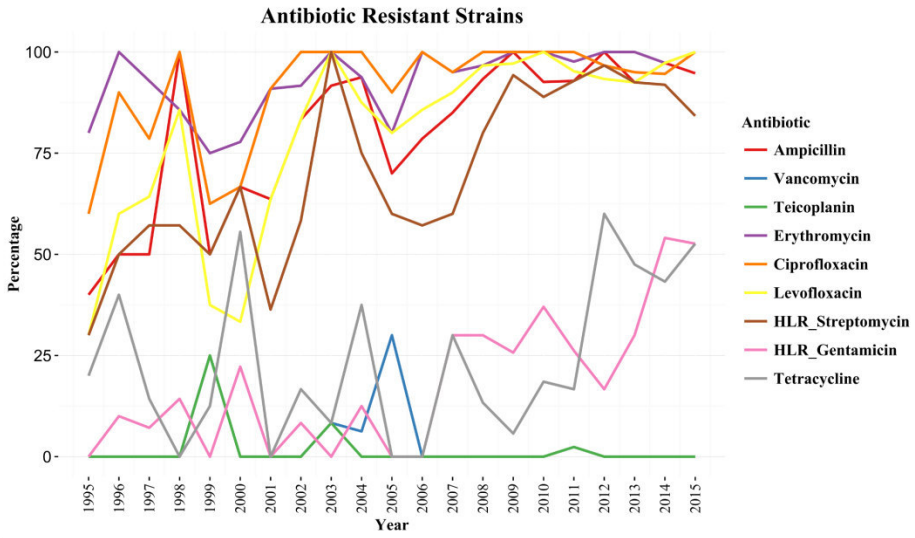

Figure 2. Trend of antibiotic resistance in *E. faecium* BSI.

A 32.7% of *E. faecium* BSI were polymicrobial. *E. coli* (n=36), *Staphylococcus* (n=30), *Klebsiella* (n=15), other *Enterococcus* (n=13), *Pseudomonas* (n=10), *Streptococcus* (n=6), *Morganella* (n=5) and yeasts (n=11) were the most common microorganisms isolated with *E. faecium.*

Genotypic heterogeneity of *E. faecium* isolates: dominance of a few lineages

The 413 *E. faecium* isolates were classified in 53 STs, 12 of these STs (22.6%) being described here for the first time. Most STs correspond to major hospital associated lineages 78 (n=154, 6 STs), 17 (n=119, 5 STs) and 18 (n=82, 2 STs) originated from ST17, ST78 and ST18 respectively.[11] Other STs less frequently detected are shown in Figure 3.

The 53 STs were partitioned into 6 BAPS groups (supplementary text and Figure S2). The most prevalent were BAPS 3 (54.3%) and BAPS 2 (40.8%), whereas BAPS 1, BAPS 7, BAPS 6 and BAPS 9 (3.2%, 1.0%, 0.5%, and 0.2% respectively) were sporadically detected (Figures 4 and 5).

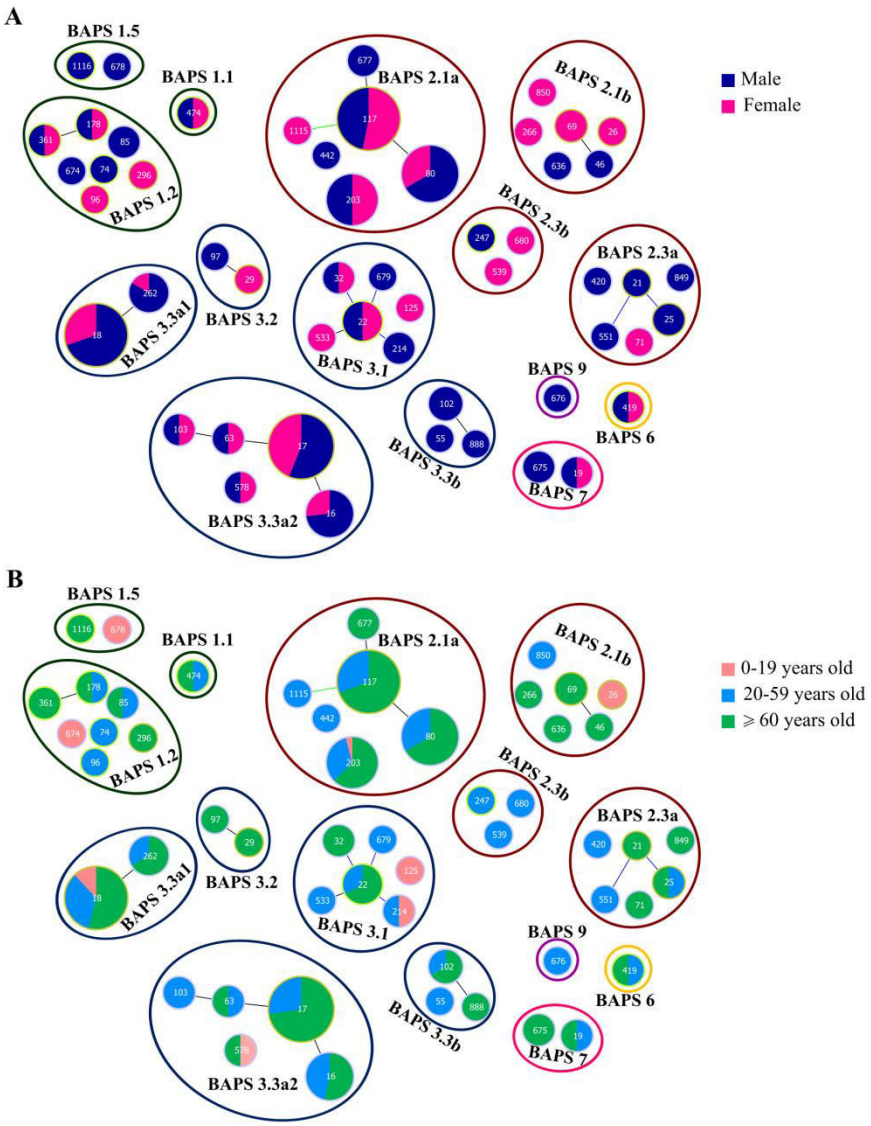

Figure 3. Population structure of *E. faecium* by sex (A) and by age group (B).

BAPS 3 comprises both ampicillin resistant *E. faecium* (AREfm, 90.5%) and ampicillin susceptible *E. faecium* (ASEfm, 9.5%) isolates, some recovered for long periods of time. BAPS 3.3a1 (37.1%; e.g. lineage 18) and BAPS 3.3a2 (53.8%; e.g. lineage 17) were often MDR strains[22] that contained IS*16* (97.5% and 92.8%, respectively), putative virulence traits, as hyl_{Efm} (50.0% and 72.3%, respectively) or *esp* genes (37.8% and 66.4%, respectively). They account for 52.4% of *E. faecium* BSI in elderly patients at medical wards. The other BAPS 3 subgroups, namely BAPS 3.3b (2.3%, 3 STs; e.g. ST102), BAPS 3.1 (5.9%, 6 STs; e.g. ST22) and BAPS 3.2 (0.9%, 2 STs), comprise mostly ASEfm corresponding to diverse STs and PFGE-types. They were susceptible to the majority of antibiotics tested and only sporadically harboured putative virulence genes or IS*16* (Figures 4-5; Figure S4 supplementary data).

BAPS 2 also comprises both AREfm (91.0%) and ASEfm (9.0%) isolates. While ASEfm were recovered during the whole study period, most of AREfm isolates were detected since 2006. Within BAPS 2, subgroup BAPS 2.1a was predominant (89.8%). It includes the major hospital associated lineage 78, and comprises MDR isolates, enriched in *esp* (59.7%) and IS*16* (91.2%), which eventually harboured hyl_{Efm},

(42.3%). Isolates of BAPS subgroups 2.1b, 2.3a, and 2.3b were scarcely identified in our sample (Figure 4-5; Figure S4 supplementary data).

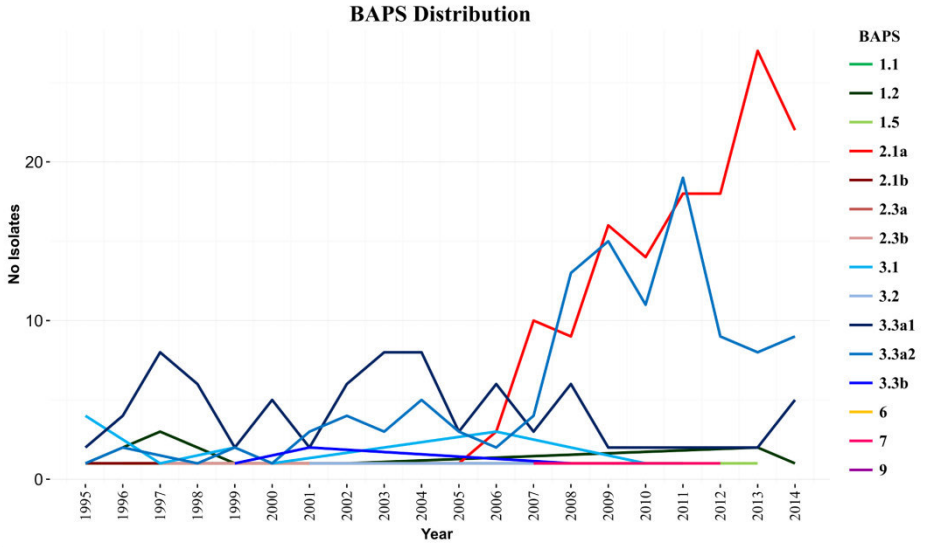

Figure 4. Distribution of BAPS groups and subgroups in Hospital Ramón y Cajal per year.

BAPS 1 (BAPS 1.2, BAPS 1.5 and BAPS 1.1, 10 STs), one of the most abundant enterococcal groups among colonized healthy individuals [12], was only sporadically represented in this study and most isolates were susceptible to antibiotics and/or lacked virulence genes and IS*16* (Figure 4-5; Figure S4 supplementary data).

Clonal dynamics of *E. faecium* causing BSI.

Figures 5 and 6 showed the clonal dynamics of *E. faecium* in our institution. We identified apparent "waves" of *E. faecium* clones belonging to different lineages. Genetically diverse ASEfm (STs of BAPS 1, BAPS 3.1, BAPS 3.3b) which commonly colonized non-hospitalized persons in our setting [12], and also AREfm ST18 isolates (PFGE-types AREF-A and AREF-D) were recovered throughout the period of study, particularly from 1995 to 2006. The low numbers of isolates of these STs since then parallel the emergence in our setting of AREfm clones belonging to the 78 and 17 lineages.

BAPS 3.3a1 (ST16 and ST17) strains were also detected in early years. While the peak of ST16 was due to clonal spread (AREF-T) in the Gastroenterology ward between 2001 and 2006 [17], the rise of ST17 isolates in 2007 reflects the emergence of different clones (AREF-38, AREF-73, AREF-74, AREF-75) in several wards (manly Gastroenterology, Oncology, Haematology and Internal Medicine).

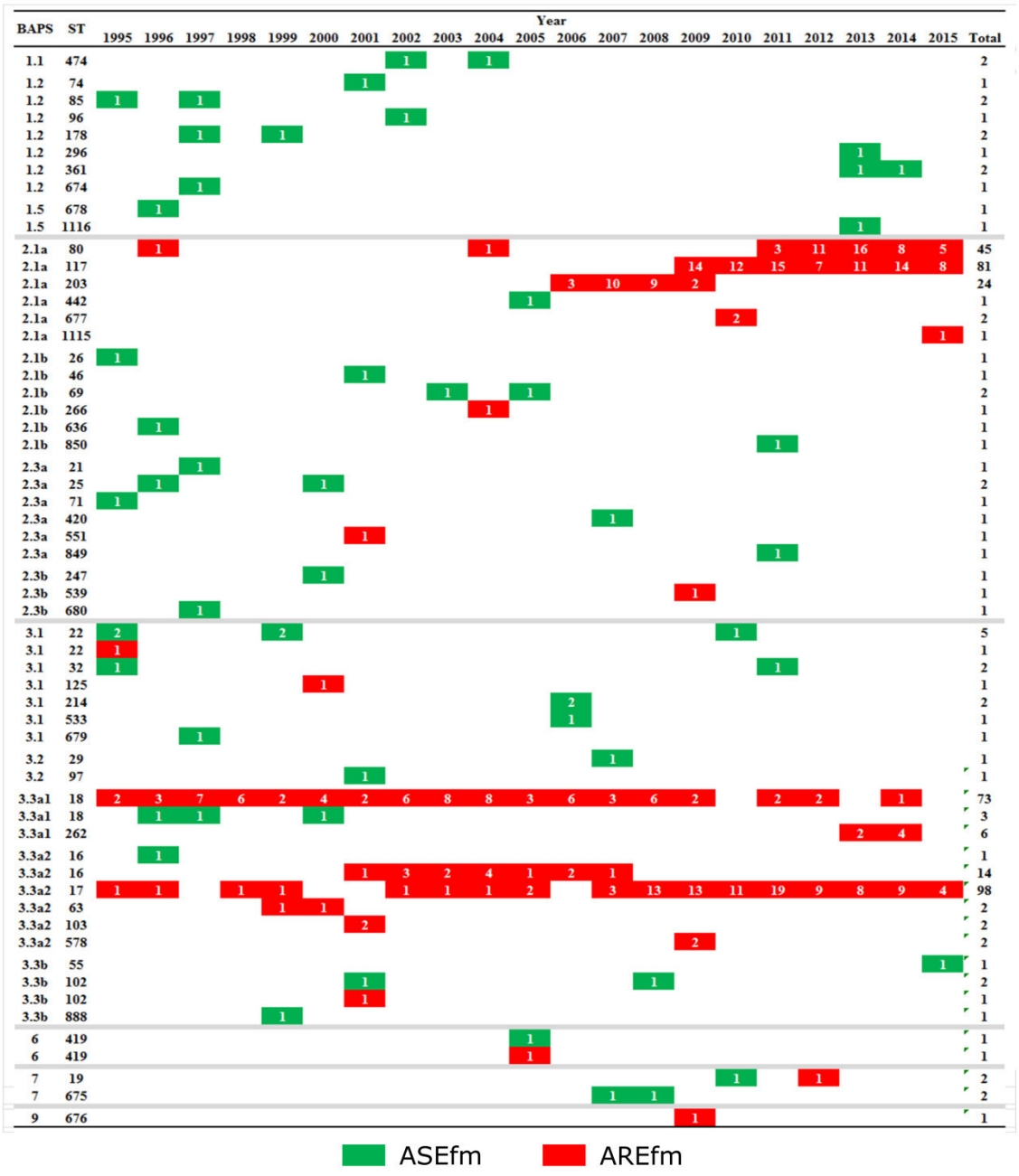

Figure 5. Distribution of AREfm and ASEfm by ST per year in Hospital Ramón y Cajal.

The steady increase in the number of *E. faecium* BSI (Figure 1) in our hospital appears to be also linked to subsequent outbreaks of clones belonging to lineage 78. After the blooming of different ST203 clonal variants (AREF-9 and later also AREF-15 and AREF-20) in 2006, a ST117 strain (AREF-25) became endemic in our hospital from 2009 to 2015. An ST80 clone (AREF-68) firstly identified in 2011, is frequently detected in our hospital nowadays. Isolates exhibiting related PFGE-types (up to six bands of difference) and persistently recovered for years (e.g. ST117-AREF-25, ST203-AREF-9, ST16-AREF-T, ST18-AREF-D among others) differed in the number, size and type of plasmids they harboured (data not shown).

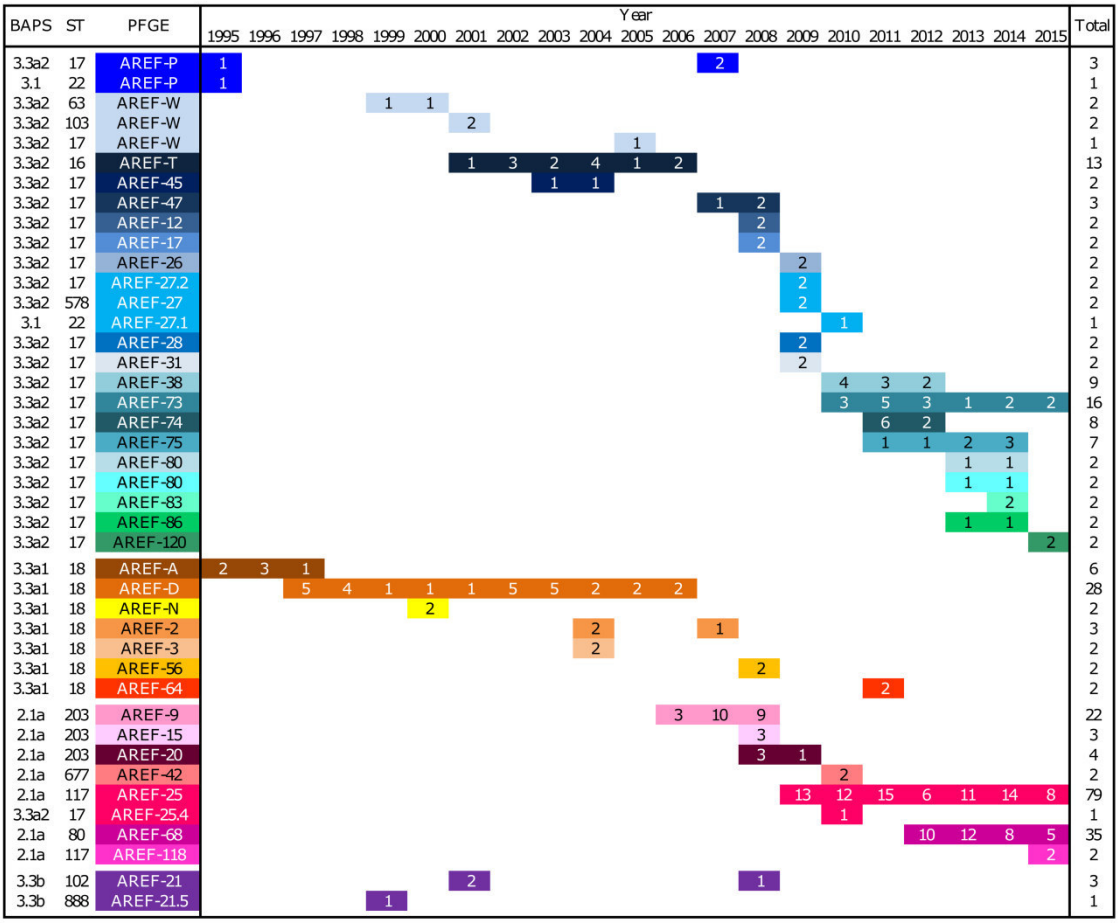

Figure 6. Distribution of prevalent PFGE-types in Hospital Ramón y Cajal throughout the study period.

It is of note that some ASEfm strains were also identified over the entire period of study as ASEF-21 (1999-2008, corresponding to ST102 and SLV ST888 within BAPS 3.3b) or ASEF-2 (1995-2011; ST22, BAPS 3.1). These strains have previously been identified in non-hospitalized colonized individuals.[12]

DISCUSSION

This work constitutes one of the few long-term longitudinal studies addressing the occurrence of enterococcal infections in the hospital setting during 20 years and documents a remarkable increase of *E faecium* BSI in the last decade in our Hospital. The coincidental increase in the number of *E. faecium* BSI cases and the number of cancer patients with BSI *E. faecium* episodes with the "blooming" of certain AREfm lineages in our hospital during the 2006-2015 period, can also be indirectly inferred from the data of multicentre studies[1,23], studies confined to a single institution[13],or surveys focused on VREf, MDR isolates, or particular groups of patients (e.g. cancer patients).[9]

Previous antibiotic treatment is traditionally considered as one of the main risk factors for acquisition of BSI by enterococci.[24] Nowadays, therapy with broad spectrum β-lactams (including carbapenems) or levofloxacin, to treat or prevent infections caused by MDR *Enterobacteriaceae* in onco-haematological patients with febrile neutropenia[9] has dramatically increased since the mid-2000s in many hospitals, including ours[25], and is recognized as one of the main risk factors for acquisition of BSI by MDR Gram

positive organisms.[9,26,27] Differences in chemotherapy composition or clinical practice guidelines also seem influence acquisition and intestinal persistence of AREfm.[25]

The recovery of *E. faecium* of 7 BAPS groups that are normal components of the human gut microbiota from blood cultures[12], suggest that bacterial translocation and invasive processes of stochastic nature can eventually be enhanced when an increase in the populations size occurs (classically known as "colonization pressure")[28]. However, STs of major hospital associated lineages ST17, ST18 and ST78, previously designed as "high-risk clonal complexes" (HiRCCs) in most clinical surveys[29,30] were also predominant in our series when the whole sample was considered. At a first glance, the high rate of *E. faecium* isolates would be the result of clonal "waves" dynamics involving different HiRCCs as suggested.[10,11] This trend, initially observed for *Staphylococcus aureus*[31], seems to imply the successive selection, expansion, and evolution of certain clones, following hospital microepidemics.[2] Besides clonal expansion of specific strains, an increasingly high intra-clonal diversification of *E. faecium* strains belonging to ST17, ST18 and ST78 lineages, was found in this and also in other works[8], reflecting the "*ex unibus plurum*" evolutionary dynamics.[32]

The contemporary increase of isolates within BAPS 2.1a (ST117, ST192, ST203) and BAPS 3.3a2 (ST17) in the hospital environment is intriguing. Recent studies have recently highlighted the relevance of microbial inheritance in basic processes of infectious diseases[33] and the impact of antibiotic treatments in the composition and structure of microbial communities.[34] Although cross transmission among individuals remains a key factor in the epidemiology of *E. faecium*, the importance of the various resident *E. faecium* populations, eventually changing in frequency with antibiotic exposure and age, comes to light in this and other works.[12] It has been recently demonstrated that heterogeneity of *S. aureus* populations that colonize humans, designated as "clouds", leads to a possible global adaptive benefit for certain clones and finally for the overall species, that is enhanced by horizontal gene transfer (HGT).[35,36] This might also explain what was observed in *E. faecium*. One of the most remarkable features of major *E. faecium* lineages of this species is the content of mobile genetic elements.[37] HGT among co-occurring related clones assuring a common pool of adaptive plasmids interacting between clones and clonal variants is increasingly documented.[38]

In summary, the increasing occurrence of invasive infections caused by MDR *E. faecium* strains in hospitalized elderly patients and particularly among cancer patients, associated with high mortality rates, constitutes an emerging problem that is becoming as serious antibiotic resistance threat as VRE.[39] Detailed longitudinal studies of changes in the *E. faecium* population structure will be required to associate different clones and subclones with particular types of patients and drug exposures and to understand the relative risks of the different enterococcal populations.

FUNDING

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REFERENCES

1. Hidron AI, Edwards JR, Patel J, et al. *NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007*. Infect Control Hosp Epidemiol 2008; 29: 996–1011.

2. Arias CA, Murray BE. *The rise of the Enterococcus: beyond vancomycin resistance*. Nat Rev Microbiol 2012; 10: 266–78.

3. Fortun J. *Risk factors associated with ampicillin resistance in patients with bacteraemia caused by Enterococcus faecium*. J Antimicrob Chemother 2002; 50: 1003–9.

4. Maki DG, Agger WA.. Medicine (Baltimore) 1988; 67: 248–69. *Enterococcal bacteremia: clinical features, the risk of endocarditis, and management*

5. Grayson ML, Eliopoulos GM, Wennersten CB, et al. *Increasing resistance to beta-lactam antibiotics among clinical isolates of Enterococcus faecium: a 22-year review at one institution*. Antimicrob Agents Chemother 1991; 35: 2180–4.

6. National Nosocomial Infections Surveillance System. *National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004, issued October 2004*. Am J Infect Control 2004; 32: 470–85.

7. Treitman AN, Yarnold PR, Warren J, Noskin GA. *Emerging incidence of Enterococcus faecium among hospital isolates (1993 to 2002)*. J Clin Microbiol 2005; 43: 462–3.

8. Lam MMC, Seemann T, Tobias NJ, et al. *Comparative analysis of the complete genome of an epidemic hospital sequence type 203 clone of vancomycin-resistant Enterococcus faecium*. BMC Genomics 2013; 14: 595.

9. Gudiol C, Ayats J, Camoez M, et al. *Increase in bloodstream infection due to vancomycin-susceptible Enterococcus faecium in cancer patients: risk factors, molecular epidemiology and outcomes*. PLoS One 2013; 8: e74734.

10. Werner G, Coque TM, Hammerum AM, et al. *Emergence and spread of vancomycin resistance among enterococci in Europe*. Euro Surveill 2008; 13.

11. Willems RJL, Top J, van Schaik W, et al. *Restricted gene flow among hospital subpopulations of Enterococcus faecium*. MBio 2012; 3: e00151–12.

12. Tedim AP, Ruiz-Garbajosa P, Corander J, et al. *Population Biology of Intestinal Enterococcus Isolates from Hospitalized and Nonhospitalized Individuals in Different Age Groups*. Appl Environ Microbiol 2015; 81: 1820–31.

13. Johnson PDR, Ballard SA, Grabsch EA, et al. *A sustained hospital outbreak of vancomycin-resistant Enterococcus faecium bacteremia due to emergence of vanB E. faecium sequence type 203*. J Infect Dis 2010; 202: 1278–86.

14. Homan WL, Tribe D, Poznanski S, et al. *Multilocus sequence typing scheme for Enterococcus faecium*. J Clin Microbiol 2002; 40: 1963–71.

15. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. *eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data*. J Bacteriol 2004; 186: 1518–30.

16. Nesse RM, Bergstrom CT, Ellison PT, et al. *Evolution in health and medicine Sackler colloquium: Making evolutionary biology a basic science for medicine*. Proc Natl Acad Sci U S A 2010; 107 Suppl : 1800–7.

17. Coque TM, Willems RJL, Fortún J, et al. *Population structure of Enterococcus faecium causing bacteremia in a Spanish university hospital: setting the scene for a future increase in vancomycin resistance?* Antimicrob Agents Chemother 2005; 49: 2693–700.

18. CLSI. *Performance Standards for Antimicrobial Susceptibility Testing*; Twenty-Second Informational Supplement. Clin Lab Stand Inst 2013; 32: 1–184.

19. Werner G, Fleige C, Geringer U, van Schaik W, Klare I, Witte W. *IS element IS16 as a molecular screening tool to identify hospital-associated strains of Enterococcus faecium.* BMC Infect Dis 2011; 11: 80.

20. Vankerckhoven V, Van Autgaerden T, Vael C, et al. *Development of a multiplex PCR for the detection of asa1, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among European hospital isolates of Enterococcus faecium*. J Clin Microbiol 2004; 42: 4473–9.

21. RStudio Team. *RStudio: Integrated Development Environment for R*. 2015.

22. Magiorakos A-P, Srinivasan A, Carey RB, et al. *Multi-drug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance*. Clin Microbiol Infect 2012; 18: 268–81.

23. European Centre for Disease Prevention and Control. *Antimicrobial resistance surveillance in Europe 2013. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net)*. Stockholm; 2014.

24. Rice LB. *Antibiotics and gastrointestinal colonization by vancomycin-resistant enterococci*. Eur J Clin Microbiol Infect Dis 2005; 24: 804–14.

25. Sánchez-Díaz AM, Cuartero C, Rodríguez JD, Lozano S, Alonso JM, Rodríguez-Domínguez M, Tedim AP, Campo R del, Cantón R, Ruiz-Garbajosa P. 2016. *The rise of ampicillin-resistant Enterococcus faecium high-risk clones as a frequent intestinal colonizer in oncohaematologic neutropenic patients on levofloxacin prophylaxis: a risk for bacteraemia?* Clin Microbiol Infect. 22:59.e1-8.

26. Holland T, Fowler VG, Shelburne SA. *Invasive gram-positive bacterial infection in cancer patients*. Clin Infect Dis 2014; 59 Suppl 5: S331–4.

27. Taur Y, Xavier JB, Lipuma L, et al. *Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation*. Clin Infect Dis 2012; 55: 905–14.

28. Bonten MJ, Willems R, Weinstein RA. *Vancomycin-resistant enterococci: why are they here, and where do they come from?* Lancet Infect Dis 2001; 1: 314–25.

29. Baquero F, Coque TM. *Multilevel population genetics in antibiotic resistance*. FEMS Microbiol Rev 2011; 35: 705–6.

30. Willems RJL, Hanage WP, Bessen DE, Feil EJ. *Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance*. FEMS Microbiol Rev 2011; 35: 872–900.

31. Chambers HF, Deleo FR. *Waves of resistance: Staphylococcus aureus in the antibiotic era*. Nat Rev Microbiol 2009; 7: 629–41.

32. Baquero F. *The 2010 Garrod Lecture: the dimensions of evolution in antibiotic resistance: ex unibus plurum et ex pluribus unum*. J Antimicrob Chemother 2011; 66: 1659–72.

33. Faith JJ, Colombel J-F, Gordon JI. *Identifying strains that contribute to complex diseases through the study of microbial inheritance*. Proc Natl Acad Sci U S A 2015; 112: 633–40.

34. Nobel YR, Cox LM, Kirigin FF, et al. *Metabolic and metagenomic outcomes from early-life pulsed antibiotic treatment.* Nat Commun 2015; 6: 7486.

35. Golubchik T, Batty EM, Miller RR, et al. *Within-host evolution of Staphylococcus aureus during asymptomatic carriage*. PLoS One 2013; 8: e61319.

36. Stanczak-Mrozek KI, Manne A, Knight GM, Gould K, Witney AA, Lindsay JA. *Within-host diversity of MRSA antimicrobial resistances*. J Antimicrob Chemother 2015; 70: 2191–8.

37. Lebreton F, Willems RJL, Gilmore MS. *Enterococcus Diversity, Origins in Nature, and Gut Colonization*. *In*: Gilmore MS, Clewell DB, Ike Y, Shankar N, eds. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Boston: Massachusetts Eye and Ear Infirmary: Massachusetts Eye and Ear Infirmary, 2014.

38. Freitas AR, Novais C, Tedim AP, et al. *Microevolutionary Events Involving Narrow Host Plasmid Influences Local Fixation of Vancomycin-Resistance in Enterococcus*. PLoS One 2013: e60589.

39. Centers for Disease Control and Prevention. *Antibiotic Resistance Threats in the United States*. 2013.

SUPPLEMENTARY MATERIAL

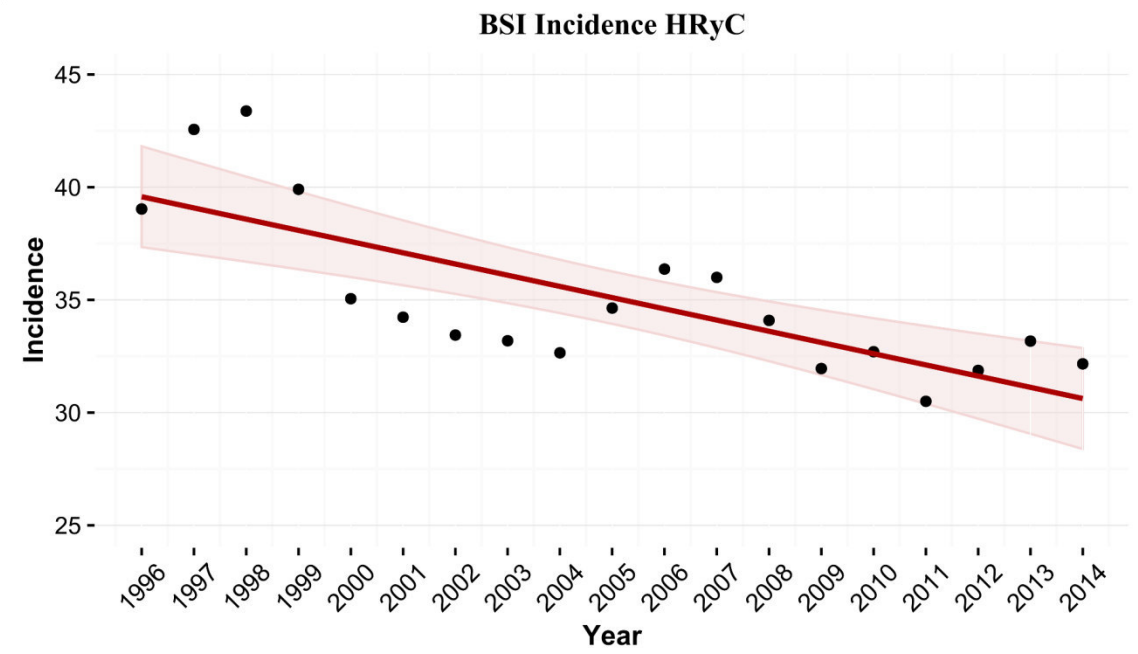


Figure S1. Trend line throughout the study period of BSI incidence in Hospital Ramón y Cajal.

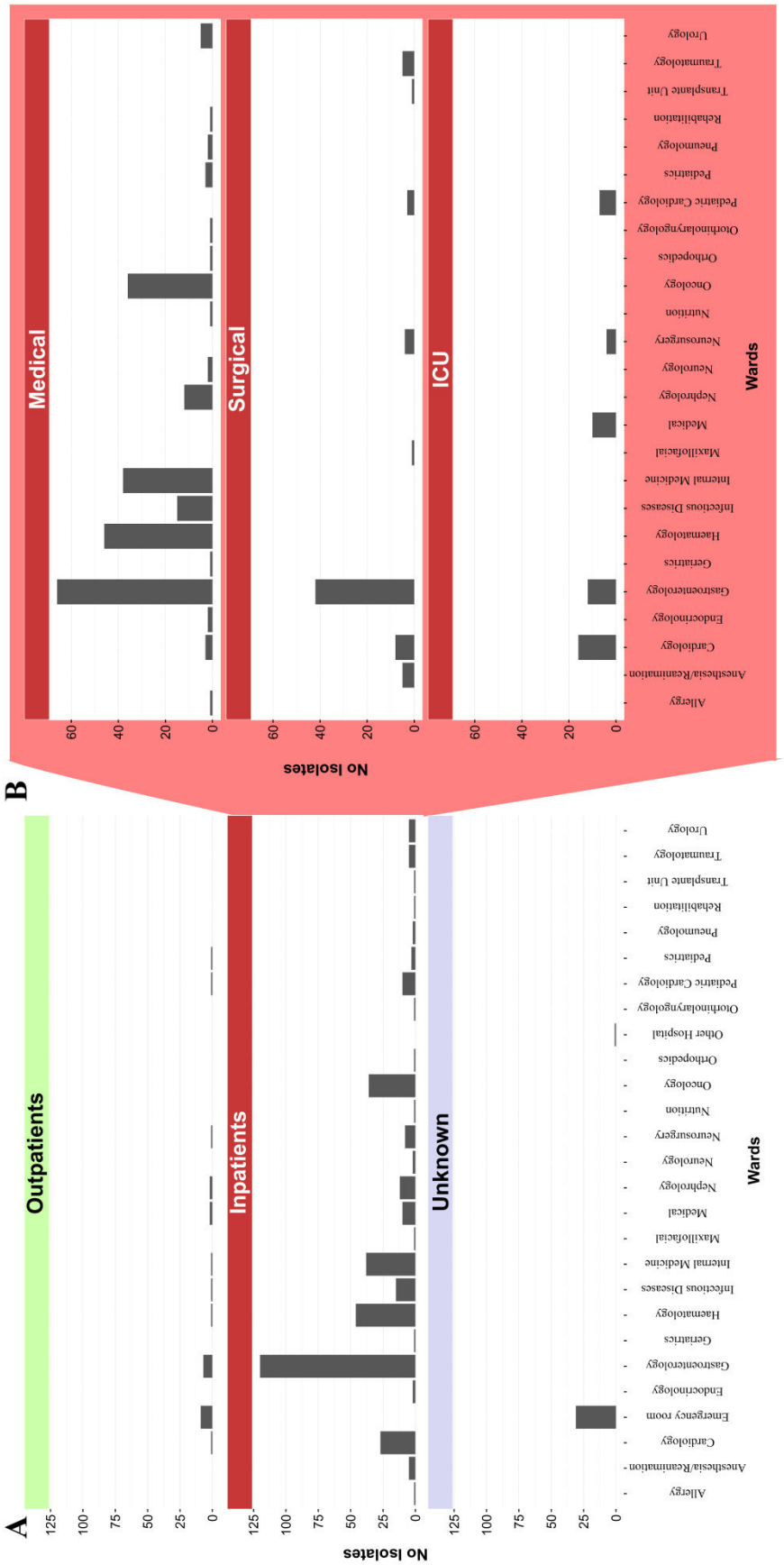


Figure S2. Distribution of *E. faecium* BSI infections. A) Distribution among inpatients and outpatients. B) Distribution of inpatients BSI among different wards.

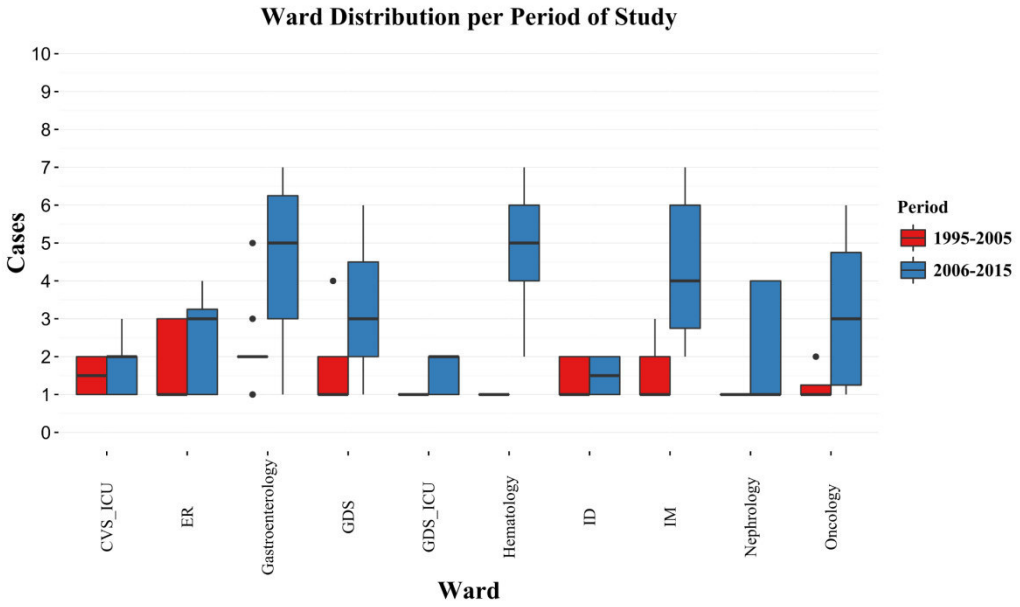


Figure S3. Wards with significant increase in *E. faecium* BSI in the period of 2006 to 2015.

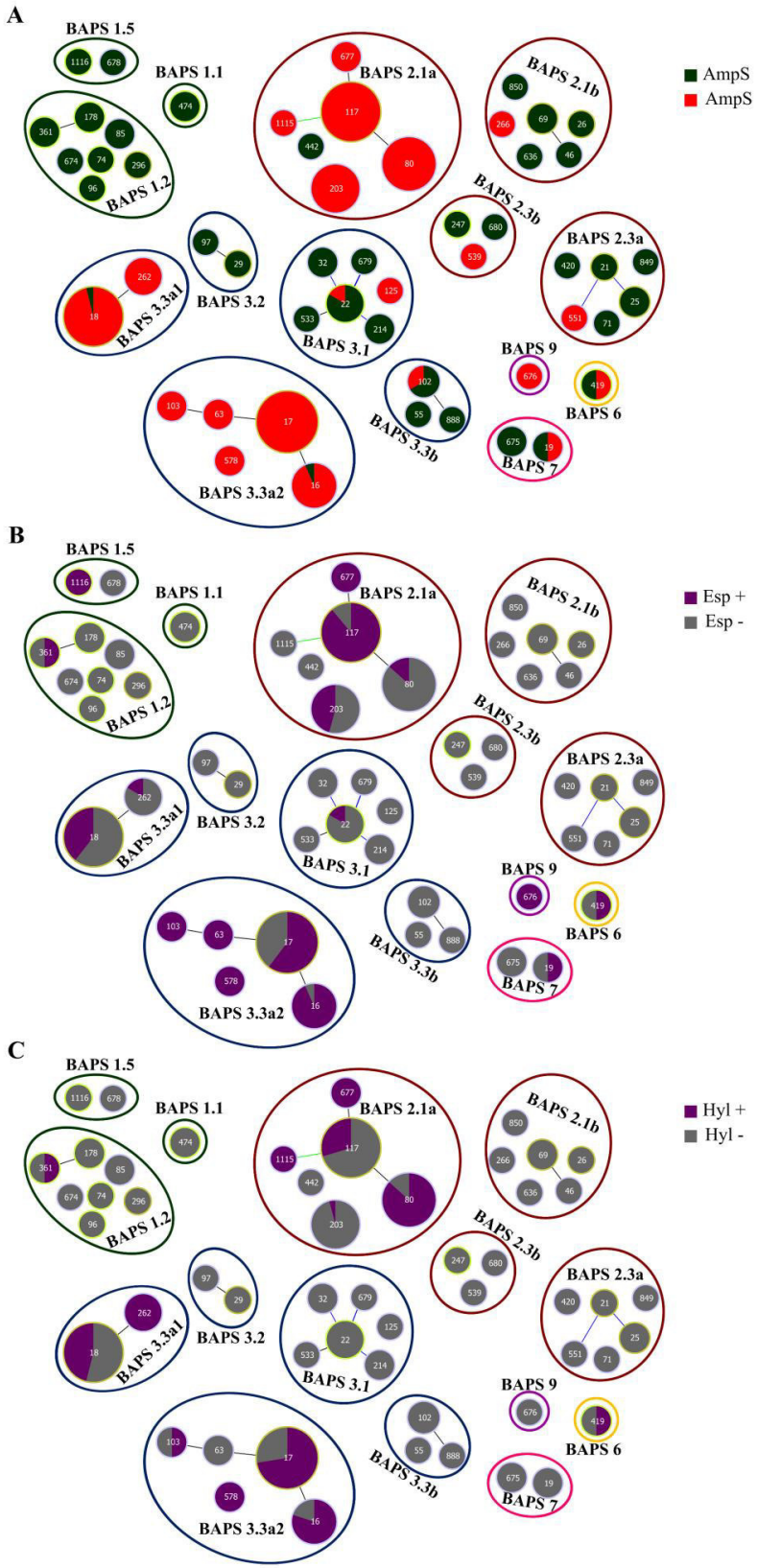


Figure S4. Population structure of *E. faecium* by A) ampicillin susceptibility, B) by presence/absence *esp*; and C) by presence/absence hyl_{Efm} (C).

E. faecium population BAPS analysis

BAPS software was used to probabilistically assign *E. faecium* STs to non-overlapping evolutionary groups as previously described using a dataset corresponding to 1115 STs.[1–3] ST998 was excluded from the analysis because its *ddl* allele (*ddl*_67) was truncated by an IS*Efm1* and the consequent change in the fragment size greatly influenced the analysis. The accuracy of this BAPS analysis for establishing *E. faecium* population structure was determined by comparison with the most recent dataset described by Tedim *et al* (see supplementary data Figure S4). Correlation analysis was performed using Microsoft Excel 2010.[3]

A hierarchical BAPS clustering analysis of the currently available 1115 *E. faecium* STs yield 9 BAPS groups. The majority of STs grouped in BAPS 1, BAPS 2, BAPS 3 and BAPS 7 (15.9%, 39.2%, 30.4%, and 7.0%, respectively) while BAPS 4, BAPS 5, BAPS 6, BAPS 8 and BAPS 9 were much more infrequently detected (1.2%, 1.5%, 1.1%, 1.2% and 2.5%, respectively). BAPS nested analysis subdivided BAPS 1 in six subgroups (BAPS1.1-1.6), BAPS 2 in four subgroups (BAPS 2.1a, 2.1b, 2.3a, 2.3b), and BAPS 3 in 5 subgroups (3.1, 3.2, 3.3a1, 3.3a2, 3.3b) (Figure S5 supplementary data). The main change regarding Tedim *et al* BAPS analysis is the split of BAPS 3.3a in to subgroups BAPS 3.3a1 corresponding to lineage 17 and BAPS 3.3a2 corresponding to lineage 18 and the split of BAPS 7 originating the new BAPS group BAPS 9. BAPS 7 from this study comprises all BAPS 7.1 isolates from Tedim *et al* and BAPS 9 comprises all isolates from BAPS 7.2-7.4 and one BAPS 1.6 isolate [3].

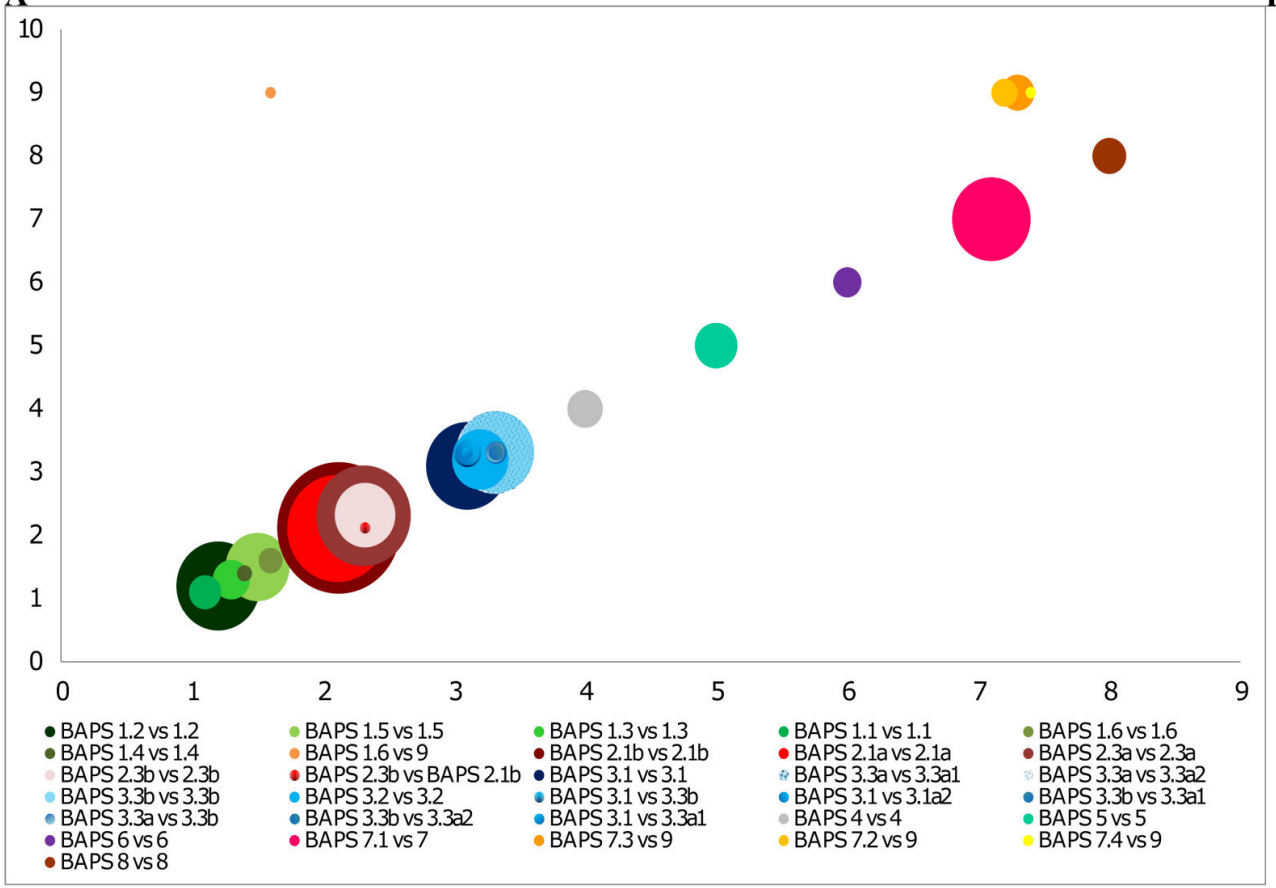
We analyzed the congruence between the BAPS grouping of 837 STs using the BAPS assignment as described previously by Tedim *et al* [3] and the BAPS grouping from this study. A correlation coefficient of 0.9795 indicates an almost perfect correlation between these two analysis, indicating that only a small number of changes occurred (41/1115 STs, 3.7%) in BAPS assignment, either at a group or subgroup level, when the number of STs analyzed was significantly increased. This result further confirms the conclusion from the last BAPS analysis for *E. faecium* performed by our group highlighting this tool is both reproducible and robust and may accurately describe the *E. faecium* population structure.

References

1. Corander J, Marttinen P, Sirén J, Tang J. Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. BMC Bioinformatics 2008; 9: 539.

2. Willems RJL, Top J, van Schaik W, et al. Restricted gene flow among hospital subpopulations of Enterococcus faecium. MBio 2012; 3: e00151–12.

3. Tedim AP, Ruiz-Garbajosa P, Corander J, et al. Population Biology of Intestinal Enterococcus Isolates from Hospitalized and Nonhospitalized Individuals in Different Age Groups. Appl Environ Microbiol 2015; 81: 1820–31.



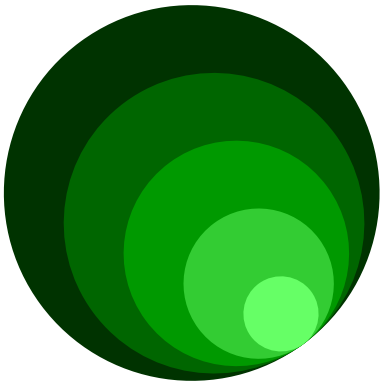
B

BAPS			
Group	Subgroup	No STs	% STs
BAPS 1	1.1	11	6,2%
	1.2	81	45,8%
	1.3	17	9,6%
	1.4	3	1,7%
	1.5	58	32,8%
	1.6	7	4,0%
Total		177	15,9%
BAPS 2	2.1a	143	32,7%
	2.1b	156	35,7%
	2.3a	97	22,2%
	2.3b	41	9,4%
Total		437	39,2%
BAPS 3	3.1	74	21,8%
	3.2	42	12,4%
	3.3a1	72	21,2%
	3.3a2	80	23,5%
	3.3b	72	21,2%
Total		340	30,5%
BAPS 4		13	1,2%
BAPS 5		17	1,5%
BAPS 6		12	1,1%
BAPS 7		78	7,0%
BAPS 8		13	1,2%
BAPS 9		28	2,5%

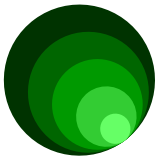
Figure S5. A) Correlation between the *E. faecium* BAPS groups obtained using the *E. faecium* dataset (837 STs) (Y) or using an updated database (1115 STs) (X). The size of each circle represents the population size included in each group.[2,3]. B) *E. faecium* BAPS analysis data.

I don't pretend we have all the answers. But the questions are certainly worth thinking about

Arthur C. Clarke



Chapter 3



The Plasmidome of *Firmicutes*: Impact on the Emergence and the Spread of Resistance to Antimicrobials

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ABSTRACT

The phylum *Firmicutes* is one of the most abundant groups of prokaryotes in the microbiota of humans and animals and includes genera of outstanding relevance in biomedicine, health care, and industry. Antimicrobial drug resistance is now considered a global health security challenge of the 21st century, and this heterogeneous group of microorganisms represents a significant part of this public health issue.

The presence of the same resistant genes in unrelated bacterial genera indicates a complex history of genetic interactions. Plasmids have largely contributed to the spread of resistance genes among *Staphylococcus*, *Enterococcus*, and *Streptococcus* species, also influencing the selection and ecological variation of specific populations. However, this information is fragmented and often omits species outside these genera. To date, the antimicrobial resistance problem has been analyzed under a "single centric" perspective ("gene tracking" or "vehicle centric" in "single host-single pathogen" systems) that has greatly delayed the understanding of gene and plasmid dynamics and their role in the evolution of bacterial communities.

This work analyzes the dynamics of antimicrobial resistance genes using gene exchange networks; the role of plasmids in the emergence, dissemination, and maintenance of genes encoding resistance to antimicrobials (antibiotics, heavy metals, and biocides); and their influence on the genomic diversity of the main Gram-positive opportunistic pathogens under the light of evolutionary ecology. A revision of the approaches to categorize plasmids in this group of microorganisms is given using the 1,326 fully sequenced plasmids of Gram-positive bacteria available in the GenBank database at the time the article was written.

INTRODUCTION

Firmicutes constitutes one of the dominant bacteria phyla of human and animal gut microbiota. It comprises a number of genera of outstanding relevance in health care and industry such as *Staphylococcus*, *Listeria*, and lactic acid bacteria (LAB), a group of microorganisms that ferment carbohydrates into lactic acid and that includes the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, and *Weisella*. Furthermore, species of *Negativicutes* (*Selenomonas*, *Veillonella*) and *Clostridium* have clinical interest for humans and animals (Table 1).

Antibiotic resistance (AbR) in this heterogeneous group of organisms constitutes a significant part of the public health problem. The most recent report by the Centers for Disease Control and Prevention in the United States provides a ranking list of AbR human pathogens according to their threat level to society and the attention that such a problem requires. Gram-positive organisms were grouped in the categories of "urgent" (*Clostridium difficile*), "serious" (methicillin-resistant *Staphylococcus aureus* [MRSA], antibiotic-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus* [VRE]), and "concerning" (erythromycin-resistant *Streptococcus pyogenes* and clindamycin-resistant *Streptococcus agalactiae*) on the basis of the limited therapeutic options to treat infections caused by these bacteria-resistant variants (1).

LAB, which are used as probiotics and in the preparation of various products (dairy, fermented meat and seafood, fermented cereals and vegetables, wine), are defined as "generally regarded as safe" (GRAS) microorganisms by the U.S. Food and Drug Administration. However, the potential risk to transfer acquired AbR genes recently found in LAB species to animal and human pathogens is a cause for concern. AbR LAB may also contaminate industrial processes, leading to economic losses (2). In addition, the possibility that opportunistic or commensal bacteria and non-pathogen organisms could serve as reservoirs of AbR genes is increasingly recognized (3). Consequently, several European and American regulatory agencies have recently recommended the mandatory screening of some species such as *Enterococcus faecalis* and *Enterococcus faecium* as indicators of the presence of AbR in foods and food animals and as a mirror of the patterns of antibiotic use in veterinary medicine and agriculture (4, 5). Finally, it is worth mentioning that AbR in a context of the wide use of antibiotics favors the selection of clonal lineages of multihost species with zoonotic potential (e.g. *S. aureus*, *E. faecium, Clostridium perfringens*) as well as emblematic zoonotic species such as *Listeria monocytogenes* (see below).

The presence of the same AbR genes in ecologically connected (but also in unconnected) bacterial genera, mentioned above, indicates a complex history of genetic interactions in which AbR genes have parasitized the natural circuits of adaptive gene flow. Plasmids have largely contributed to the spread of AbR and other adaptive genes among members of *Staphylococcus*, *Enterococcus*, and to a lesser extent, species of the *Streptococcus* pyogenic group (6–8), thus influencing the selection of particular subspecies populations due to the acquisition of AbR (8–10). However, the global adaptive role of plasmids of other genera remains largely unexplored outside single pathogens colonizing or infecting

single “relevant” hosts. The “single centric” perspective, focusing on “gene tracking” or “vehicle centric” (plasmid, transposon, or other mobile genetic elements [MGEs]) in “single host-single pathogen” systems hampers a comprehensive view of gene and plasmid dynamics and their role in the evolvability of bacterial communities. An integrative view of plasmid ecology is needed to understand community evolvability.

Table 1. Fully characterized plasmids from low G+C bacteria available in GenBank database

Phylum/class	Order	Family	Plasmid			
			Total	AbR	Met^R	AbR+Met^R
Actinobacteria			**252**	**16**	**40**	**7**
	Actynomicetales	Streptomycetaceae	56	2	4	2
		Corynebacteriaceae	38	10	7	3
		Nocardiaceae[a]	30	0	9	0
		Mycobacteriaceae[b]	29	2	7	2
		Micrococcaceae[c]	27	1	7	0
		Pseudonocardiaceae[d]	9	0	1	0
		Propionibacteriaceae	7	0	0	0
		Gordonaciaee	6	0	3	0
		Microbacteriaceae[e]	4	0	0	0
		Streptosporangiaceae[f]	2	0	0	0
		Nocardiopsaceae	1	0	0	0
		Nocardioidaceae	1	0	0	0
		Promicromonosporaceae[g]	1	0	0	0
		Micromonosporaceae	1	0	0	0
		Kineosporiineae	2	0	1	0
		Brevibacteriaceae	2	0	0	0
		Frankiaceae	3	0	0	0
		Tsukamurellaceae	1	0	0	0
	Bifidobacteiales	Bifidobacteriaceae	29	0	0	0
Firmicutes			**1073**	**244**	**178**	**85**
Negativicutes	Selenomonadales		17	0	1	0
Clostridia			86	7	4	0
Erysipelotrix			1	0	0	0
Bacilli			969	237	121	85
	Lactobacillales					
		Lactobacillaceae	172	16	19	3
		Streptococcaceae	133	15	4	0
		Enterococacceae	74	24	3	2
		Leuconostoc	29	0	9	0
		Carnobacteriaceae	6	0	1	0
		Oenococcus	6	0	0	0
		Weisella	4	0	0	0
	Bacillales	Staphylococcaceae	275	175	118	80
		Bacillaceae	223	5	9	0
		Listeriaceae	14	1	8	0
		Paenobacillaceae	9	0	0	0
		Macrococcus	8	1	0	0
		Planococaceae	6	0	1	0
		Bacillales group XII	4	0	0	0
		Alyciclobacillaceae	3	0	0	0
		Bhargaceae	2	0	0	0

[a]Nocardiaceae (4 Nocardia 25 Rhodococcus); [b]Mycobacteriaceae (Mycobacterium plus Amycolicicoccus.); [c]Micrococcaceae (4 Micrococcus 23 Arthrobacter); [d]Pseudonocardiaceae (2 Amycolatopsis, 6 Pseudonocardia, 1 Saccharomonospora); [e]Microbacteriaceae (4 Clavibacter); [f]Streptosporangiaceae (1 Planobispora, 1 ;Streptosporangium); [g]Promicromonosporaceae (Xylanimonas); (updated September 2014).

In this work, we analyze the development of AbR in *Firmicutes* within an ecological framework using gene exchange networks. We also discuss the role of plasmids in the emergence, spread, and maintenance of genes encoding resistance to antimicrobials (antibiotics, heavy metals, and biocides) and their influence on the genomic diversity of the main Gram-positive opportunistic pathogens in the light of evolutionary ecology. Finally, a critical revision of plasmid classifications in this group of

microorganisms is also provided under this eco-evo perspective by analyzing the 1,326 fully sequenced plasmids of Gram-positive bacteria (*Firmicutes* and *Actinobacteria*) available in the GenBank database at the time this article was written.

AN ECO-EVO PERSPECTIVE TO ANALYZE HGT IN FIRMICUTES

Recent phylogenomic analyses using networks revealed a history of horizontal gene transfer (HGT) events even among highly structured and ecologically disconnected groups of bacteria (11–13). These events are more likely to occur in the case of donors and recipients with a similar G+C content (differing in <5% for 86% of connected pairs) (14) and involving plasmids able to mediate exchange of information between close or distant chromosomal backgrounds (12, 15). Although limited by the current number of available genome sequences, such studies evidenced sound differences in "betweeness" among different bacterial groups and plasmids of *Firmicutes*. LAB frequently undergo HGT events among similar species (11), with streptococci acting as a hub for interactions with more distant ecological groups (12), and some plasmids of the Inc18 family possibly contributing to the spread of AbR genes among different bacterial species (15). To analyze this situation in more detail, we constructed a gene exchange network that comprises all genes conferring resistance to antibiotics and heavy metals described in *Firmicutes* so far (Fig. 1 and 2). This network clearly shows that many resistance genes in different bacterial genera can present plasmid and/or chromosomal locations, illustrating the diversity of interactions, often plasmid mediated, within bacterial communities (Fig. 1 and 2). Available (and often fragmented) knowledge from different fields enabled us to state that the dynamics of bacterial populations are influenced by the interplay of selection processes at different levels of organization (genes, MGEs, clones, species) and their associated environments (16–20). Because of that, the complexity resulting from such interplay cannot be understood using either single centric studies or the above-mentioned phylogenomic analysis of HGT networks.

The presence of the same genes in different genetic contexts implies contacts and exchanges between bacteria belonging to different genera, probably facilitated in complex biofilms and environments allowing high local bacterial densities. HGT via transduction or conjugative mechanisms has been extensively documented in *Lactobacillales* and is a prominent process for niche-specific adaptation in different genera (12, 21–23), with plasmids and conjugative transposons being the most relevant providers of communal adaptive gene pools in microbial ensembles sharing complex niches.

An important question is if resistance genes contributed to the recombination between different replicons and, consequently, to their evolvability. The frequent association of resistance genes with site-specific recombination systems and insertion sequences located either in plasmids or in chromosomes favors homologous recombination and therefore different events of integration or excision, as well as the interplay among different elements (19, 24–28). Restriction-modification (RM) systems and clustered regularly interspaced short palindromic repeats (CRISPR) are the main post-transfer barriers protecting a given host cell from invasion by foreign DNA either by conjugation, transformation or

transduction (9, 29, 30). Some RM systems specifically limit the acquisition of plasmids to some pathogens which may influence their clonal structure (e.g., RM types I, III, and IV in *S. aureus*) (31, 32). This may also explain the lack of plasmids in certain species such as *S. pneumonia* or the narrow host range of plasmids from some *Clostridium* species (33, 34). Anti-RM systems such as analogues of ArdA (alleviation of restriction of DNA) proteins that act against type I restriction systems (detected in Tn*916* and CTn*6000*) or other genes predicted to be involved in methylation (e.g., in CTn*6000* and Tn*1721*) are involved in the restricted spread of certain MGEs, as well as in certain clonal expansions. There is evidence that the presence of complete CRISPR loci is inversely proportional to the presence of MGEs in *Clostridium* and *Staphylococcus* (34, 35), a situation that has also been suggested to occur for *Streptococcus* and *Enterococcus* (34, 36, 37). In agreement with this statement, Fig. 1 and 2 reflect a heterogeneous distribution of AbR and heavy metal and biocide resistance (MetR and BcR) genes in different genera. Particularly interesting is the confinement of vancomycin resistance within enterococci and of some AbR, MetR, and BcR genes within staphylococci and clostridia, a situation that is in part due to the barriers shaping different populations (see next sections).

Fluctuating environments, concentration gradients, and high population sizes, all frequent in different "source-sink" ecologies such as bacterial populations under antimicrobial selective pressure, favor DNA transfer and the selection of some clonal and plasmid variants (38–42). Such selective processes favor the emergence of novel variants, resulting from genetic drift, or migration to heterogeneous environments. Recent studies demonstrated that plasmid transfer occurs *in vivo* more frequently than in experimental evolution assays (43), and that gradients in populations under selection pressure by diverse antimicrobials favor selection of multi-drug resistance (MDR) plasmids (44, 45).

The influence of plasmids has been extensively discussed in the literature from different evolutionary perspectives (20, 46–48), but only limited information about plasmid ecology and the specific roles that plasmids actively play within microbial systems *in situ* is available. Mosaic MGEs have often been documented in *Firmicutes*, reflecting a different epidemiological history of contacts with strains of the same or different species. Some mosaic MGEs have been fixed, making the interpretation of results by using traditional classification schemes extremely difficult (49).

PLASMID DIVERSITY AND CLASSIFICATION SYSTEMS

Efforts in plasmid characterization and classification are justified for the understanding of plasmid biology. Nowadays, plasmid categorization is relevant from the public and environmental health perspective to follow the movement of genes coding for resistance to antimicrobials (antibiotics, heavy metals, biocides), colonization and virulence factors for humans and animals, and/or other adaptive traits that drive ecological success (bacteriocins, metabolic traits) and consequently increase the population size of bacteria harboring MGEs. In fact, only a "representative diversity" of bacterial plasmids has been systematically analyzed in a few genera of multihost opportunistic pathogens of

interest in biomedicine, with a particular emphasis on species of the *Enterobacteriaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, and *Enterococcaceae* families (7, 50–53). The diversity of plasmids from *Lactococcus* (54), *Lactobacillus* (55), *C. perfringens* (56), *Micrococcus* (57), and *Bifidobacterium* (58) has also been analyzed from different perspectives.

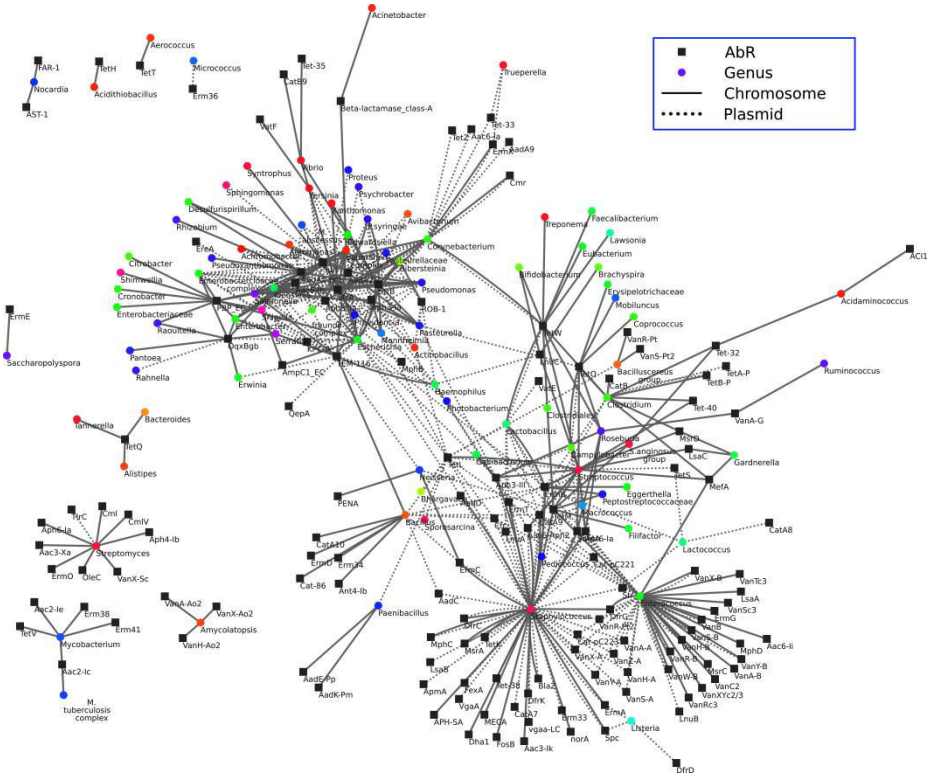

Figure 1. Protein content network (PCN) of AbR proteins found in plasmids and chromosomes of *Firmicutes* and *Actinobacteria*.

To determine the AbR protein catalog of Gram-positive strains (chromosomes and plasmids), a Blastp search was performed of all their proteomes against the ARG-ANNOT database (http://en.mediterranee-infection.com/article.php?laref=283&titre=arg-annot) using a cut-off of 1e-30 and 85% of identity. The presence of the Gram-positive AbR proteins identified above in all bacterial species (only complete sequences, not partial) was determined using a similar Blast search (blastp, 1e-30 E-value and 85% identity) against the NCBI GenBank database. The nodes correspond to bacterial species (circular nodes; each color indicates one genus) and AbR proteins (square nodes). Nodes were connected by an edge when a positive hit between AbR proteins and one or more strains of a given species were identified. Edges further indicate the location of the AbR genes associated with each AbR protein of the Gram-positive catalog. Solid lines represent chromosomal location, and dotted lines represent plasmid location. When an AbR gene was located in both chromosomes and plasmids, both lines were plotted.

Plasmid diversity within a particular bacterial species in the *Firmicutes* phylum started to be comprehensively analyzed in the 1960s just after the discovery of staphylococcal plasmids. These elements were initially categorized into three main classes designated by roman numerals on the basis of size, replication machinery, ability to be transferred, phenotypic and functional characteristics, and host range (7, 51, 53, 59, 60). Class I comprised high copy number plasmids (10 to 60 copies per cell) of less than 5 kb with a rolling circle replication (RCR) mechanism that often harbored one or two AbR genes (usually conferring resistance to tetracycline, chloramphenicol, macrolides, and trimethoprim). Class II comprised low copy number plasmids (4 to 6 copies per cell) of 15 to 40 kb, with a theta replication mechanism, which typically carried resistance to antibiotics (β-lactams, aminoglycosides, and

macrolides), heavy metals (arsenic, cadmium, and mercury), and/or antiseptics (quaternary ammonium compounds). Class III comprised plasmids similar to those found in class II which were transferred by conjugation (61). Afterward, Richard Novick and others classified staphylococcal plasmids in 15 incompatibility (Inc) groups based on the finding that two plasmids with the same replication (rep) proteins cannot be stably maintained in the same cell (50, 62, 63). Plasmids of most Inc groups correspond to class I (10 Inc groups of apparently closely related plasmids) and class II (diverse plasmids that belong to the same Inc group) (53). Following the same Inc numeral designation criteria, Brantl *et al* categorized a few streptococcal plasmids that replicated via a theta mechanism and that were regulated by an antisense RNA that mediated transcriptional attenuation, such as the Inc18 family (64) (see below). Pheromone-responsive plasmids of enterococci were also subgrouped into different incompatibility groups on the basis of distinct responses to small peptides or pheromones which are secreted by plasmid-free donors (65).

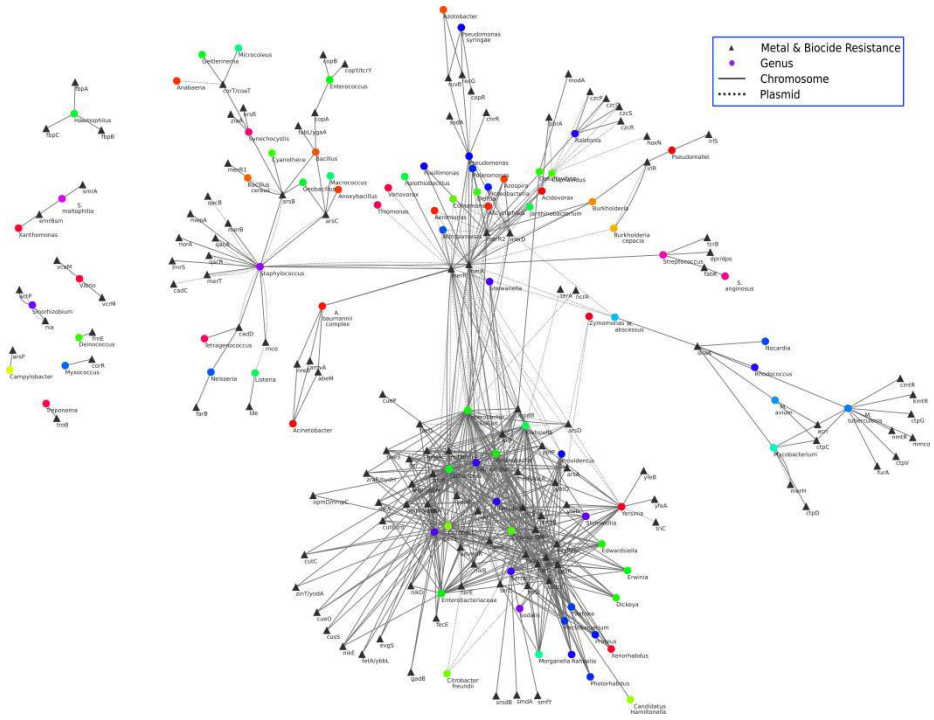

Figure 2. PCN of metal-biocide (MetR/BcR) proteins found in plasmids and chromosomes of *Firmicutes* and *Actinobacteria*.

To determine the MetR/BcR protein catalog of Gram-positive strains (chromosomes and plasmids), a Blastp search was performed of all their proteomes against the BacMet database (http://bacmet.biomedicine.gu.se/) using a cut-off of 1e-30 and 85% of identity. The presence of the Gram-positive MetR/BcR proteins identified above in all bacterial species (only complete sequences, not partial) was determined using a similar Blastp search (blastp, 1e-30 evalue and 85% identity) against the NCBI GenBank database. The nodes correspond to bacterial species (circular nodes) and MetR/BcR proteins (triangular nodes). Nodes were connected by an edge when a positive hit between MetR/BcR proteins on one or more strains of a given species was identified. Edges further indicate the location of the MetR/BcR genes associated with each MetR/BcR protein of the Gram-positive catalog. Solid lines represent chromosomal location, and dotted lines represent plasmid location. When a MetR/BcR gene was located in both chromosomes and plasmids, both lines were plotted.

A multiplex-PCR typing system based on the diversity of replication initiator proteins (RIPs) developed by Jensen *et al* (59) has recently been applied for the characterization of *Firmicutes* plasmids, mainly staphylococci (66) and enterococci (67–71) of human, animal, and environmental origin. According to

this typing system, RIP variants are designated as "rep" followed by a subindex number and are arbitrarily called Rep families. Although this system is very useful to enlarge the knowledge of scarcely explored plasmid diversity in contemporary isolates of enterococci and staphylococci, its application is limited to known plasmids, mainly AbR plasmids of these genera, as illustrated in various surveys and this study (59, 66, 68, 71).

The diversity of mobilization (MOB) systems has also recently been used to classify plasmids and other conjugative elements in different bacterial groups including *Firmicutes* (72, 73). The approach relies on the variability of relaxases (RELs), which form part of the plasmid MOB region, are involved in the initiation of DNA transfer, and that, aside from the origin of transfer (*oriT*), are present in both conjugative and mobilizable plasmids as well as in other conjugative elements (74). To date, only five (MOB_P, MOB_Q, MOB_V, MOB_C, and MOB_T) out of seven known REL families have been identified in *Firmicutes* (7, 72, 74). MOB_Q, MOB_C, and MOB_T are present in conjugative elements, and MOB_V is present in mobilizable plasmids. MOB_P has been identified in both conjugative and mobilizable elements (7, 72, 73). The application of this PCR-based classification scheme is obviously limited to the typing of known RELs. Frequent plasmid mosaicism, redundancy, and coexistence of different "core" genes, and the interplay of plasmids with other conjugative elements that contain homologs of RIPs and RELs, complicates the establishment of a robust plasmid core ontology and precludes the use of typing approaches similar to those used in Gram-negative organisms such as plasmid multilocus sequence type (http://pubmlst.org/plasmid/).

Whole-genome (plasmid/chromosome) sequencing provides accurate and non-biased information on plasmid backbones. Although the number of fully sequenced plasmids in databases is still limited, we used a plasmid homology network analysis of the 1,326 fully sequenced plasmids of *Firmicutes* and *Actinobacteria* to study the diversity of plasmids carrying genes coding for AbR and MetR/BcR and the impact of plasmids in the evolvability of contemporary AbR bacterial populations of *Firmicutes*.

Figures 3 and 4 illustrate the existence of group-specific plasmid populations, with a number of plasmids being shared between *Lactobacillales* (mainly *Enterococcus* and *Streptococcus*) and *Bacillales* (*Staphylococcus*), which are greatly implicated in the spread of AbR and MetR/BcR. These shared plasmids include RCR and theta-replicating plasmids of different families, which have been recently analyzed at the molecular level (7, 75, 76). Next in this section, we will analyze the diversity of these groups and highlight the usefulness of current typing systems for each group. However, it is of note that the main genera of *Firmicutes* carry a variable number of plasmids containing several replication and transfer systems, some of them being able to be transferred. The interplay between genes, plasmids, and populations will be analyzed under an ecological perspective in the section "Gene and Plasmid Flow Shapes the Evolutionary Ecology of Firmicutes."

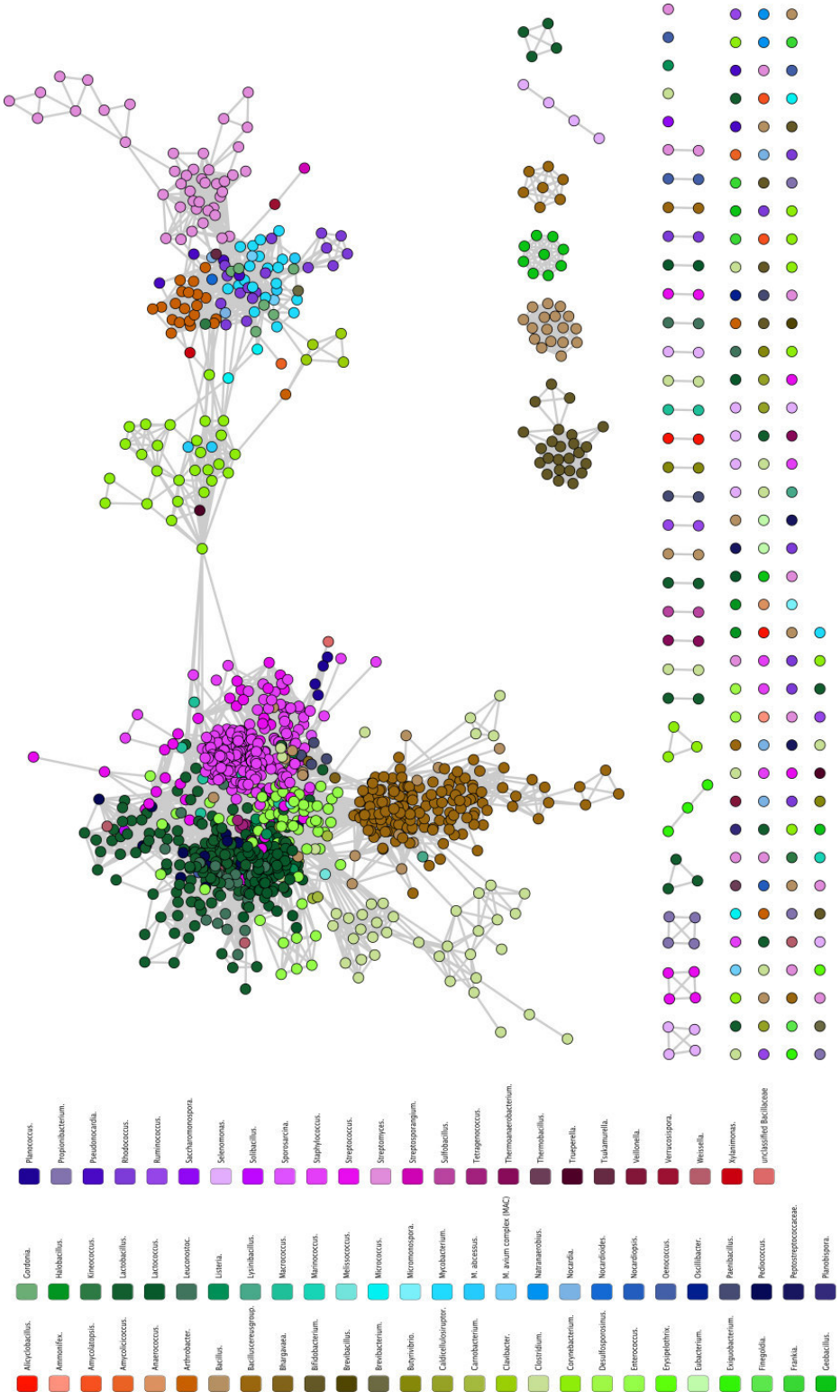

Figure 3. Plasmid homology network.

The genomic homology network was performed using "All-*versus*-All" genomic Megablast (239) of 1,326 fully sequenced plasmids from low G+C bacterial species (*Firmicutes* and *Actinobacteria* phyla) available at public gene databases. The nodes correspond to bacterial plasmids (circular nodes; different colors representing different genera). Two nodes are connected by an edge if they share homologous DNA.

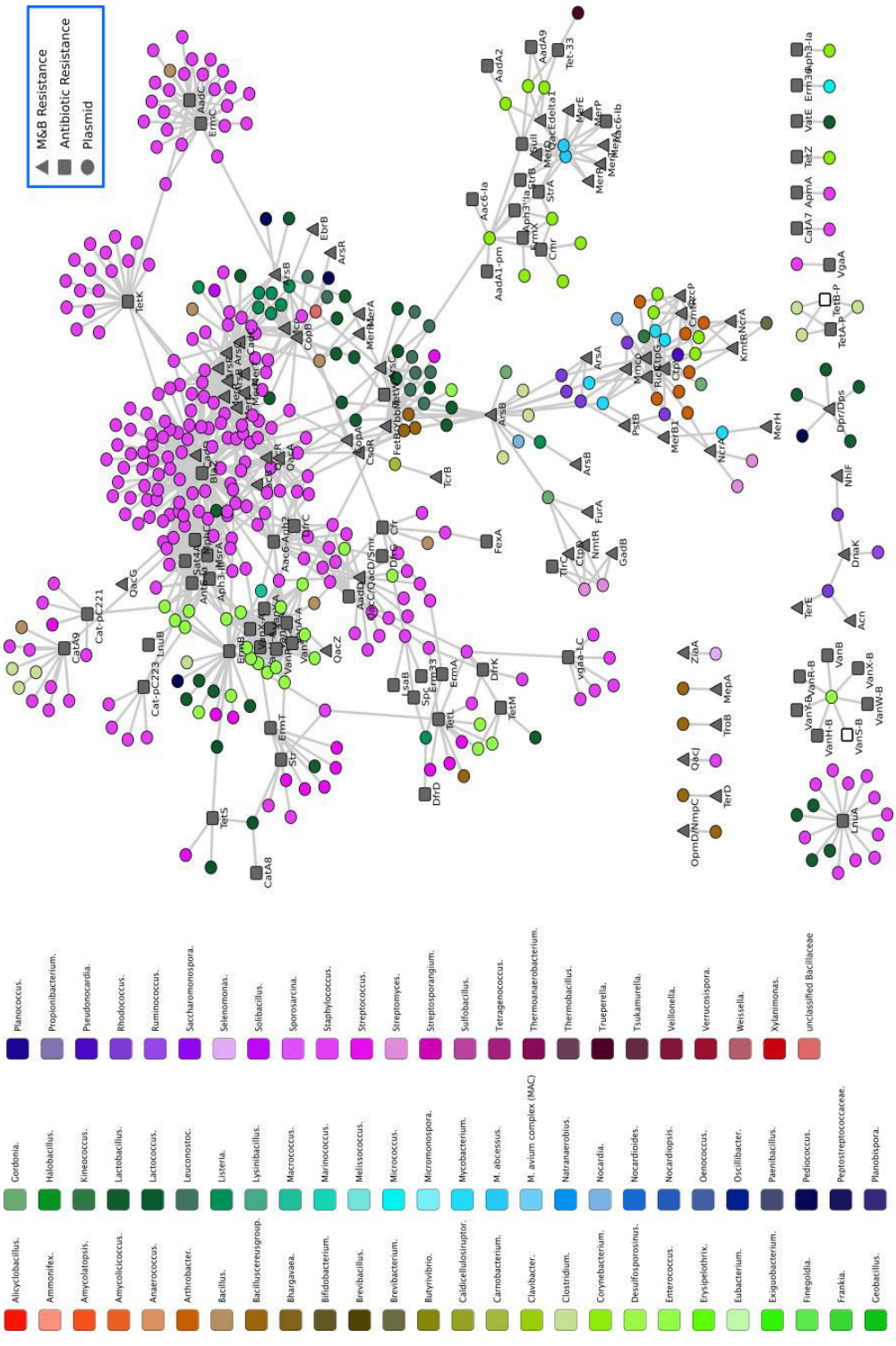

Figure 4. PCN of AbR and MetR/BcR proteins located on plasmids of *Firmicutes* and *Actinobacteria*.

We formed the PCN by representing plasmids as circular nodes, AbR as square nodes, and MetR/BcR as triangular nodes, connecting two nodes (plasmid and AbR or MetR/BcR) if one plasmid has this AbR or MetR/BcR. The presence of the MetR/BcR gene was determined by blasp of all plasmid proteins against the BacMet database. The presence of the AbR gene was determined by blasp of all plasmid proteins against the ARG-ANNOT database. The colors for the genus are the same as those in Fig. 3.

Rolling Circle Replication Plasmids

RCR plasmids are classified in a few families according to the RIP and the double origin of replication (*dso*) (see comprehensive reviews in references) (75, 77–79). Most of the RCR plasmids known to date have been found in species of *Firmicutes*, *Proteobacteria*, *Cyanobacteria*, and *Spirochaetes*, and some of them have been identified in genetically distant hosts. The production of single-stranded DNA and the mechanism of replication of these plasmids enhance their ability to recombine, by either homologous recombination or illegitimate recombination with other RCRs and theta replicating plasmids (Fig. 5 to 10 and text below). RCR plasmids are also frequently integrated into chromosomes (e.g., pUB110 within SCC*mec* cassettes in methicillin-resistant *S. aureus* or pC194/pUB110 [*catA*] in *S. pneumonia* genomes) (80).

In *Firmicutes*, four groups of RCR plasmids have been defined according to RIP similarity, namely Rep_trans (PF025486), Rep_1 (PF14046), Rep_2 (PF01719), and Rep_L, which are historically represented by plasmids pT181, pUB110, pMV158, and pSN2, respectively (53, 75, 77, 78). Within these families, some members have been fixed by selection and might be maintained by the vertical expansion of certain clones, aside from HGT, with the emergence of variants from time to time. Figures 11 to 14 and Supplementary Table S1 show the similarity of genes encoding RIPs of all available fully sequenced plasmids and the correspondence to the Rep families described by Jensen *et al*. (59). These plasmids may contain different adaptive genes (AbR, heat shock proteins, or bacteriocins), although most of them are classified as “cryptic,” without any clear adaptive function.

The Rep_1 family

The Rep_1 family comprises plasmids with RIPs of the families rep_{13} (associated with *catA7*, which encodes resistance to chloramphenicol), rep_{21} (cryptic or eventually carrying *lnuA*, coding for resistance to lincosamides), rep_{22} (carrying a variety of AbR genes), and other underrepresented members categorized as $rep_{Unique7}$. However, the available typing systems are unable to classify relevant Rep_1 plasmid members including plasmids containing heat shock proteins in *Streptococcus thermophilus*, plasmid-borne bacteriocins in *S. pyogenes* (82), or *S. pneumoniae* plasmids (80, 83), among others (Fig. 11). Remarkably, RIPs of this Rep_1 group are often detected in mosaic plasmids of staphylococci and enterococci (Fig. 5 and 7), some plasmid chimeras being fixed and persistently recovered for years. For example, emblematic mosaics theta/RCR plasmids of staphylococci (e.g., cointegrates of RepA_N/pSK41 and Rep_1/pUB110, which encode resistance to gentamicin) and *E. faecalis* (e.g., pAMα1) have both been selected in those lineages since the early 1970s (7, 71, 84).

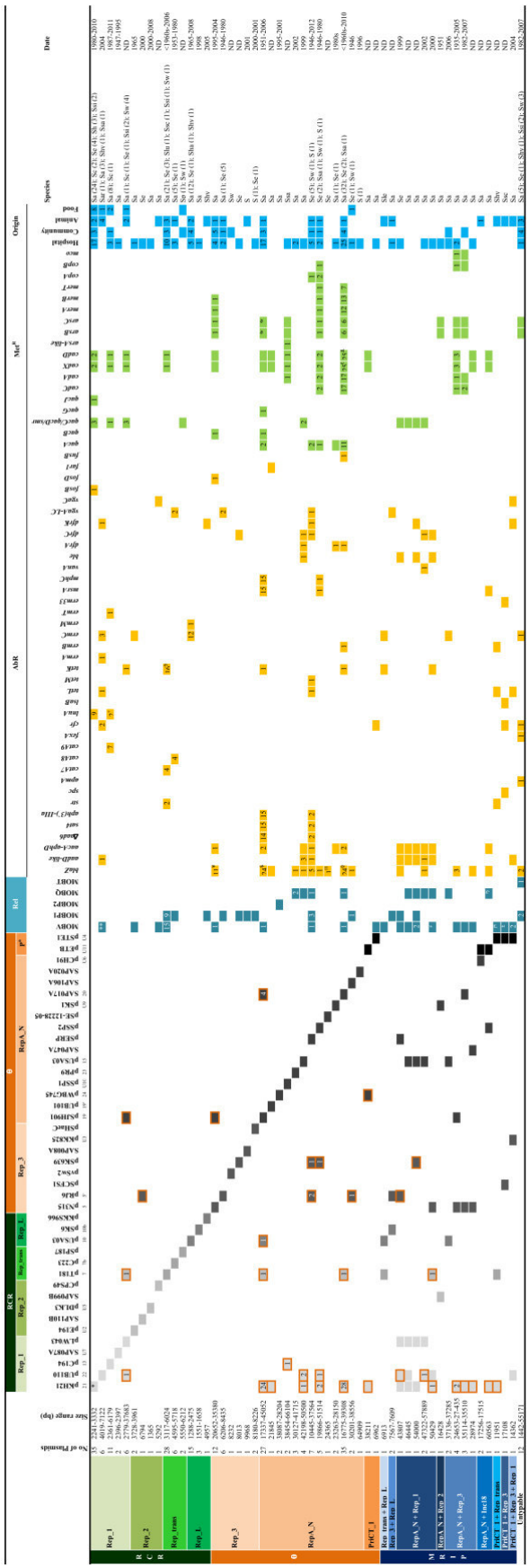

Figure 5. Plasmids from *Staphylococcus* spp.

The presence of an orange border in the RIP family indicates tha corresponding RIP is truncated. [a]PriCT_1; One of these plasmids (GenBank accession number NC_013381) has a truncated rep_pKH21 (rep_1) gene, and no other known RIPs were identified. **Two of these plasmids (GenBank accession number NC_016054 and NC_019144) appear to have two copies of the MOBV gene. [+]One of these plasmids (GenBank accession number NC_008354) has two copies of the *lnuA* gene. [§]One plasmid (GenBank accession number NC_001393) has a truncated copy of the *tetK* gene. [¥]One plasmid (GenBank accession number NC_010419) has a truncated copy of the *blaZ* gene. [£]The plasmid (GenBank accession number NC_005076) appears to have two copies of the MOB$_V$ gene. [$]One plasmid (GenBank accession number NC_018959) has a truncated copy of the *blaZ* gene. [&]Two plasmids (GenBank accession numbers NC_007931 and NC_016942) have two copies of the *arsB* and *arsC* genes. [Ç]This plasmid (GenBank accession number NC_013320) appears to have two copies of the MOB$_V$ gene. [ñ]This plasmid (GenBank accession number NC_005004) has a truncated copy of the *blaZ* gene. [ð]Three plasmids (GenBank accession numbers NC_013321, NC_019007, and NC_018976) have a truncated copy of the *blaZ* gene. [Ð]Eleven of these plasmids have a truncated copy of the *cadD* gene (GenBank accession numbers NC_020531, NC_020538, NC_013337, NC_020534, NC_020565, NC_020567, NC_020530, NC_020539, NC_017352, NC_013323, and NC_022610). [ß]One plasmid (GenBank accession number NC_013334) has a truncated copy of the *cadX* gene. [ª]This plasmid (GenBank accession number NC_020237) appears to have two copies of the MOB$_V$ gene. [Fr]This plasmid (GenBank accession number NC_022598) appears to have two copies of the MOB$_V$ gene. **Abbreviations:** MRIP, Multi-RIP; S, *Staphylococcus* spp; Sar, *Staphylococcus arlettae*; Sa, *S. aureus*; Sc, *Staphylococcus chromogenes*; Se, *Staphylococcus epidermidis*; Sha, *Shaemolyticus haemolyticus*; Shy, *Staphylococcus hyicus*; Sle, *Staphylococcus lentus*; Slu, *Staphylococcus lugdunensis*; Sp, *Staphylococcus pasteuri*; Ssa, *Staphylococcus saprophyticus*; Ssc, *Staphylococcus sciuri*; Ssi, *Staphylococcus simulans*; Sw, *Staphylococcus warneri*.

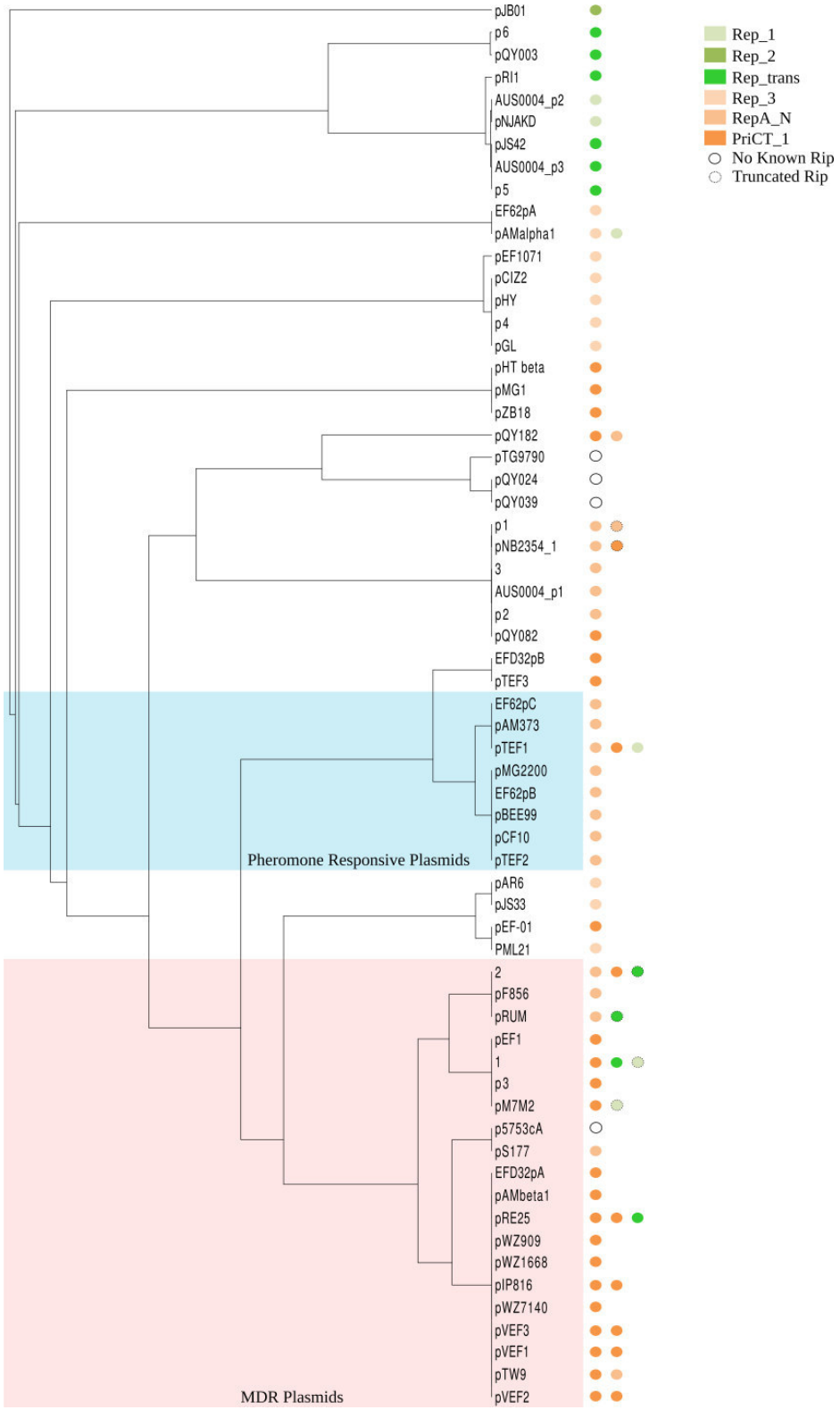

Figure 6. Hierarchical clustering dendrogram of plasmids from enterococci.

The matrix distance used for building the UPGMA dendrogram is based on the Raup-Crick distance of the orthologous protein profile of each plasmid. For each plasmid, a presence/absence protein profile was made using cut-off values of 80% identity and 80% coverage. Protein clustering was made by using CD-HIT (81). Different background colors are used to emphasize branches of related plasmids and are the same as those defined in Fig. 5. Names to the left of the dendrogram indicate the RIP family. Background colors were used to point out plasmid groups frequently involved in mobility of AbR genes and *E. faecalis* pheromone-responsive plasmids. Circles indicate RIPs identified in each plasmid according to data shown in Fig. 7.

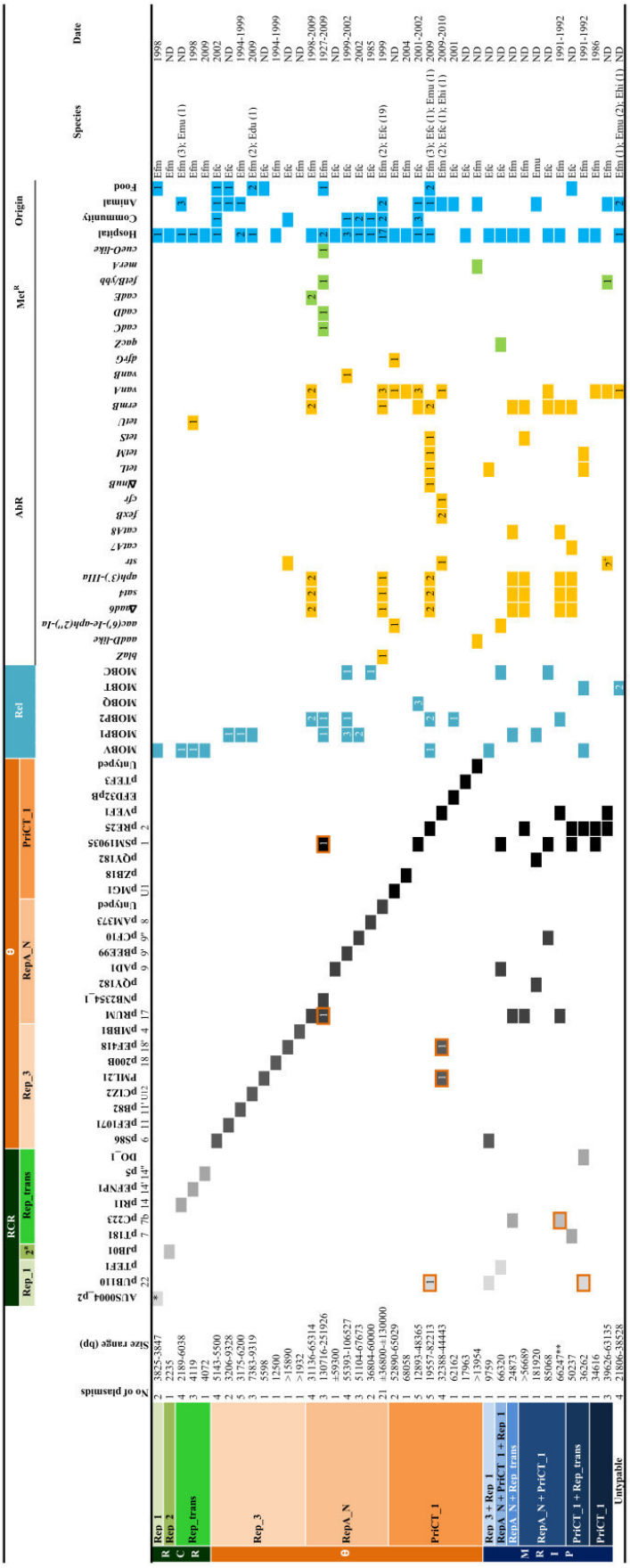

Figure 7. Plasmids from *Enterococcus* spp.

The presence of an orange border in the RIP family indicates that the corresponding RIP is truncated. [a]Rep_2; *One of these plasmids (GenBank accession number NC_015849) has a truncated rep_AUS0004_p2 (Rep_1) gene, and no other known replication initiator proteins were found. **This plasmid (GenBank accession number NC_017962) has two copies of Tn*401*; in one of them the *add(6)* gene is not truncated; this plasmid also appears to have two copies of the MOBP1 gene. [+]These two plasmids (GenBank accession numbers NC_008768 and NC_008821) have a truncated copy of the *str* gene. **Abbreviations:** MRIP, multi-RIP; Efm, *E. faecium*; Efc, *E. faecalis*; Emu, *E. mundtii*; Edu, *E. durans*; Ehi, *E. hirae*.

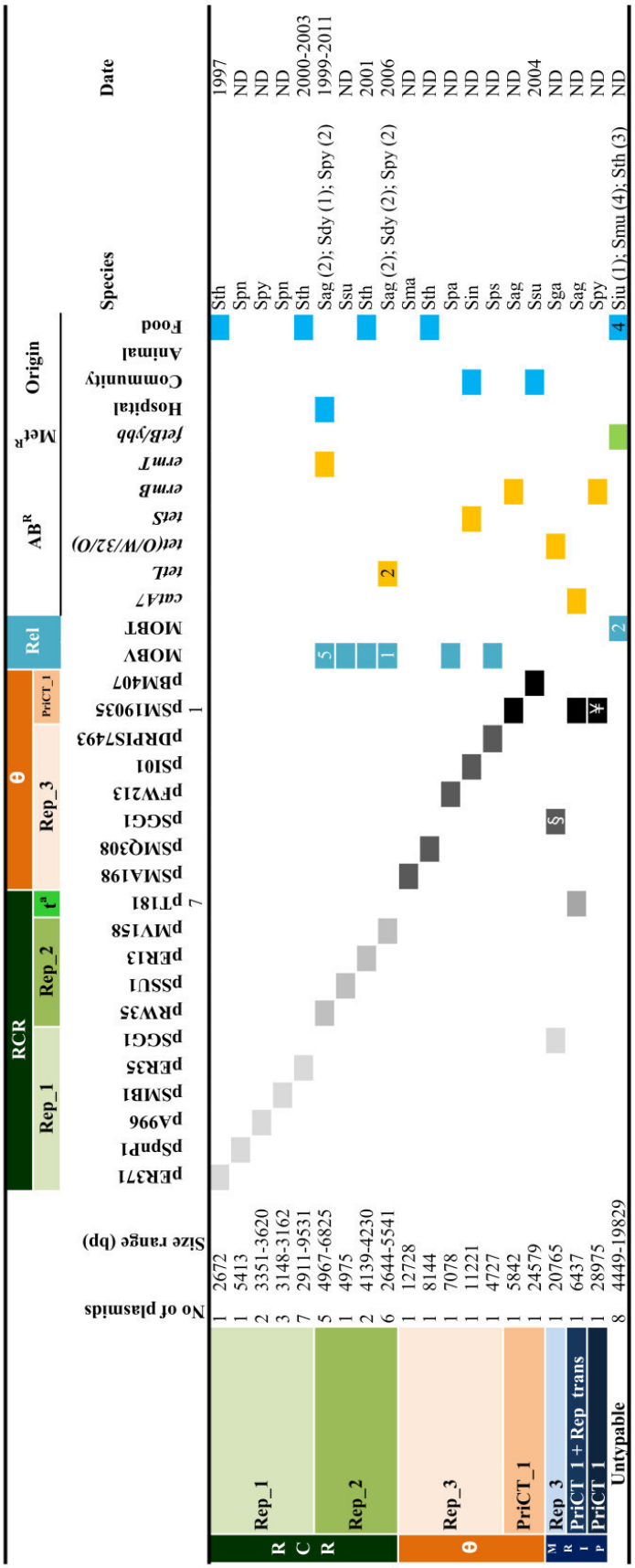

Figure 8. Plasmids from Streptococcus spp.

[a]Rep_trans; [§]This plasmid (GenBank accession number NC_015219) has two similar replication genes belonging to the PriCT_1 family. [¥]This plasmid (GenBank accession number NC_006979) has two similar replication genes belonging to the PriCT_1 family. **Abbreviations:** MRIP, Multi-RIP; Sag, *S. agalactiae*; Sdy, *Streptococcus dysgalactiae*; Sga, *Streptococcus gallolyticus*; Siu, *Streptococcus infantarius*; Sin, *Streptococcus infantis*; Sma, *Streptococcus macedonicus*; Smu, *Streptococcus mutans*; Spa, *Streptococcus parasanguinis*; Spn, *S. pneumoniae*; Sps, *Streptococcus pseudopneumoniae*; Spy, *S. pyogenes*; Ssu, *Streptococcus suis*; Sth, *Streptococcus thermophilus*.

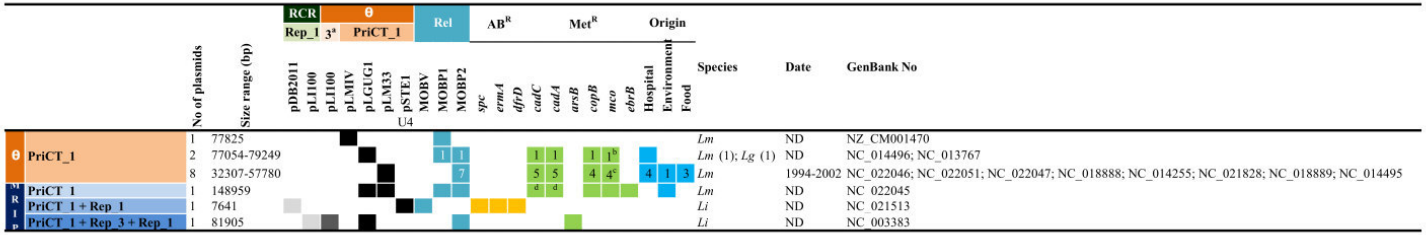

Figure 9. Plasmids from *Listeria* spp.

[a]rep_3. [b]One plasmid (GenBank NC_013767) has two copies of the *mco* gene, one of which is truncated. [c]One plasmid (GenBank NC_018888) has a truncated copy of the *mco* gene. [d]This plasmid (GenBank NC_022045) has two copies of the *cadC-cadA* operon. Abbreviations: MRIP, multi-RIP; Lm, *L. monocytogenes*; Lg, *Listeria grayi*; Li, *Listeria innocua*.

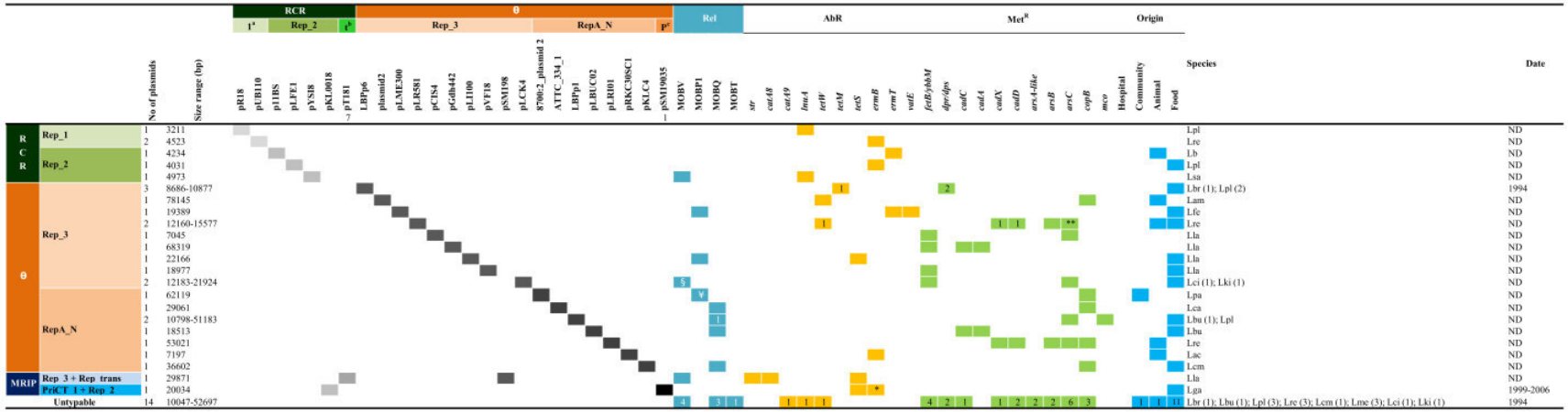

Figure 10. Plasmids from LAB.

[a]Rep_1. [b]Rep_trans. [c]PriCT_1. *This plasmid (GenBank accession number NC_010540) has two copies of the *ermB* gene. **One of these plasmids (GenBank accession number NC_010603) has a truncated copy of the *arsC* gene. [§]One of these plasmids (GenBank accession number NC_014133) appears to have three copies of the MOB_V gene. [¥]One of these plasmids (GenBank accession number NC_022123) appears to have two copies of the MOB_{P1} gene. **Abbreviations:** MRip, multi-RIP; Lb, *Lactobacillus* spp; Lca, *Lactobacillus acidophilus*; Lam, *Lactobacillus amylovorus*; Lbr, *Lactobacillus brevis*; Lbu, *Lactobacillus bucheneri*; Lca, *Lactobacillus casei*; Lfe, *Lactobacillus fermentum*; Lpa, *Lactobacillus paracasei*; Lpl, *Lactobacillus plantarum*; Lre, *Lactobacillus reuteri*; Lsa, *Lactobacillus sakei*; Lga, *Lactococcus garvieae*; Lla, *Lactococcus lactis*; Lcm, *Leuconostoc carnosum*; Lci, *Leuconostoc citreum*; Lki, *Leuconostoc kimchii*; Lme, *Leuconostoc mesenteroides*.

The Rep_trans group

Plasmids of the Rep_trans group are clustered in two large branches (Fig. 12). One branch comprises plasmids of *Staphylococcus* that harbor *tetK* (rep_7) and *catA8*/*catA7* (rep_{7b}) with different MOB genes. Such plasmids have been reported in *S. aureus* since their first detection in the early 1950s (84) and were eventually described in contemporary *E. faecalis* isolates (68, 85). A second branch contains pRI1-like plasmids (rep_{14}), which correspond to plasmids of different enterococcal species (*E. faecium*, *Enterococcus hirae*, *Enterococcus mundtii*) isolated from foodborne animals and hospital patients (7, 59, 71, 86). These plasmids can be mobilized by other AbR conjugative theta replicating plasmids present in the same cell (71, 87), and it seems they are widely spread among enterococcal populations.

The Rep_2 group

The Rep_2 group (Fig. 13) comprises numerous promiscuous elements able to replicate in distant hosts which have been extensively analyzed at the molecular level by Espinosa *et al.* using pMV158 as a model (75). Plasmids carrying *ermT* (an inducible methylase conferring resistance to first-line macrolide-lincosamide antibiotics such as erythromycin and clindamycin), from group A Streptococci (GAS) and group B Streptococci (GBS), are the sole representatives of AbR in this group. They appear to be responsible for the rise of macrolide resistance among GAS and GBS in hospitals since the mid-1990s (8).

The Rep_L group

In contrast to the above-mentioned RCR plasmid groups, proteins within the Rep_L family (Fig. 14) are represented in public gene databases by a very few RIPs of *Staphylococcus*, *Selenomonas* (class *Negativicutes*), and *Butyrivibrio* (*Clostridia*) species, all these genera being frequent components of the oral flora of humans and the rumen of some animal species. These plasmids are responsible for the widespread *ermC* in staphylococci (rep_{10}). Interestingly, the emergence of both *ermT*-Rep_2 and *ermC*-Rep_L plasmids seems to be associated with the abusive use of tylosin in cattle, amplified by the location of these AbR genes in RCR plasmids, and further transferred to other populations of *Firmicutes* (8, 88, 89).

RCR plasmids were associated with REL of the group MOB_{V1} (72, 73, 75), although representatives of all the RCR groups mentioned above that lack REL were detected in databases. Interestingly, RELs of MOB_{P1} and MOB_T families were also found, and their presence is probably due to the co-integration of RCR with theta-replicating plasmids (see below).

Figure 11. Similarity of *rep*-like sequences encoding RIPs of the Rep_1 family.

A neighbor-joining tree of gene sequences coding for RIPs of the Rep_1 family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different backgrounds colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10.

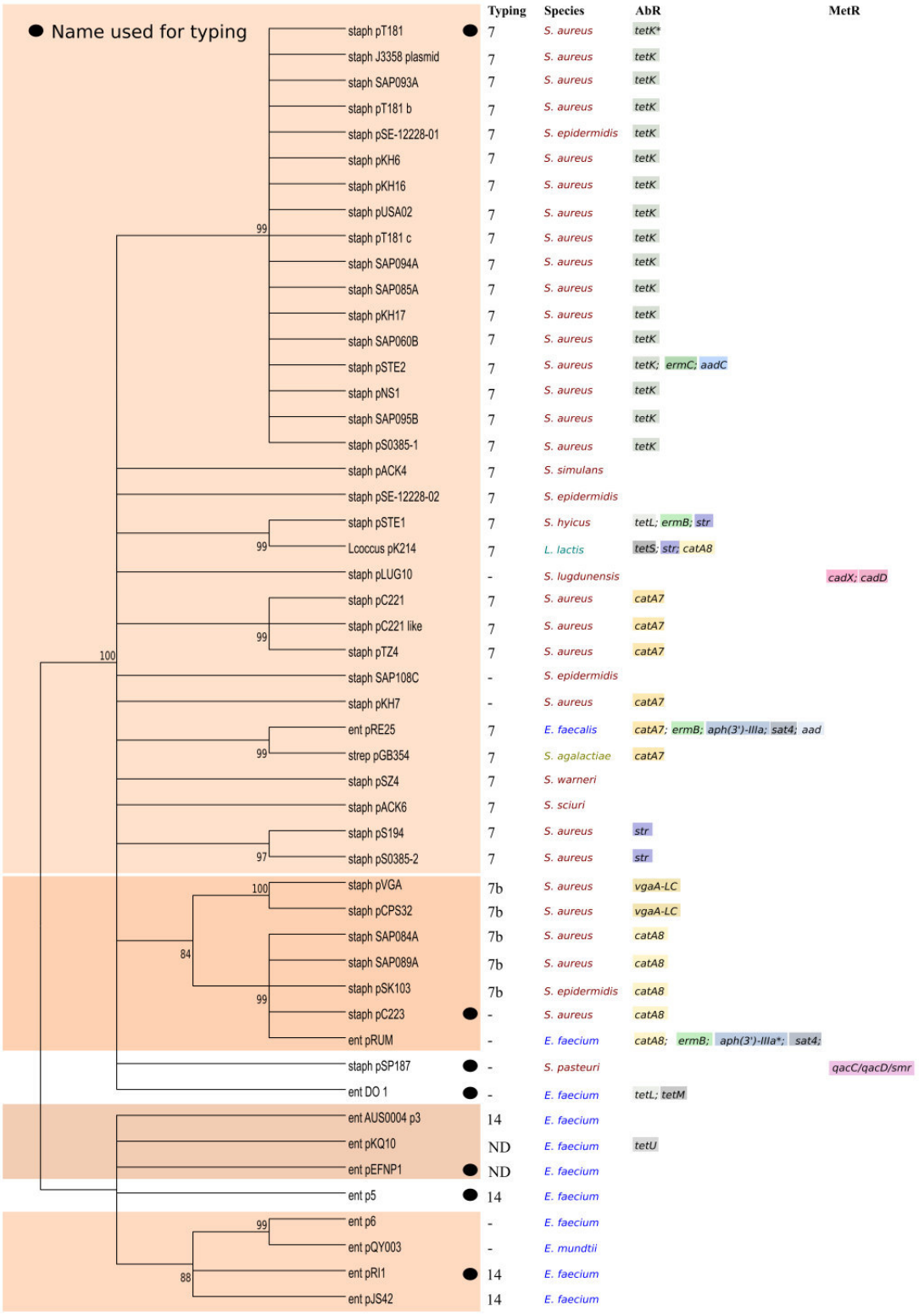

Figure 12. Similarity of *rep*-like sequences encoding RIPs of the Rep_trans family.

A neighbor-joining tree of gene sequences coding for RIPs of the Rep_trans family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different backgrounds colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. *Truncated gene. **Similar to *E. faecalis ant6-Ia* and *aadE*. **Abbreviations:** ND, not determined.

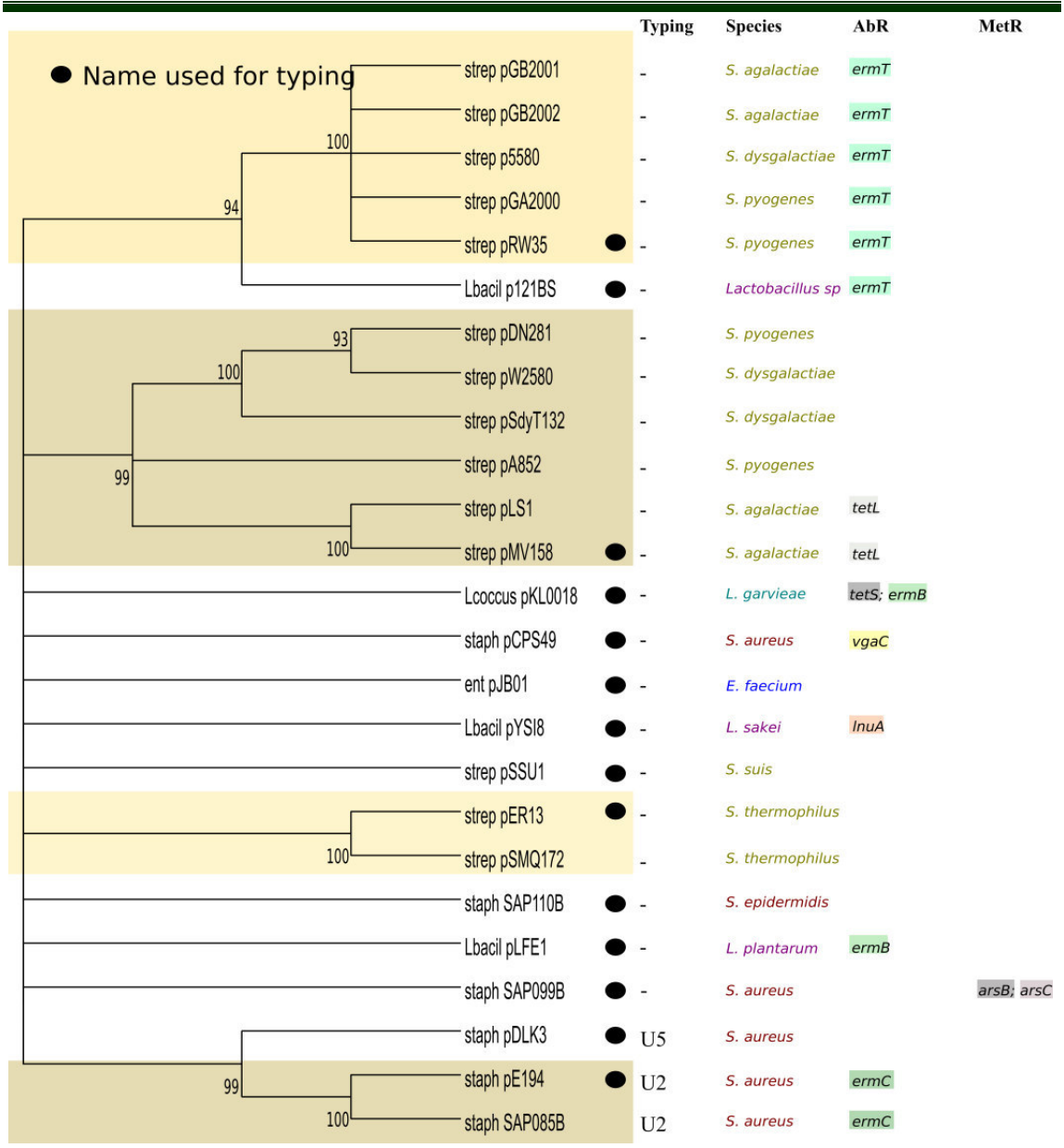

Figure 13. Similarity of *rep*-like sequences encoding RIPs of the Rep_2 family.

A neighbor-joining tree of gene sequences coding for RIPs of the Rep_2 family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different background colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10.

Theta-Replicating Plasmids

Four families of plasmids that replicate by a theta mechanism, three that comprise conjugative plasmids (RepA_N, Inc18, and pMG1) and one in groups of small non-conjugative elements (Rep_3), are involved in the capture, spread, and maintenance of AbR among different genera of *Firmicutes*. Members of the RepA_N and Inc18 families are often enriched in insertion sequences, mainly IS*257*, IS*256*, IS*1216*, IS*L3*, and IS*431*, that facilitate co-integration, rearrangements, and deletions among elements of *Staphylococcus*, *Enterococcus*, LAB, and *Clostridium* of different origins (6, 7, 28, 90–95). Such recombination events seem to have facilitated the origin of the great mosaicism of MDR plasmids that

often carry more than one RIP, lack transfer and maintenance modules, and eventually carry more than one REL (Fig. 5 and 7). The transfer mechanisms of RepA_N pSK41-like plasmids and the Inc18-like plasmids are similar and are categorized as type IV secretion systems (96).

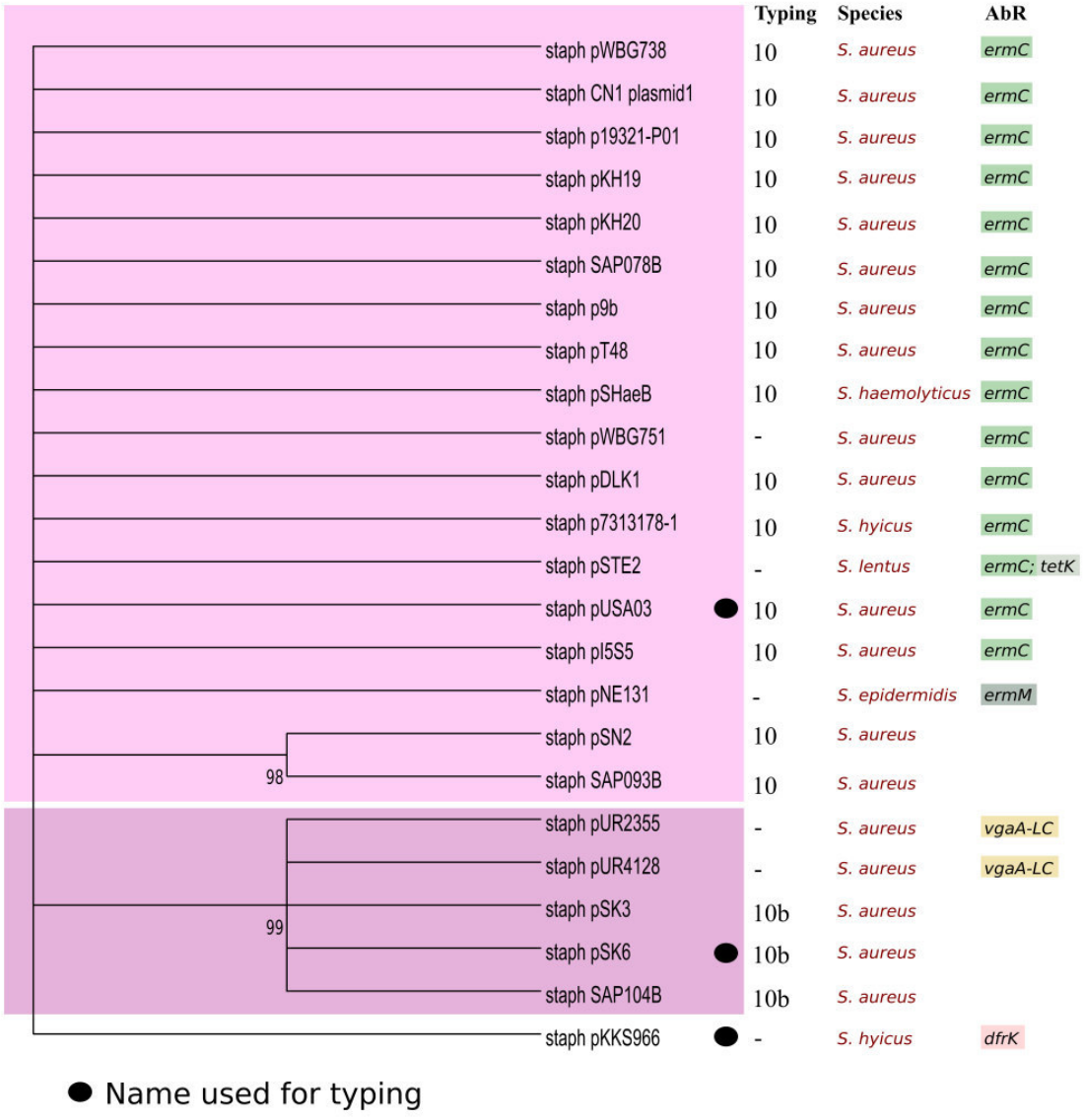

Figure 14. Similarity of *rep*-like sequences encoding RIPs of the Rep_L family.

A neighbor-joining tree of gene sequences coding for RIPs of the Rep_L family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different background colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10.

The Rep_3 family

Plasmids containing RIPs with the Rep_3 domain (Fig. 15) are common among disparate bacterial genera including *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Lactobacillus*, and *Enterobacteriaceae* (7). Figure 15 shows the diversity of RIPs among fully sequenced plasmids of *Firmicutes*, and Fig. 5 to 10 reflect the features of known members of this family within each genus of biomedical interest. In enterococci,

Rep_3 plasmids (<15 kb) have been found in isolates recovered from hospitalized patients, animals (pigs, cows), cheese, milk, and dry-fermented sausage, frequently associated with the production of bacteriocins that are active against a variety of Gram-positive genera (7). In *Lactobacillus* and *Lactococcus*, they harbor bacteriocins and, eventually, AbR genes. Rep_3 plasmids play a relevant role as vehicles of AbR among staphylococci. Plasmids from *S. aureus* are overrepresented by closely related variants containing rep_5, which are associated with genes coding for penicillinase and resistance to heavy metals (cadmium and arsenic) (51, 53, 66, 84). Staphylococcal plasmids within this group include AbR plasmids from coagulase-negative strains of animal origin, some of them with RIPs that would not be detected by current typing systems (97, 98).

The Inc18 family

First described in the 1990s, the Inc18 family comprises a highly heterogeneous group of broad host range, low copy number plasmids (<10 per cell) that replicate by a theta mechanism, regulated by an antisense RNA that mediates transcriptional attenuation and that are able to conjugate on solid media at high frequencies (64, 99). The transfer system of pIP501 has been extensively studied and constitutes a paradigm of conjugation systems, showing significant similarity with the *tra* regions of RepA_N plasmids pGO1 and pSK41 from *S. aureus* and pMRC01 from *Lactococcus* lactis (96, 100).

The Inc18 group is traditionally represented by three emblematic plasmids: pSM19035 (101) and pIP501 from *S. agalactiae* and pAMβ1 from *E. faecalis* (64, 101–104). It gets its name from the apparent incompatibility of these plasmids with each other described in seminal studies in the field and following the nomenclature of Inc groups started by Richard Novick for staphylococcal plasmids (50, 60, 64, 105). Inc18 plasmids frequently carry the post-segregational killing systems, εç, and type I partition cassette *prgPprgO*, which are associated with a variety of RIPs and seem to contribute to their persistence in different populations in the absence of antibiotic selection pressure (7, 106, 107). Detailed molecular characterization of such plasmids is described elsewhere (64, 99, 108) and shows a remarkably high modular interplay among different Inc18 plasmids, leading to the high modularity observed in plasmid sequences (see Fig. 5 to 10 and text below).

Inc18 plasmids have contributed remarkably to the spread of AbR (macrolides, chloramphenicol, aminoglycosides, and glycopeptides) and MetR (copper and mercury) among streptococci and other phylogenetically distant genera of Gram-positive (*S. aureus*, *Listeria*, *Bacillus subtilis*, *Lactobacillus*, *Leuconostoc*, various *Clostridium* species) and Gram-negative bacteria (108–111).

Plasmid relatives of pAMβ1 (harboring *ermAM*, later on recognized as *ermB*, and conferring resistance to macrolides, lincosamides, and streptogramines) and pIP501 (carrying *ermB* and $catA7_{pC221}$, which confers resistance to chloramphenicol) were rapidly spread during the 1970s and have frequently been detected among streptococci of groups A, B, and D (enterococci) since then (110, 112–114) (see also Supplementary Table S1 for contemporary representatives of this plasmid group).

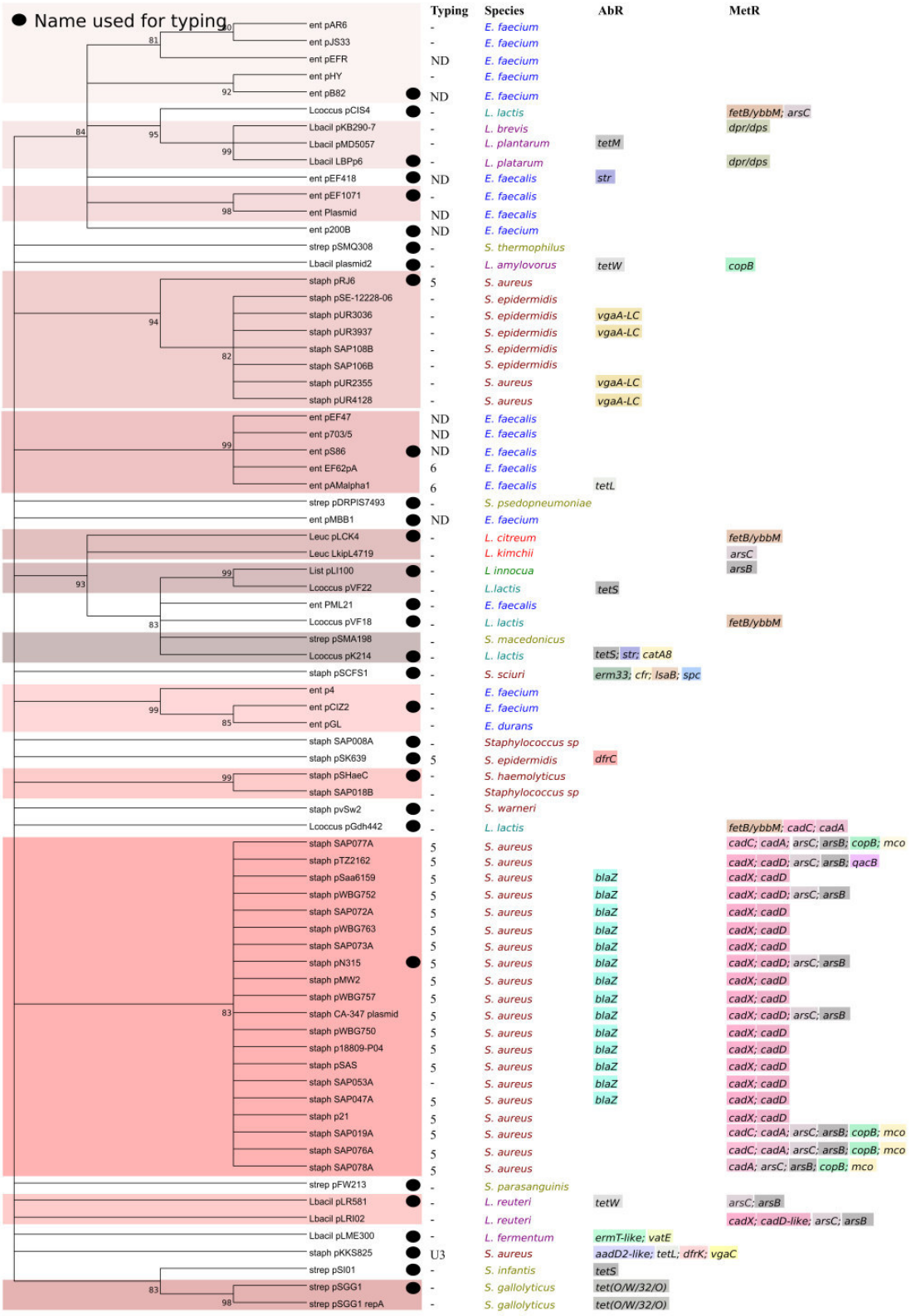

Figure 15. Similarity of *rep*-like sequences encoding RIPs of the Rep_3 family.

A neighbor-joining tree of gene sequences coding for RIPs of the Rep_3 family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different background colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. **Abbreviations:** ND, not determined.

Initially, the successful spread of intact AbR plasmids among clones of various streptococcal genera, including *S. pneumoniae*, and *S. aureus* was reported, despite the lack of stability in these last two clonal backgrounds (110, 113). Inc18 plasmids conferring resistance to aminoglycosides (kanamycin, streptomycin, and neomycin) and to macrolides were also detected in 1972, in the emblematic Streptococcus (*Enterococcus*) *faecalis* strain JH1 that carried pJH1 (an MDR plasmid, presumably Inc18) and pJH2 (a RepA_N pheromone-responsive plasmid carrying hemolysin and bacteriocins). pJH1 represented the first description of conjugative transfer of AbR plasmids in enterococci (114). Aminoglycoside resistance in pJH1 relatives was due to the presence of Tn*5405*, a transposon comprising three genes in tandem (an aminoglycoside 6-adenyltransferase [*aad*], a streptothricin acetyltransferase [*sat*], and an aminoglycoside-phosphotransferase [*aph3*]). These genes were identified later on in *S. pyogenes*, *S. agalactiae*, *S. aureus*, *Campylobacter coli*, *C. perfringens*, and *C. difficile* (now *Peptoclostridium difficile*).

More recently, diverse Inc18 plasmids carrying Tn*1546* in enterococci and staphylococci have emerged in different locations. In Europe, Inc18::Tn*1546* plasmids (such as pVEF1, pEVF2, pVEF3, and pVEF4) seem to have evolved from pIP816 (the first Inc18::Tn*1546* was isolated in France in 1987). They lack a transfer system and appear to be confined to *E. faecium* (70, 115, 116). Inc18::Tn*1546* plasmids from the United States are linked to *E. faecalis* isolates (pWZ909, pWZ1668, pWZ7140) and contain a complete transfer system (117, 118). A plethora of multiresistant mosaic Inc18 plasmids containing up to three RIPs, including RepR of pIP501 (CAA35647.1) and RepS of pRE25 (YP_783890.1), have been described in different *Firmicutes* (70, 71, 116, 119). These plasmids have an arsenal of insertion sequences, mainly IS*1216* and IS*L3*, which facilitate genetic exchange with different genetic elements of different origins and the acquisition of different AbR (*tetS*) and MetR (*tcrB*, mer operon). These ISs also facilitate the co-integration with other RCR (e.g., pC221, which is cointegrated in pRE25) or theta replication plasmids as pheromone responsive (115, 119–121) or some pSK41-like elements. Figure 16 shows the diversity of Inc18 RIPs that can be identified by typing systems. All these RIPs have a primase domain PriCT_1 that allowed their identification as belonging to the Inc18 family. Fig. 6, 7, and 9 illustrate the mosaicism of Inc18 plasmids in enterococci and Listeria.

The pMG1/pHT plasmids

The pMG1/pHT plasmids are related to those of the Inc18 family (RIP homolog approximately 30% identical to Inc18 initiators, including the PriCT_1 domain [Fig. 16]) (122), although they also show high homology with the pXO2 virulence plasmid from *Bacillus anthracis*. Because many open reading frames of pHT and pMG1 plasmids do not show significant homology with any reported proteins, they used to be categorized as a new type of highly efficient conjugative plasmids with a MOB_P family REL.

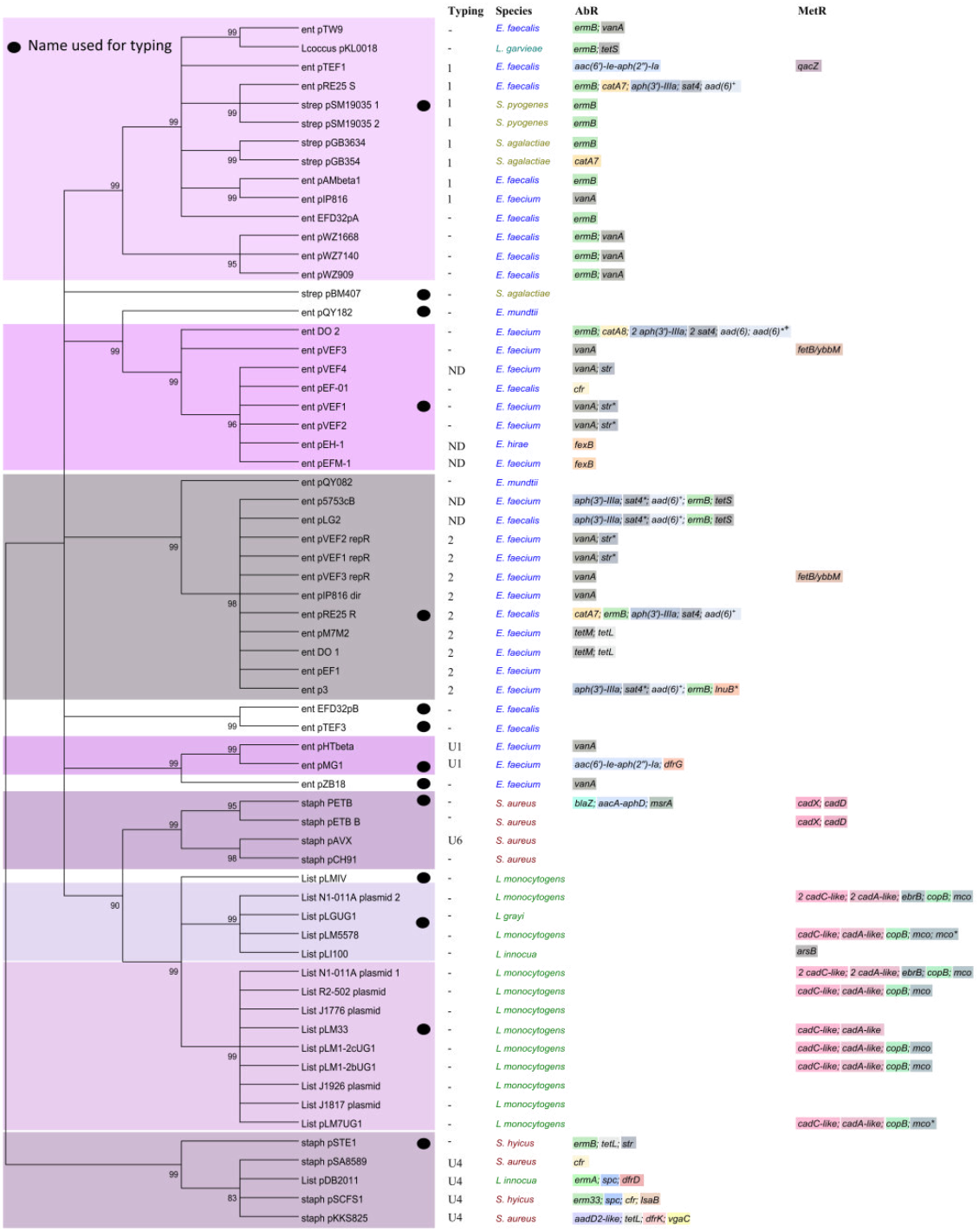

Figure 16. Similarity of *rep*-like sequences encoding RIPs with the PriCT_1 domain.

A neighbor-joining tree of gene sequences coding for RIPs with PriCT_1 domains was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different background colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. **Abbreviations:** ND, not determined.

This plasmid group is represented by relatives of pHT (pHTα, pHTβ, and pHTγ) and pMG1, which have greatly facilitated the dissemination of resistance to glycopeptides (Tn*1546*-*vanA*) and high-level resistance to aminoglycosides (Tn*4001*-like elements) among human *E. faecium* and *E. avium* isolates from the United States and Japan (123, 124) and, to a lesser extent, European countries (7, 70, 71, 122).

The RepA_N family

This is a large family of plasmids (also including a few phages) that are widespread among the low G+C Gram-positive bacteria and which possess RIP homologs to the RepA protein of pAD1 (76). The five groups of RepA homologs identified are phylogenetically congruent with their host background (Fig. 17), suggesting that the replicons have evolved along with their current hosts and that inter-genus movement of RepA_N plasmids does not often occur. Such RepA_N clusters correspond to plasmids from *Staphylococcus* (MetR/*bla* pSK1 and pSK41 MDR plasmids), plasmids from *Enterococcus* (*E. faecalis* pheromone-responsive plasmids and *E. faecium* non-pheromone-responsive plasmids related to pRUM, pLG1, or untypeable megaplasmids), plasmids from *Lactobacillus* and *Lactococcus*, phage homologs from *Streptococcus* (*S. pneumoniae*, *S. thermophilus*), and plasmids from *B. subtilis* (e.g., pLS32). Staphylococcal and enterococcal RepA_N plasmids have greatly contributed to the spread of AbR genes among humans and, eventually, animals and will be further described below. They also facilitate the movement of other non-conjugative plasmids and large genomic regions (36, 125, 126).

RepA_N staphylococcal plasmids (Fig. 5)

Large staphylococcal MDR plasmids use evolutionarily related theta-mode replication, although they can be further divided into three types: the MetR/beta-lactamase-producing plasmids, the pSK1 family, and pSK41-like conjugative elements. All these are compatible and can be identified as the rep_{19}, rep_{20}, and rep_{15} families, respectively, according to Jensen's plasmid typing system (59, 127, 128). The pSK41 family (rep_{15}) is the largest group of conjugative plasmids in staphylococci, traditionally represented by pSK41, pG01, and pJE1, which emerged in the early 1980s associated with resistance to gentamicin due to the presence of Tn*4001* (84, 129). They often confer resistance to other antibiotics such as neomycin, tobramycin and kanamycin (due to the integration of pUB110 plasmids that harbor the *aadD* gene), antiseptics (due to the presence of *qac* genes) (130), and eventually trimethoprim (mediated by Tn*4001*), penicillins (due to the presence of Tn*552*::*blaZ*), and others. Plasmids of this group may also confer resistance to mupirocin (131–133) and vancomycin (134, 135), represented by pUSA03 (which harbors *ileS* and *tetK*) and pWL1043 (which contains Tn*1546*, Tn*4001*, Tn*4002*, Tn*552*, and *qacC*). The pSK41-like plasmids are able to mobilize other plasmids present in the same bacterial cell (133, 136, 137). The pSK1 and MetR/beta-lactamase plasmids belong to the same incompatibility groups and are also compatible with pSK41 plasmids. Despite their inability to self-transfer, these groups of plasmids have been detected in many staphylococcal species.

RepA_N enterococcal plasmids

This cluster groups pheromone-responsive plasmids of *E. faecalis* and pRUM- and pLG1-like plasmids of *E. faecium* (7) (Fig. 6 and 7).

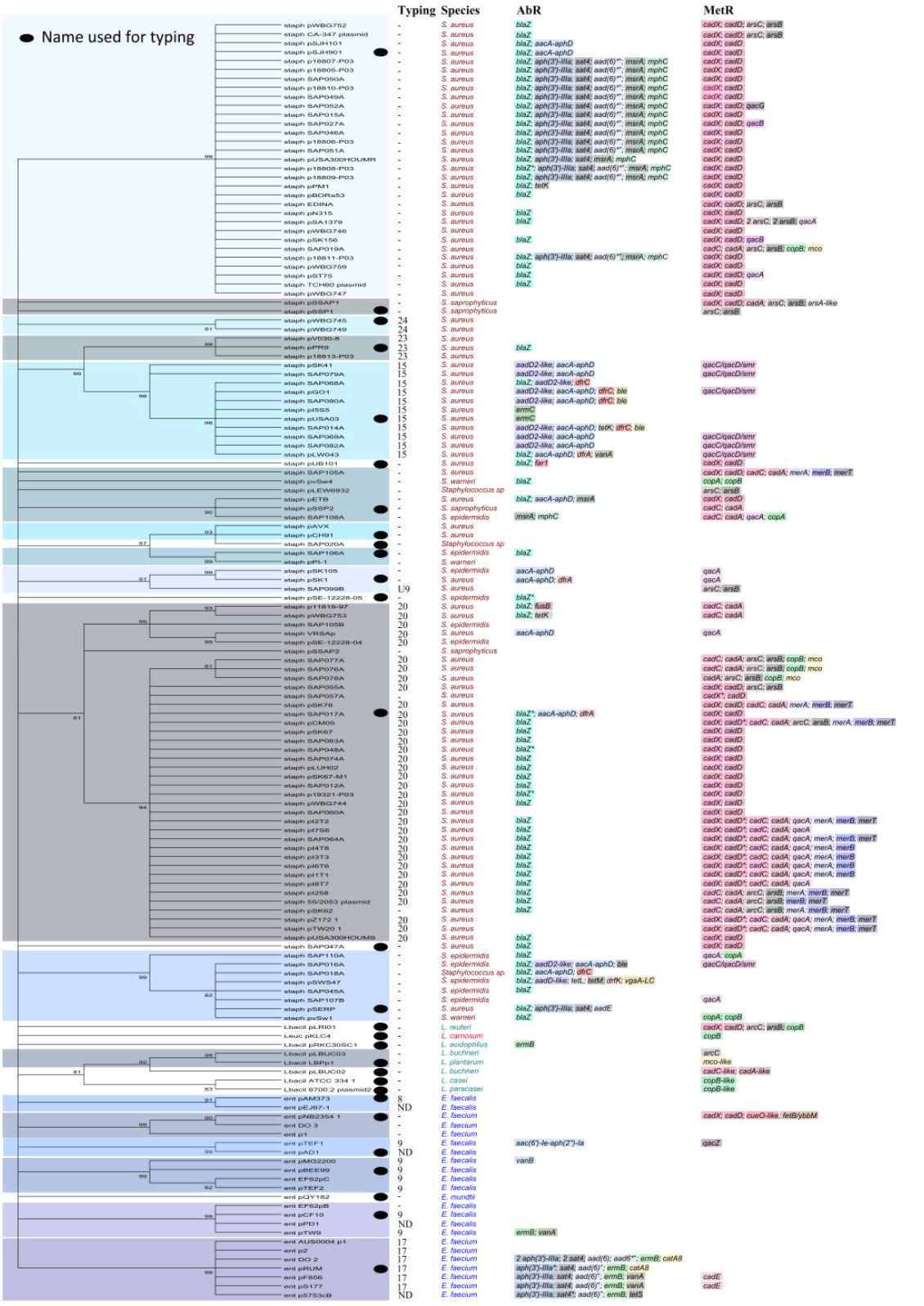

Figure 17. Similarity of *rep*-like sequences encoding RIPs of the RepA_N family.

A neighbor-joining tree of gene sequences coding for replication initiator proteins of the RepA_N family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different background colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. *Truncated gene. [+]Similar to *E. faecalis ant6-Ia* and *aadE*. **Abbreviations:** ND, not determined.

Pheromone-responsive plasmids

Pheromone-responsive plasmids represent a paradigm of elements in the biology of MGEs and are, together with Inc18 plasmids, the best-known plasmids described to date. For details about the mechanism of replication, conjugation, and evolvability of this plasmid group see references (7, 49, 65, 92, 138). Plasmids that respond to pheromones are present in most contemporary *E. faecalis* isolates from humans and birds but are only occasionally found among *E. faecium*. Synthesis of pheromones is confined to *E. faecalis*, although *Enterococcus hirae*, *S. aureus*, and *Streptococcus gordonii* may secrete cAM373-like peptides that facilitate the conjugation of pAM373 from *E. faecalis* (139). The description of cAM373-responsive plasmids coding for resistance to glycopeptides (Tn*1546*-*vanA*) highlights the potential risk of the spread of glycopeptide resistance in staphylococci in institutions where VRE are endemic (134, 140). Although pheromone plasmids are unable to replicate in *S. aureus*, their transference and establishment in this host might occur by co-integration with other plasmids able to replicate in this species. In addition, some pAD1 relatives enhance the rate of mobilization of plasmids, conjugative transposons, and PAIs (125).

Plasmids of this family can be classified on the basis of responses to pheromones in different incompatibility groups (139) or according to RIP diversity (59, 68) within $rep_{8(pAM373)}$ and rep_{9} (further split into subgroups $rep_{9a(pAD1)}$ and $rep_{9b(pTEF2)}$) families (59, 68). Transfer systems of MOB_C or MOB_P families have been detected in plasmids of this family.

Pheromone-responsive plasmids may encode putative virulence traits (aggregation substance, hemolysin/bacteriocin) and a diversity of AbR elements located on transposable elements such as Tn*916*-like (*tetM*), Tn*4001* (*aac-aph*), Tn*1546* (*vanA*), Tn*1549* (*vanB*), and a composite transposon containing a β-lactamase gene flanked by two IS*4* copies (7). The *par* locus encodes a unique antisense-regulated toxin-antitoxin system present in the plasmid pAD1, but *par* homologs have been detected on other plasmids and chromosomes of *E. faecalis* and *Staphylococcus*, *Clostridium*, *Listeria*, and *Lactobacillus* species (141). Toxin-antitoxin systems associated with other plasmid families such as εç and *relBE* have been detected on members of this plasmid group, reflecting rearrangements with representatives of other plasmid families (7). Even though to date, only a few members of pheromone-responsive plasmids have been fully sequenced, typing surveys reveal a wide diversity of plasmids among populations, often containing RIPs, RELs, or regions from plasmids of different origins (68, 71).

pRUM-like plasmids

pRUM-like plasmids (represented by pRUM, p5373c, pS177, and pDO2) are mosaic plasmids of variable size (>30 kb) that comprise diverse genetic elements of different origins (transposons, insertion sequences, small theta-replicating plasmids, bacteriocin clusters). They can be identified as the rep_{17} family according to PCR-based typing systems (59) but differ in the RIP sequence, the MOB system, and the presence of the toxin-antitoxin Axe-Txe locus (71, 142, 143). Both Inc18 and pRUM plasmids are

driving the spread of glycopeptide resistance among contemporary isolates of *E. faecium* by carrying Tn*1546* (*vanA*) or Tn*1549* (*vanB*). Two types of pRUM plasmids are currently widespread among VRE and vancomycin-susceptible *E. faecium* isolates from different hosts. One contains RepA and Axe-Txe from pRUM and, eventually, the mobilization system of pC223 from *S. aureus* (70, 71, 142–144). The other type is characterized by a RepA protein that is 95% identical to RepA-pRUM, lacking postsegregational killing Axe-Txe and the presence of a MOB_{P7} relaxase originally detected in pEF1, a plasmid with an environmental origin. Tn*1546* is frequently located on both types of pRUM plasmids, frequently containing replicons of other plasmid families (author's unpublished results).

Large plasmids

Plasmids larger than 150 kb are widely distributed among *E. faecium*, *Enterococcus durans*, and *E. hirae* from different origins, but they have not been detected among *E. faecalis* (71, 144–150). To date, only a handful of *E. faecium* megaplasmids have been fully sequenced (AUS0085_p1 [NC_021987], pNB2354_1 [NC_020208], DO_3 [NC_017963], and pLG1, although this last one has not been closed (148)). All of them contain a RIP similar to $RepA_{pAD1}$, making them part of the RepA_N family (Fig. 7 and 17, Supplementary Table S1) (59, 71, 148). A similar RIP has also been found in a 130-kb plasmid (NC_021987) from a VRE ST203 *E. faecium* strain isolated in 2009 in Australia (151). Although RIP sequences of pLG1 plasmids are often identified among enterococcal megaplasmids, most of them do not hybridize with known RIP genes included in published schemes (71, 148, 152). Enterococcal megaplasmids carry genes involved in sugar metabolism (mannitol, glycerol, sorbitol, raffinose, complex carbohydrates), AbR (macrolides, glycopeptides, aminoglycosides), MetR (copper-*tcrYAZB*), and enhanced adhesion (71, 126, 144, 147–149, 152–154). They are frequently involved in the acquisition or persistence of AbR among *E. faecium* isolates from food animals (148, 150).

GENE AND PLASMID FLOW SHAPES THE EVOLUTIONARY ECOLOGY OF FIRMICUTES

As described in previous sections, the acquisition of novel traits encoding adaptive resistance to antimicrobials in *Firmicutes* is mainly due to genes located on plasmids and transposable elements. This acquisition is, certainly, regulated by interactions at genetic and ecological (social) levels. Interplay between genes, mobile genetic elements, and microbial populations and their relation with the host population and local or global environments shapes the plasmid flow. Such flow can be modified by "external" (supra-cellular) changes, including variations in the host population structure and size (e.g., mass rearing, crowding) and their associated chemical or behavioral landscape (e.g., use of different antimicrobials, immunization, global food supply, international travel). These changes ultimately determine the density and diversity of particular bacterial populations in particular habitats, leading to ecological specialization, clonalization, and gradual emergence of gene flow barriers (23, 155, 156) , a process that mimics the general dynamics of speciation, as bacterial clones and species constitute ecological units of microbial biodiversity.

The challenge to define "units of biodiversity" in microbial community ecology has approached the concept of genes as "defining elements of networks and metacommunities" (155). In such a context, extra-chromosomal elements greatly influence the HGT interactions between microbial organisms and are the building forces for the establishment of "gene exchange communities" (155, 157). The selective power of antimicrobials (antibiotics [Ab], heavy metal, biocides) may then shape this multilevel bacterial population biology (158, 159), involving genes, plasmids (MGEs), bacterial clones and species, and gene exchange communities. The evolutionary tradeoff between early and late stages of adaptation to such selective pressures may determine the local evolvability of clonal and plasmid populations by increasing the number of genotypes resulting from chromosomal and plasmid recombination processes that facilitate further ecological differentiation (18). To establish effective public health interventions to fight the AbR problem in its eco-biological dimension, we then need to define the gene exchange communities relevant for the acquisition, evolution, and spread of resistance (160, 161). Below, we will specifically discuss the role of AbR genes and plasmids in the ecological differentiation of bacterial populations of the main *Firmicutes* genera.

Antimicrobial Resistance Genes and Bacterial Population Ecology

The environmental origin of AbR genes has been extensively discussed, but very few AbR genes identified in the environment are found in human or animal pathogens, which indicates severe bottlenecks for their acquisition and transmission (162, 163). However, the gut microbiota is increasingly considered a significant reservoir of AbR genes (3), which is supported by studies that associate widely spread AbR genes of relevance in clinical therapy, such as *ermB*, *ermT*, *ermC* (encoding resistance to macrolides), *vanB* (coding for resistance to glycopeptides), and *cfr* (coding for resistance to different antimicrobials), with members of the normal microbiota such as species of the *Clostridium* group XIVa now reclassified as family *Lachnospiraceae* (*Clostridium bolteae*, *Clostridium innocuum*–like, *Clostridium lavalense*, *Clostridium symbiosum*) and some lactic bacteria (3, 88, 164–168).

Recent work demonstrates that a given AbR gene (or genetic element such as Tn*1549*-*vanB*) may be independently acquired by different clonal populations in the intestine of a particular host (165). Once an AbR gene is present in gut commensals (independent of the origin of the gene), members of the normal intestinal flora of humans and animals can exchange such genes among themselves or with bacterial pathogens, which might be present in low numbers or just be passing through the intestine after being transferred from other body sites or with food intake, using different intermediates in the case of distant bacteria (3, 165, 169).

The rapid emergence in *Firmicutes* of genes coding for AbR, MetR, and BcR immediately after their introduction and significant (often massive) use in different settings has been demonstrated for chloramphenicol (*catA*), tetracyclines (*tetL*), macrolides (*ermB*), neomycin (*aad*), gentamicin (*aac6-aph2*), trimethoprim (*dfr*), beta-lactams (*blaZ*), and antiseptics (*qac*) in hospitals during the 1950s to 1970s, and for tylosin (*ermC*, *ermT*), phenicols (*fex*), pleuromutilins (*cfr*), and zinc-bacitracin in animals

during the 1990s, thus supporting the hypothesis of the existence of a previous gastrointestinal reservoir of genes that were selected for the first time as AbR genes (gene exaptation) (84, 88, 91).

Plasmids and Bacterial Population Ecology

The number and types of *Firmicutes* plasmids and integrative-conjugative elements [currently considered as plasmids under the perspective of evolutionary biology (22)] greatly vary with the different bacterial species, certainly as a result of both ecological specialization and selective events resulting from exposure to different anthropogenic activities. Most (if not all) of the contemporary isolates belonging to different species of staphylococci, enterococci, lactobacilli, and others contain plasmids of different families in a consistent pattern (for instance, RCR, small theta, or megaplasmids in *E. faecium*; pheromone plasmids in *E. faecalis*) (7, 68, 71). Such frequent plasmid-bacteria host correspondence indicates a basic co-adaptive evolutionary relation between two different types of organisms.

For a long time, plasmids were considered as “organisms,” units of a continuous lineage with an individual evolutionary history, and hence producing evolved populations, in line with the Luria and other seminal works in the field (46, 170). However, plasmids are not necessarily discrete units or individuals as classically considered in evolutionary theory (20, 170, 171). Organisms are units of selection, evolutionary units in a sense “evolutionary individuals,” defined as any entity that, independently from the number of elements that enters into its composition or from its hierarchical level of complexity, is selected and evolves as a unit (171, 172). The frequent out-of-equilibrium events that characterize the interplay between bacterial hosts, plasmids, and gene populations is explained because selective events might act independently on these different evolutionary individuals, as predicted in the “levels of selection” conceptual frame (20, 173–175). However, it is of note that we should recognize “levels of individuality”; for instance, a substantial number of *Firmicutes* plasmids have a lower-level self-identity than their bacterial hosts (18, 155), because of the more complex genetic interplay with other mobile genetic elements which in turn are also “leaky individuals,” frequently mosaics of individuals with a partial or contingent self-identity, produced under the effect of adaptive challenges when confronting variable environments (155, 176). Even if this problem of “individual constancy” (177) makes it difficult to study the network of plasmids and hosts in *Firmicutes*, and such a network were biased by sampling, we should accept the existence of a certain interactive frame.

Valeria Souza, still following Maynard Smith's ideas about the population structure of bacteria, proposed in 1997 to classify plasmid-bacteria interactions in four patterns, namely, (i) the plasmid-host clonal pattern, where the plasmid phylogeny is mirrored by host phylogeny; (ii) the limited transfer pattern, in which the plasmid flow is limited to closely related (genetically and/or ecologically) strains; (iii) panmictic plasmid spread, in the case of plasmids that circulate among a variety of hosts (the stability of the association being dependent on the benefits and costs of plasmid carriage); and (iv) epidemic plasmid dispersal, in which “successful” plasmids spread in bacterial populations because they provide a

clear advantage in high-potency selective landscapes (49, 171). Although illustrative and useful for epidemiological purposes, this single centric view should not replace the complex interplay between different elements that may result in the emergence of different chimeric configurations (49, 178). Therefore, these "patterns" should be currently understood as possible interactive states, even though some of them could be more ephemeral than others, depending on the co-evolutionary history, the adaptive demands of the plasmids, and the bacterial populations and communities.

Plasmids and Population Biology of Firmicutes

This section will focus on the genera of *Firmicutes* that are relevant to the problem of AbR (1).

Streptococcus

The genus *Streptococcus*, a main hub in gene networks in this and other studies (11, 12), is one of the most heterogeneous groups within the phyla *Firmicutes*. Remarkably, the 138 known species of streptococci found as opportunistic pathogens or commensals (many of them zoonotic pathogens) in humans, horses, pigs, cows, and fish have recently been divided into seven species groups on the basis of 16S rDNA gene sequencing, chemotaxonomic approaches, and DNA hybridization, namely the bovis, pyogenic, mitis, mutans, salivarius, anginosus, and unknown groups (179–181). HGT seems to play a relevant role in the adaptation and cohesiveness of the groups (180). Available information about streptococcal plasmids is scarce, with only a few plasmids being fully sequenced, representing an unbalanced sample of species and ecological groups (Supplementary Table S1). Figure 10 illustrates the 20 AbR plasmids currently found in streptococci.

The streptococcal groups bovis and mutans rarely harbor plasmids, although they can be relevant in the adaptation of particular species. *S. thermophilus*, a non-pathogenic species in the bovis group that is used in the dairy industry (182), contains a set of plasmids harboring heat shock proteins; *Streptococcus mutans*, a member of the human indigenous flora that is transmitted mostly from mother to child, often carries 5- to 6-kb cryptic plasmids that parallel the evolution of lineages associated with racial cohorts and geographical locations (183). Megaplasmids in the group salivarius coding for different lantibiotics favor their persistence in the oral cavity (184). Conversely, the pyogenic group, which is represented by species of clinical interest such as *S. agalactiae* and *S. pyogenes* (also called GAS and GBS, respectively), frequently carry plasmids that code for AbR genes aside from bacteriocins. Inc18 plasmids are widely spread among streptococci and seem to have determined the selection of certain populations resistant to chloramphenicol, aminoglycosides, and macrolides since the late 1970s in different groups of streptococci and enterococci (105, 110, 185). Rep_2 plasmids carrying *erm*(T) seem to have recently spread among GAS and GBS clinical isolates of different countries, having contributed to the increase of macrolide resistance rates in these species since the mid-1990s, either by clonal expansion, in the case of GAS, or by plasmid transference among unrelated clonal backgrounds, in the case of GBS (8, 186).

These *erm(*T)-containing plasmids are also spread among other non-streptococcal species, such as *Enterococcus*, *Staphylococcus*, and *Lactobacillus* (89, 187, 188). Often, streptococcal plasmids are mobilized by coresident integrative-conjugative elements belonging to the ICESa2603 family (189). Resistance to macrolides (*ermB*, *mefA*), tetracyclines (*tetM*, *tetS*, and other mosaic *tet* genes), aminoglycosides (*aph3*, *aadA6*, Tn*4001*), or vancomycin (*vanA*, *vanB*) is commonly detected among isolates of this group, but the location of determinants seems to be linked to transposable elements often involving insertion sequences (reviewed in reference (182)). *Streptococcus suis*, a particularly virulent emerging zoonotic pathogen that remains an outlier to the mitis, sanguinis, and anginosus groups is known to carry plasmids, although they have been scarcely characterized (190, 191). Relevant AbR genes coding for chloramphenicol (*cfr* and *fexA*) and lincosamides (*lnu*) embedded in composite regions similar to those present in plasmids of *E. faecalis* have been located in streptococcal plasmids of approximately 100 kb (192). Smaller plasmids carrying *tetB* associated with Gram-negative species have been described (193).

Enterococcus

The genus *Enterococcus* comprises different species, members of the intestinal flora of animals and humans able to cause disease in their hosts (194). Although seminal works in the field of plasmid biology focus on particular enterococcal plasmids and transposons, such as pheromone-responsive plasmids or Tn*916*, which became paradigms of different mechanisms of conjugation, the plasmidome of enterococcal species has scarcely been analyzed (7). Recent studies revealed that most strains of *E. faecium* and *E. faecalis*, the two main species detected in humans and animals, carry a number of plasmids of different families that include species-specific plasmids (e.g., narrow host range RCRs and RepA_N plasmids such as megaplasmids in *E. faecium* and pheromone-responsive plasmids in *E. faecalis*) and broad host range plasmids (e.g., Inc18), plasmid chimeras being abundant and still difficult to classify (Fig. 6 and 7; see previous section and comprehensive reviews in references (7, 141)). Megaplasmids of *E. faecium* or pheromone-responsive *E. faecalis* plasmids enhance the ability to colonize, invade, and form biofilms (65, 126, 154). Conjugative plasmids may influence the mobilization of non-conjugative elements and chromosomal regions and facilitate the acquisition of different adaptive traits and genome evolvability (71, 125, 126). Most enterococcal plasmids are able to acquire and disseminate AbR genes by different mechanisms of genetic exchange. However, the role of plasmids in the population structure and evolvability of these enterococcal species has been poorly addressed (195–198) due to the overrepresentation of recent clinical and animal isolates of specific lineages commonly associated with AbR included in most studies (7, 141) and due to the lack of available plasmid sequences. Similar plasmids have been found in *E. faecium* and other enterococcal species that may play equivalent functional roles in the gastrointestinal tract such as *Enterococcus avium*, *Enterococcus raffinosus*, *E. durans*, and *E. hirae* (196, 199).

AbR genes are located on plasmids that often contain different replicons associated with different narrow (RCR, RepA_N) and broad host range (Inc18) plasmids. Inc18 streptococcal plasmids greatly influenced the worldwide increase of aminoglycoside-macrolide resistance among *E. faecalis* isolates from humans and animals during the 1970s (200). They also contributed to the spread of vancomycin resistance among *E. faecium* of animal origin in Europe and *E. faecalis* from hospitalized patients in the United States (7, 70, 71). Diverse narrow host range plasmids have been involved in local expansions of enterococci conferring resistance to first-line antibiotics such as gentamicin (Tn*4001*) or beta-lactams (ΔTn*552*-*blaZ*) (152) and beta-lactamase-producing *E. faecalis* and *E. faecium* (152, 201–203), which highlights the role of endogenous plasmids and recombination in the adaptation of particular lineages (*E. faecium* ST17, ST18, ST78 and *E. faecalis* ST6 and ST16) (7, 67, 144, 204).

Analysis of the same AbR genes in different species (*cfr*, *bac*, lincosamide resistance genes) reflects the impact of recombination events between genes, MGEs, and different populations of *Firmicutes* (*Staphylococcus*, *Clostridium*, *Lactobacillus*, *Lactococcus*, and *Enterococcus*) and other Gram-negative organisms (201, 205) in the gastrointestinal tract of animals and humans (120, 144, 206, 207).

Staphylococci

These organisms are opportunistic pathogens and members of the commensal flora of skin and mucous membranes of humans and animals (208–210) and, thus, are part of a microbial community with limited contact with members of other main genera of *Firmicutes* that inhabit distinct body sites (211). Figures 1 and 2 show the limited plasmid connectivity of staphylococci with other genera. However, HGT and the acquisition of AbR and MetR is relevant in the evolvability of this genus, mainly due to genetic exchange events between closely related species (Fig. 5) (9, 212–214). Comprehensive reviews address the essentially clonal population structure of *S. aureus* (215–217) and other staphylococcal species (208) and also the impact of HGT in the evolutionary history of staphylococcal populations (9, 218–220), with emphasis on the description of the plasmids associated with AbR genetic elements (9, 51, 84, 221) and their influence on the variability of lineages (218, 220, 222–225).

Plasmids, transposons, and staphylococcal chromosomal cassettes (SCC*mec*) are infrequently transferred among isolates of a different origin. A close association of MGE and particular staphylococcal lineages has been suggested (31, 226), with country-specific variations (209, 227). This highlights the relevance of local conditions and the emergence of gene flow barriers in the ecological differentiation of staphylococcal lineages such as in the case of *S. aureus* CC30 (220, 228). The origin, rapid spread, and evolution of staphylococcal populations resistant to beta-lactams was mainly influenced by the interplay of genetic elements including plasmids (84, 178).

Clostridium

Clostridium is a large and extremely heterogeneous genus that has traditionally grouped more than 100 species widely distributed in the gut microbiota of mammals, amphibians, and insects and in soils. An

extensive update of clostridial classification is included in the latest edition of Bergey's Manual, although many unrelated species still retain the *Clostridium* name, causing major confusion in the clostridial taxonomy (229). To date, only 60 plasmids have been fully sequenced, mainly corresponding to *C. perfringens*, *Clostridium botulinum*, and other group I clostridia species (1 *Clostridium butyricum*, 2 *Clostridium kluyveri*, 3 *Clostridium acetobutylicum*). Some species in which plasmids were analyzed have been moved to other genera such as *Clostridium aciduricidi* (now *Anaerococcus prevotii* type XII) and *Clostridium thermocellum* (now belonging to the family *Ruminococcaceae*). Several sequenced plasmids correspond to the same strain and are mostly from contemporary isolates, thus limiting the possible knowledge about the role of plasmids in the evolution of these species (Supplementary Table S1). Only narrow host range conjugative plasmids of *C. perfringens* (CpCP) or linear megaplasmids from *C. butyricum* have been associated with AbR.

CpCP plasmids belong to the pCW3 family and are widely spread among isolates of *C. perfringens*, carrying genes encoding AbR (tetracycline [*tetAB(P)*], chloramphenicol [*catP*-Tn*4451*], lincomycin [*lnuP*-tIS*Cpe8*]) and/or enterotoxins, ε-toxin, or iota-toxin production that determine different toxinotypes (56, 230–232). All pCW3-like plasmids have a conjugative transfer locus of 11 open reading frames (*orfs*) (*tcp* [transfer *C. perfringens*]) that includes an integrase and a T4CP protein but lacks relaxase (73, 232). A transposable origin similar to that of Tn916 has been suggested for the *tcp* module of pCW3-like plasmids, which would have acquired a replication machinery specific to this species. Often, *C. perfringens* isolates harbor more than one pCW3 plasmid, which carry different adaptative traits and partition machineries. The presence of different partition systems explains the coexistence of different plasmids with the same type of RIP in the same cell (56, 232–234).

These plasmids can be transferred (and eventually serve as donors of AbR genes) but cannot replicate in other species such as *P. difficile*, *Clostridium sordelli*, or *Clostridium septicum*, which could explain the confinement of some AbR genes in these populations (235). An evolutionary scenario for CpCP has been reported, with pCW3 (*tetAB-P*) and pIP401 (*tetAB-P* and Tn*4451*) being suggested as the precursors of this family, which would have acquired different toxins by homologous recombination involving composite transposons flanked by insertion sequences (56). Large linear plasmids containing AbR have recently been described in neurotoxigenic *C. butyricum*, one of the six phylogroups able to produce the botulinum toxin (34, 236). These plasmids contain four beta-lactamase genes, transcriptional regulators and two-component regulatory systems, involved in the regulation of expression of the *bont/A* gene and a region with a functional CRISPR-*cas* locus that provides a defense against invading genetic elements present in the intestinal environment.

Acquired resistance to tetracyclines (*tetM*, *tetL*, *tetK*, *tetO*, *tetW*), chloramphenicol, macrolides (*ermB*, *lnu*), and bacitracin (a bacitracin efflux pump and an overproduced undercaprenol kinase gene located on a genetic island flanked by copies of IS*1216*) has been reported in human and animal clostridium species including *C. perfringens*, often associated with conjugative transposons and plasmids

widespread in other species (235, 237, 238). A detailed analysis of AbR networks suggests further ecological connections with mobile genetic elements of other prokaryotic groups (Fig. 1 and 2).

CONCLUSION

This work offers for the first time an integrated and comprehensive analysis of the dynamics of AbR genes in Gram-positive bacteria and highlights the need for a population view to analyze the problem of antibiotic resistance. The article analyzed the relevance of the plasmidome in the emergence, spread, and maintenance of genes encoding resistance to antimicrobials (antibiotics, heavy metals, and biocides) and their influence on the structure of bacterial populations in the light of evolutionary ecology. A critical revision of plasmid typing systems highlights the limitation of available knowledge about plasmid diversity in this group of bacteria.

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REFERENCES

1. Centers for Disease Control and Prevention. 2013. *Antibiotic Resistance Threats in the United States*.

2. Murphree CA, Heist EP, Moe LA. 2014. *Antibiotic resistance among cultured bacterial isolates from bioethanol fermentation facilities across the United States*. Curr Microbiol 69:277–85.

3. Salyers AA, Gupta A, Wang Y. 2004. *Human intestinal bacteria as reservoirs for antibiotic resistance genes*. Trends Microbiol 12:412–6.

4. European Food Safety Authority. 2012. *Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in Salmonella, Campylobacter and indicator Escherichia coli and Enterococcus spp. bacteria transmitted through food*. EFSA J 2012 10:2742.

5. European Food Safety Authority. 2012. *Technical specifications for the analysis and reporting of data on antimicrobial resistance (AMR) in the European Union Summary Report*. EFSA J 2012 10:2587.

6. Schwarz S, Shen J, Wendlandt S, Feßler AT, Wang Y, Kadlec K, Wu C. 2014. *Plasmid-Mediated Antimicrobial Resistance in Staphylococci and Other Firmicutes*. Microbiol Spectr 2:PLAS–0020–2014.

7. Clewell DB, Weaver KE, Coque TM, Dunny GM, Francia MV, Hayes F. 2014. *Extrachromosomal and Mobile Elements in Enterococci: Transmission, Maintenance, and Epidemiolog*. *In* Gilmore, MS, Clewell, DB, Ike, Y, Shankar, N (eds.), *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary, Boston.

8. DiPersio LP, DiPersio JR, Beach JA, Loudon AM, Fuchs AM. 2011. *Identification and characterization of plasmid-borne erm(T) macrolide resistance in group B and group A Streptococcus*. Diagn Microbiol Infect Dis 71:217–23.

9. Lindsay JA. 2014. *Staphylococcus aureus genomics and the impact of horizontal gene transfer*. Int J Med Microbiol 304:103–9.

10. Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems RJL, Earl AM, Gilmore MS. 2013. *Emergence of epidemic multi-drug-resistant Enterococcus faecium from animal and commensal strains*. MBio 4: e00534-13.

11. Beiko RG, Harlow TJ, Ragan M a. 2005. *Highways of gene sharing in prokaryotes*. Proc Natl Acad Sci U S A 102:14332–7.

12. Halary S, Leigh JW, Cheaib B, Lopez P, Bapteste E. 2010. *Network analyses structure genetic diversity in independent genetic worlds*. Proc Natl Acad Sci 107:127–32.

13. Popa O, Dagan T. 2011. *Trends and barriers to lateral gene transfer in prokaryotes*. Curr Opin Microbiol 14:615–23.

14. Popa O, Hazkani-Covo E, Landan G, Martin W, Dagan T. 2011. *Directed networks reveal genomic barriers and DNA repair bypasses to lateral gene transfer among prokaryotes*. Genome Res 21:599–609.

15. Tamminen M, Virta M, Fani R, Fondi M. 2012. *Large-scale analysis of plasmid relationships through gene-sharing networks*. Mol Biol Evol 29:1225–40.

16. Nogueira T, Rankin DJ, Touchon M, Taddei F, Brown SP, Rocha EPCC. 2009. *Horizontal gene transfer of the secretome drives the evolution of bacterial cooperation and virulence*. Curr Biol 19:1683–1691.

17. Mc Ginty SE, Rankin DJ, Brown SP. 2011. *Horizontal Gene Transfer and the Evolution of Bacterial Cooperation*. Evolution (N Y) 65:21–32.

18. Friedman J, Alm EJ, Shapiro BJ. 2013. *Sympatric speciation: when is it possible in bacteria?* PLoS One 8:e53539.

19. Hobman JL. 2015. *Molecular Life Sciences*. Springer, New York.

20. Eberhard WG. 1990. *Evolution in bacterial plasmids and levels of selection*. Q Rev Biol 65:3–22.

21. Kloesges T, Popa O, Martin W, Dagan T. 2011. *Networks of gene sharing among 329 proteobacterial genomes reveal differences in lateral gene transfer frequency at different phylogenetic depths*. Mol Biol Evol 28:1057–74.

22. Guglielmini J, Quintais L, Garcillán-Barcia MP, de la Cruz F, Rocha EPC. 2011. *The repertoire of ICE in prokaryotes underscores the unity, diversity, and ubiquity of conjugation*. PLoS Genet 7:e1002222.

23. Shapiro BJ, Friedman J, Cordero OX, Preheim SP, Timberlake SC, Szabó G, Polz MF, Alm EJ. 2012. *Population genomics of early events in the ecological differentiation of bacteria*. Science 336:48–51.

24. DeMaere MZ, Williams TJ, Allen MA, Brown M V, Gibson JAE, Rich J, Lauro FM, Dyall-Smith M, Davenport KW, Woyke T, Kyrpides NC, Tringe SG, Cavicchioli R. 2013. *High level of intergenera gene exchange shapes the evolution of haloarchaea in an isolated Antarctic lake*. Proc Natl Acad Sci U S A 110:16939–44.

25. Toussaint A, Chandler M. 2012. *Bacterial Molecular Networks.Methods in molecular biology* (Clifton, N.J.). Springer New York, New York, NY.

26. Shen J, Wang Y, Schwarz S. 2013. *Presence and dissemination of the multiresistance gene cfr in Gram-positive and Gram-negative bacteria.* J Antimicrob Chemother 68:1697–706.

27. Bonafede ME, Carias LL, Rice LB. 1997. *Enterococcal transposon Tn5384: evolution of a composite transposon through cointegration of enterococcal and staphylococcal plasmids*. Antimicrob Agents Chemother 41:1854–8.

28. Hung W-C, Takano T, Higuchi W, Iwao Y, Khokhlova O, Teng L-J, Yamamoto T. 2012. *Comparative genomics of community-acquired ST59 methicillin-resistant Staphylococcus aureus in Taiwan: novel mobile resistance structures with IS1216V*. PLoS One 7:e46987.

29. Marraffini LA, Sontheimer EJ. 2010. *CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea*. Nat Rev Genet 11:181–90.

30. Johnston C, Martin B, Polard P, Claverys J-P. 2013. *Postreplication targeting of transformants by bacterial immune systems?* Trends Microbiol 21:516–21.

31. McCarthy AJ, Lindsay JA. 2012. *The distribution of plasmids that carry virulence and resistance genes in Staphylococcus aureus is lineage associated*. BMC Microbiol 12:104.

32. Roberts GA, Houston PJ, White JH, Chen K, Stephanou AS, Cooper LP, Dryden DTF, Lindsay JA. 2013. *Impact of target site distribution for Type I restriction enzymes on the evolution of methicillin-resistant Staphylococcus aureus (MRSA) populations*. Nucleic Acids Res 41:7472–84.

33. Attaiech L, Olivier A, Mortier-Barrière I, Soulet A-L, Granadel C, Martin B, Polard P, Claverys J-P. 2011. *Role of the single-stranded DNA-binding protein SsbB in pneumococcal transformation: maintenance of a reservoir for genetic plasticity*. PLoS Genet 7:e1002156.

34. Iacobino A, Scalfaro C, Franciosa G. 2013. *Structure and genetic content of the megaplasmids of neurotoxigenic clostridium butyricum type E strains from Italy*. PLoS One 8:e71324.

35. Hatoum-Aslan A, Maniv I, Samai P, Marraffini LA. 2014. *Genetic characterization of antiplasmid immunity through a type III-A CRISPR-Cas system*. J Bacteriol 196:310–7.

36. Palmer KL, Gilmore MS. 2010. *Multi-drug-resistant enterococci lack CRISPR-cas*. MBio 1:e00227–10.

37. Serbanescu MA, Cordova M, Krastel K, Flick R, Beloglazova N, Latos A, Yakunin AF, Senadheera DB, Cvitkovitch DG. 2015. Role of the Streptococcus mutans CRISPR-Cas Systems in Immunity and Cell Physiology. J Bacteriol 197:749–761.

38. Hermsen R, Deris JB, Hwa T. 2012. *On the rapidity of antibiotic resistance evolution facilitated by a concentration gradient*. Proc Natl Acad Sci U S A 109:10775–80.

39. Shu C-C, Chatterjee A, Hu W-S, Ramkrishna D. 2013. *Role of intracellular stochasticity in biofilm growth. Insights from population balance modeling*. PLoS One 8:e79196.

40. Król JE, Wojtowicz AJ, Rogers LM, Heuer H, Smalla K, Krone SM, Top EM. 2013. *Invasion of E. coli biofilms by antibiotic resistance plasmids*. Plasmid 70:110–9.

41. Hermsen R, Hwa T. 2010. *Sources and sinks: a stochastic model of evolution in heterogeneous environments*. Phys Rev Lett 105:248104.

42. Nielsen KM, Bøhn T, Townsend JP. 2014. *Detecting rare gene transfer events in bacterial populations*. Front Microbiol 4:415.

43. McCarthy AJ, Loeffler A, Witney AA, Gould KA, Lloyd DH, Lindsay JA. 2014. *Extensive horizontal gene transfer during Staphylococcus aureus co-colonization in vivo*. Genome Biol Evol 6:2697–708.

44. Gullberg E, Albrecht LM, Karlsson C, Sandegren L, Andersson DI. 2014. *Selection of a multi-drug resistance plasmid by sublethal levels of antibiotics and heavy metals*. MBio 5:e01918–14.

45. Baquero F, Coque TM. 2014. *Widening the spaces of selection: evolution along sublethal antimicrobial gradients*. MBio 5:e02270.

46. Datta N. 1985. *Plasmids as Organisms*, p. 3–16. In Helinski, DR, Cohen, SN, Clewell, DB, Jackson, DA, Hollaender, A (eds.), *Plasmids in Bacteria*. Springer US, Boston, MA.

47. Eberhard WG. 1989. *Why do bacterial plasmids carry some genes and not others?* Plasmid 21:167–74.

48. Souza V, Eguiarte LE. 1997. *Bacteria gone native vs. bacteria gone awry?: plasmidic transfer and bacterial evolution*. Proc Natl Acad Sci U S A 94:5501–3.

49. Kozlowicz BK, Dworkin M, Dunny GM. 2006. *Pheromone-inducible conjugation in Enterococcus faecalis: a model for the evolution of biological complexity?* Int J Med Microbiol 296:141–7.

50. Taylor D, Gibreel A, Tracz D, Lawley T. 2004. *Antibiotic Resistance Plasmids*, p. 473–492. *In* Phillips, GJ, Funnell, BE (eds.), *Plasmid Biology*. ASM Press, Washington, DC, USA.

51. Shearer JES, Wireman J, Hostetler J, Forberger H, Borman J, Gill J, Sanchez S, Mankin A, Lamarre J, Lindsay JA, Bayles K, Nicholson A, O'Brien F, Jensen SO, Firth N, Skurray RA, Summers AO. 2011. *Major families of multiresistant plasmids from geographically and epidemiologically diverse staphylococci*. G3 (Bethesda) 1:581–91.

52. Lacey RW. 1975. *Antibiotic resistance plasmids of Staphylococcus aureus and their clinical importance*. Bacteriol Rev 39:1–32.

53. Novick R. 1989. *Staphylococcal Plasmids And Their Replication*. Annu Rev Microbiol.

54. Mills S, McAuliffe OE, Coffey A, Fitzgerald GF, Ross RP. 2006. *Plasmids of lactococci - genetic accessories or genetic necessities?* FEMS Microbiol Rev 30:243–73.

55. Wang TT, Lee BH. 1997. *Plasmids in Lactobacillus*. Crit Rev Biotechnol 17:227–72.

56. Li J, Adams V, Bannam TL, Miyamoto K, Garcia JP, Uzal FA, Rood JI, McClane BA. 2013. *Toxin plasmids of Clostridium perfringens*. Microbiol Mol Biol Rev 77:208–33.

57. Dib JR, Liebl W, Wagenknecht M, Farías ME, Meinhardt F. 2012. *Extrachromosomal genetic elements in Micrococcus*. Appl Microbiol Biotechnol 97:63–75.

58. Guglielmetti S, Mayo B, Álvarez-Martín P. 2013. *Mobilome and genetic modification of bifidobacteria*. Benef Microbes 4:143–66.

59. Jensen LB, Garcia-Migura L, Valenzuela AJS, Løhr M, Hasman H, Aarestrup FM. 2010. *A classification system for plasmids from enterococci and other Gram-positive bacteria*. J Microbiol Methods 80:25–43.

60. Iordanescu S, Surdeanu M, Della Latta P, Novick R. 1978. *Incompatibility and molecular relationships between small Staphylococcal plasmids carrying the same resistance marker*. Plasmid 1:468–479.

61. Macrina FL, Archer GL. 1993. *Conjugation and broad host range plasmids in streptococci and staphylococci*, p. 319–339. *In* Clewell, DB (ed.), *Bacterial conjugation*. Plenum Press, New York, NY.

62. Novick RP. 1987. *Plasmid incompatibility*. Microbiol Rev 51:381–95.

63. Projan SJ, Novick R. 1988. *Comparative analysis of five related Staphylococcal plasmids*. Plasmid 19:203–21.

64. Brantl S, Behnke D, Alonso JC. 1990. *Molecular analysis of the replication region of the conjugative Streptococcus agalactiae plasmid pIP501 in Bacillus subtilis. Comparison with plasmids pAM beta 1 and pSM19035.* Nucleic Acids Res 18:4783–90.

65. Dunny GM. 2013. *Enterococcal sex pheromones: signaling, social behavior, and evolution*. Annu Rev Genet 47:457–82.

66. Lozano C, García-Migura L, Aspiroz C, Zarazaga M, Torres C, Aarestrup FM. 2012. *Expansion of a plasmid classification system for Gram-positive bacteria and determination of the diversity of plasmids in Staphylococcus aureus strains of human, animal, and food origins*. Appl Environ Microbiol 78:5948–55.

67. Sadowy E, Luczkiewicz A. 2014. *Drug-resistant and hospital-associated Enterococcus faecium from wastewater, riverine estuary and anthropogenically impacted marine catchment basin*. BMC Microbiol 14:66.

68. Wardal E, Gawryszewska I, Hryniewicz W, Sadowy E. 2013. *Abundance and diversity of plasmid-associated genes among clinical isolates of Enterococcus faecalis*. Plasmid 70:329–42.

69. Wardal E, Markowska K, Zabicka D, Wróblewska M, Giemza M, Mik E, Połowniak-Pracka H, Woźniak A, Hryniewicz W, Sadowy E. 2014. *Molecular analysis of vanA outbreak of Enterococcus faecium in two Warsaw hospitals: the importance of mobile genetic elements*. Biomed Res Int 2014:575367.

70. Rosvoll TCS, Pedersen T, Sletvold H, Johnsen PJ, Sollid JE, Simonsen GS, Jensen LB, Nielsen KM, Sundsfjord A. 2010. *PCR-based plasmid typing in Enterococcus faecium strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHTbeta-related replicons associated with glycopeptide resistance and stabilizing toxin-antitoxin systems.* FEMS Immunol Med Microbiol 58:254–68.

71. Freitas AR, Novais C, Tedim AP, Francia MVMV, Baquero F, Coque TM, Peixe L, Coque TM. 2013. *Microevolutionary Events Involving Narrow Host Plasmid Influences Local Fixation of Vancomycin-Resistance in Enterococcus*. PLoS One e60589.

72. Francia MV, Varsaki A, Garcillán-Barcia MP, Latorre A, Drainas C, de la Cruz F. 2004. *A classification scheme for mobilization regions of bacterial plasmids*. FEMS Microbiol Rev 28:79–100.

73. Garcillán-Barcia MP, Francia MV, de la Cruz F. 2009. *The diversity of conjugative relaxases and its application in plasmid classification*. FEMS Microbiol Rev 33:657–87.

74. Smillie C, Garcillán-Barcia MP, Francia MV, Rocha EPC, de la Cruz F. 2010. *Mobility of plasmids*. Microbiol Mol Biol Rev 74:434–52.

75. Lorenzo-Díaz F, Fernández-López C, Garcillán-Barcia MP, Espinosa M. 2014. *Bringing them together: plasmid pMV158 rolling circle replication and conjugation under an evolutionary perspective*. Plasmid 74:15–31.

76. Weaver KE, Kwong SM, Firth N, Francia MV. 2009. *The RepA_N replicons of Gram-positive bacteria: a family of broadly distributed but narrow host range plasmids*. Plasmid 61:94–109.

77. Gruss A, Ehrlich SD. 1989. *The family of highly interrelated single-stranded deoxyribonucleic acid plasmids.* Microbiol Rev 53:231–241.

78. del Solar G, Moscoso M, Espinosa M. 1993. *Rolling circle-replicating plasmids from gram-positive and gram-negative bacteria: a wall falls*. Mol Microbiol 8:789–96.

79. Khan SA. 1997. *Rolling-circle replication of bacterial plasmids*. Microbiol Mol Biol Rev 61:442–55.

80. Widdowson CA, Adrian P V, Klugman KP. 2000. *Acquisition of chloramphenicol resistance by the linearization and integration of the entire staphylococcal plasmid pC194 into the chromosome of Streptococcus pneumoniae.* Antimicrob Agents Chemother 44:393–5.

81. Li W, Godzik A. 2006. *Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequence*s. Bioinformatics 22:1658–9.

82. Heng NCK, Burtenshaw GA, Jack RW, Tagg JR. 2004. *Sequence analysis of pDN571, a plasmid encoding novel bacteriocin production in M-type 57 Streptococcus pyogenes*. Plasmid 52:225–9.

83. Romero P, Llull D, García E, Mitchell TJ, López R, Moscoso M. 2007. *Isolation and characterization of a new plasmid pSpnP1 from a multi-drug-resistant clone of Streptococcus pneumoniae.* Plasmid 58:51–60.

84. Gillespie MT, May JW, Skurray RA. 1985. *Antibiotic resistance in Staphylococcus aureus isolated at an Australian hospital between 1946 and 1981*. J Med Microbiol 19:137–47.

85. Schiwon K, Arends K, Rogowski KM, Fürch S, Prescha K, Sakinc T, Van Houdt R, Werner G, Grohmann E. 2013. *Comparison of antibiotic resistance, biofilm formation and conjugative transfer of Staphylococcus and Enterococcus isolates from International Space Station and Antarctic Research Station Concordia*. Microb Ecol 65:638–51.

86. Shiwa Y, Yanase H, Hirose Y, Satomi S, Araya-Kojima T, Watanabe S, Zendo T, Chibazakura T, Shimizu-Kadota M, Yoshikawa H, Sonomoto K. 2014. *Complete genome sequence of Enterococcus mundtii QU 25, an efficient L-(+)-lactic acid-producing bacterium.* DNA Res 21:369–77.

87. Garcia-Migura L, Hasman H, Jensen LB. 2009. *Presence of pRI1: a small cryptic mobilizable plasmid isolated from Enterococcus faecium of human and animal origin.* Curr Microbiol 58:95–100.

88. de Vries LE, Christensen H, Agersø Y. 2012. *The diversity of inducible and constitutively expressed erm(C) genes and association to different replicon types in staphylococci plasmids*. Mob Genet Elements 2:72–80.

89. Gómez-Sanz E, Kadlec K, Feßler AT, Zarazaga M, Torres C, Schwarz S. 2013. *Novel erm(T)-carrying multiresistance plasmids from porcine and human isolates of methicillin-resistant Staphylococcus aureus ST398 that also harbor cadmium and copper resistance determinants.* Antimicrob Agents Chemother 57:3275–82.

90. Firth N, Skurray RA. 1998. *Mobile elements in the evolution and spread of multiple-drug resistance in staphylococci.* Drug Resist Updat 1:49–58.

91. Charlebois A, Jalbert L-A, Harel J, Masson L, Archambault M. 2012. *Characterization of genes encoding for acquired bacitracin resistance in Clostridium perfringens.* PLoS One 7:e44449.

92. Palmer KL, Kos VN, Gilmore MS. 2010. *Horizontal gene transfer and the genomics of enterococcal antibiotic resistance.* Curr Opin Microbiol 13:632–9.

93. Montilla A, Zavala A, Cáceres Cáceres R, Cittadini R, Vay C, Gutkind G, Famiglietti A, Bonofiglio L, Mollerach M. 2014. *Genetic environment of the lnu(B) gene in a Streptococcus agalactiae clinical isolate.* Antimicrob Agents Chemother 58:5636–7.

94. Zong Z. 2013. *Characterization of a complex context containing mecA but lacking genes encoding cassette chromosome recombinases in Staphylococcus haemolyticus*. BMC Microbiol 13:64.

95. Locke JB, Zuill DE, Scharn CR, Deane J, Sahm DF, Denys GA, Goering R V, Shaw KJ. 2014. *Linezolid-resistant Staphylococcus aureus strain 1128105, the first known clinical isolate possessing the cfr multi-drug resistance gene*. Antimicrob Agents Chemother 58:6592–8.

96. Goessweiner-Mohr N, Arends K, Keller W, Grohmann E. 2013. *Conjugative type IV secretion systems in Gram-positive bacteria*. Plasmid 70:289–302.

97. Li J, Li B, Wendlandt S, Schwarz S, Wang Y, Wu C, Ma Z, Shen J. 2014. *Identification of a novel vga(E) gene variant that confers resistance to pleuromutilins, lincosamides and streptogramin A antibiotics in staphylococci of porcine origin*. J Antimicrob Chemother 69:919–23.

98. Lozano C, Aspiroz C, Rezusta A, Gómez-Sanz E, Simon C, Gómez P, Ortega C, Revillo MJ, Zarazaga M, Torres C. 2012. *Identification of novel vga(A)-carrying plasmids and a Tn5406-like transposon in meticillin-resistant*

Staphylococcus aureus and Staphylococcus epidermidis of human and animal origin. Int J Antimicrob Agents 40:306–12.

99. Brantl S. 2015. *Antisense-RNA mediated control of plasmid replication – pIP501 revisited*. Plasmid 78:4–16.

100. Grohmann E, Muth G, Espinosa M. 2003. *Conjugative plasmid transfer in gram-positive bacteria.* Microbiol Mol Biol Rev 67:277–301, table of contents.

101. Behnke D, Malke H, Hartmann M, Walter F. 1979. *Post-transformational rearrangement of an in vitro reconstructed group-A streptococcal erythromycin resistance plasmid.* Plasmid 2:605–616.

102. Clewell DB, Yagi Y, Dunny GM, Schultz SK. 1974. *Characterization of three plasmid deoxyribonucleic acid molecules in a strain of Streptococcus faecalis: identification of a plasmid determining erythromycin resistance*. J Bacteriol 117:283–9.

103. Horodniceanu T, Bouanchaud DH, Bieth G, Chabbert YA. 1976. *R plasmids in Streptococcus agalactiae (group B).* Antimicrob Agents Chemother 10:795–801.

104. Thompson JK, Collins MA. 2003. *Completed sequence of plasmid pIP501 and origin of spontaneous deletion derivatives*. Plasmid 50:28–35.

105. Horaud T, Le Bouguenec C, Pepper K. 1985. *Molecular genetics of resistance to macrolides, lincosamides and streptogramin B (MLS) in streptococci*. J Antimicrob Chemother 16 Suppl A:111–35.

106. Derome A, Hoischen C, Bussiek M, Grady R, Adamczyk M, Kedzierska B, Diekmann S, Barillà D, Hayes F. 2008. *Centromere anatomy in the multi-drug-resistant pathogen Enterococcus faecium.* Proc Natl Acad Sci U S A 105:2151–6.

107. Li X, Alvarez V, Harper WJ, Wang HH. 2011. *Persistent, toxin-antitoxin system-independent, tetracycline resistance-encoding plasmid from a dairy Enterococcus faecium isolate*. Appl Environ Microbiol 77:7096–103.

108. Abajy MY, Kopeć J, Schiwon K, Burzynski M, Döring M, Bohn C, Grohmann E. 2007. *A type IV-secretion-like system is required for conjugative DNA transport of broad-host-range plasmid pIP501 in gram-positive bacteria*. J Bacteriol 189:2487–96.

109. Krah ER, Macrina FL. 1991. *Identification of a region that influences host range of the streptococcal conjugative plasmid pIP501*. Plasmid 25:64–9.

110. Schaberg DR, Zervos MJ. 1986. *Intergeneric and interspecies gene exchange in gram-positive cocci.* Antimicrob Agents Chemother 30:817–22.

111. Teuber M, Meile L, Schwarz F. *Acquired antibiotic resistance in lactic acid bacteria from food.* Antonie Van Leeuwenhoek 76:115–37.

112. El-Solh N, Bouanchaud DH, Horodniceanu T, Roussel A, Chabbert YA. 1978. *Molecular studies and possible relatedness between R plasmids from groups B and D streptococci.* Antimicrob Agents Chemother 14:19–23.

113. Engel HW, Soedirman N, Rost JA, van Leeuwen WJ, van Embden JD. 1980. *Transferability of macrolide, lincomycin, and streptogramin resistances between group A, B, and D streptococci, Streptococcus pneumoniae, and Staphylococcus aureus*. J Bacteriol 142:407–13.

114. Clewell DB. 1981. *Plasmids, drug resistance, and gene transfer in the genus Streptococcus*. Microbiol Rev 45:409–36.

115. Sletvold H, Johnsen PJ, Simonsen GS, Aasnaes B, Sundsfjord A, Nielsen KM. 2007. *Comparative DNA analysis of two vanA plasmids from Enterococcus faecium strains isolated from poultry and a poultry farmer in Norway*. Antimicrob Agents Chemother 51:736–9.

116. Sletvold H, Johnsen PJ, Wikmark O-G, Simonsen GS, Sundsfjord A, Nielsen KM. 2010. *Tn1546 is part of a larger plasmid-encoded genetic unit horizontally disseminated among clonal Enterococcus faecium lineages*. J Antimicrob Chemother 65:1894–906.

117. Zhu W, Clark NC, McDougal LK, Hageman J, McDonald LC, Patel JB. 2008. *Vancomycin-resistant Staphylococcus aureus isolates associated with Inc18-like vanA plasmids in Michigan.* Antimicrob Agents Chemother 52:452–7.

118. Zhu W, Murray PR, Huskins WC, Jernigan JA, McDonald LC, Clark NC, Anderson KF, McDougal LK, Hageman JC, Olsen-Rasmussen M, Frace M, Alangaden GJ, Chenoweth C, Zervos MJ, Robinson-Dunn B, Schreckenberger PC, Reller LB, Rudrik JT, Patel JB. 2010. *Dissemination of an Enterococcus Inc18-Like vanA plasmid associated with vancomycin-resistant Staphylococcus aureus.* Antimicrob Agents Chemother 54:4314–20.

119. Teuber M, Schwarz F, Perreten V. 2003. *Molecular structure and evolution of the conjugative multiresistance plasmid pRE25 of Enterococcus faecalis isolated from a raw-fermented sausage*. Int J Food Microbiol 88:325–9.

120. Maki T, Santos MD, Kondo H, Hirono I, Aoki T. 2009. *A transferable 20-kilobase multiple drug resistance-conferring R plasmid (pKL0018) from a fish pathogen (Lactococcus garvieae) is highly homologous to a conjugative multiple drug resistance-conferring enterococcal plasmid.* Appl Environ Microbiol 75:3370–2.

121. Lim S-K, Tanimoto K, Tomita H, Ike Y. 2006. *Pheromone-responsive conjugative vancomycin resistance plasmids in Enterococcus faecalis isolates from humans and chicken feces*. Appl Environ Microbiol 72:6544–53.

122. Tanimoto K, Ike Y. 2008. *Complete nucleotide sequencing and analysis of the 65-kb highly conjugative Enterococcus faecium plasmid pMG1: identification of the transfer-related region and the minimum region required for replication.* FEMS Microbiol Lett 288:186–95.

123. Tomita H, Pierson C, Lim SK, Clewell DB, Ike Y. 2002. *Possible connection between a widely disseminated conjugative gentamicin resistance (pMG1-like) plasmid and the emergence of vancomycin resistance in Enterococcus faecium*. J Clin Microbiol 40:3326–33.

124. Tomita H, Tanimoto K, Hayakawa S, Morinaga K, Ezaki K, Oshima H, Ike Y. 2003. *Highly conjugative pMG1-like plasmids carrying Tn1546-like transposons that encode vancomycin resistance in Enterococcus faecium*. J Bacteriol 185:7024–8.

125. Manson JM, Hancock LE, Gilmore MS. 2010. *Mechanism of chromosomal transfer of Enterococcus faecalis pathogenicity island, capsule, antimicrobial resistance, and other traits*. Proc Natl Acad Sci U S A 107:12269–74.

126. Arias CA, Panesso D, Singh K V, Rice LB, Murray BE. 2009. *Cotransfer of antibiotic resistance genes and a hylEfm-containing virulence plasmid in Enterococcus faecium*. Antimicrob Agents Chemother 53:4240–6.

127. Firth N, Apisiridej S, Berg T, O'Rourke BA, Curnock S, Dyke KG, Skurray RA. 2000. *Replication of staphylococcal multiresistance plasmids*. J Bacteriol 182:2170–8.

128. Kwong SM, Lim R, Lebard RJ, Skurray RA, Firth N. 2008. *Analysis of the pSK1 replicon, a prototype from the staphylococcal multiresistance plasmid family.* Microbiology 154:3084–94.

129. Berg T, Firth N, Apisiridej S, Hettiaratchi A, Leelaporn A, Skurray RA. 1998. *Complete nucleotide sequence of pSK41: evolution of staphylococcal conjugative multiresistance plasmids*. J Bacteriol 180:4350–9.

130. Littlejohn TG, DiBerardino D, Messerotti LJ, Spiers SJ, Skurray RA. 1991. *Structure and evolution of a family of genes encoding antiseptic and disinfectant resistance in Staphylococcus aureus*. Gene 101:59–66.

131. Pérez-Roth E, Kwong SM, Alcoba-Florez J, Firth N, Méndez-Alvarez S. 2010. *Complete nucleotide sequence and comparative analysis of pPR9, a 41.7-kilobase conjugative staphylococcal multiresistance plasmid conferring high-level mupirocin resistance*. Antimicrob Agents Chemother 54:2252–7.

132. Pérez-Roth E, Potel-Alvarellos C, Espartero X, Constela-Caramés L, Méndez-Álvarez S, Alvarez-Fernández M. 2013. *Molecular epidemiology of plasmid-mediated high-level mupirocin resistance in methicillin-resistant Staphylococcus aureus in four Spanish health care settings*. Int J Med Microbiol 303:201–4.

133. Udo EE, Jacob LE. 1998. *Conjugative transfer of high-level mupirocin resistance and the mobilization of non-conjugative plasmids in Staphylococcus aureus*. Microb Drug Resist 4:185–93.

134. Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, Flannagan SE, Kolonay JF, Shetty J, Killgore GE, Tenover FC. 2003. *Genetic analysis of a high-level vancomycin-resistant isolate of Staphylococcus aureus*. Science 302:1569–71.

135. Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, Lin F, Lin J, Carleton HA, Mongodin EF, Sensabaugh GF, Perdreau-Remington F. 2006. *Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus*. Lancet 367:731–9.

136. Thomas WD, Archer GL. 1992. *Mobilization of recombinant plasmids from Staphylococcus aureus into coagulase negative Staphylococcus species*. Plasmid 27:164–8.

137. Projan SJ, Archer GL. 1989. *Mobilization of the relaxable Staphylococcus aureus plasmid pC221 by the conjugative plasmid pGO1 involves three pC221 loci*. J Bacteriol 171:1841–5.

138. Wardal E, Sadowy E, Hryniewicz W. 2010. *Complex nature of enterococcal pheromone-responsive plasmids*. Pol J Microbiol 59:79–87.

139. Clewell DB, Francia MV. 2004. *Conjugation in Gram-Positive Bacteria*, p. 227–256. *In* Phillips, GJ, Funnell, BE (eds.), *Plasmid Biology*. ASM Press, Washington, DC.

140. Clewell DB, Francia MV, Flannagan SE, An FY. 2002. *Enterococcal plasmid transfer: sex pheromones, transfer origins, relaxases, and the Staphylococcus aureus issue.* Plasmid 48:193–201.

141. Weaver KE. 2015. *The Type I toxin-antitoxin par locus from Enterococcus faecalis plasmid pAD1: RNA regulation by both cis- and trans-acting elements*. Plasmid 78:65–70.

142. Grady R, Hayes F. 2003. *Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multi-drug-resistant, clinical isolate of Enterococcus faecium*. Mol Microbiol 47:1419–32.

143. Halvorsen EM, Williams JJ, Bhimani AJ, Billings EA, Hergenrother PJ. 2010. *Txe, an endoribonuclease of the enterococcal Axe-Txe toxin-antitoxin system, cleaves mRNA and inhibits protein synthesis.* Microbiology 157:387–397.

144. Freitas AR, Coque TM, Novais C, Hammerum AM, Lester CH, Zervos MJ, Donabedian S, Jensen LB, Francia MV, Baquero F, Peixe L. 2011. *Human and swine hosts share vancomycin-resistant Enterococcus faecium CC17 and CC5 and Enterococcus faecalis CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids*. J Clin Microbiol 49:925–31.

145. Werner G, Klare I, Witte W. 1999. *Large conjugative vanA plasmids in vancomycin-resistant Enterococcus faecium*. J Clin Microbiol 37:2383–4.

146. Hasman H, Villadsen AG, Aarestrup FM. 2005. *Diversity and stability of plasmids from glycopeptide-resistant Enterococcus faecium (GRE) isolated from pigs in Denmark*. Microb Drug Resist 11:178–84.

147. Biavasco F, Foglia G, Paoletti C, Zandri G, Magi G, Guaglianone E, Sundsfjord A, Pruzzo C, Donelli G, Facinelli B. 2007. *VanA-type enterococci from humans, animals, and food: species distribution, population structure, Tn1546 typing and location, and virulence determinants*. Appl Environ Microbiol 73:3307–19.

148. Laverde Gomez JA, van Schaik W, Freitas AR, Coque TM, Weaver KE, Francia MV, Witte W, Werner G. 2011. *A multiresistance megaplasmid pLG1 bearing a hylEfm genomic island in hospital Enterococcus faecium isolates*. Int J Med Microbiol 301:165–75.

149. Zhang X, Vrijenhoek JEP, Bonten MJM, Willems RJL, van Schaik W. 2011. *A genetic element present on megaplasmids allows Enterococcus faecium to use raffinose as carbon source.* Environ Microbiol 13:518–28.

150. Gordoncillo MJN, Donabedian S, Bartlett PC, Perri M, Zervos M, Kirkwood R, Febvay C. 2013. *Isolation and molecular characterization of vancomycin-resistant Enterococcus faecium from swine in Michigan, USA.* Zoonoses Public Health 60:319–26.

151. Johnson PDR, Ballard SA, Grabsch EA, Stinear TP, Seemann T, Young HL, Grayson ML, Howden BP. 2010. *A sustained hospital outbreak of vancomycin-resistant Enterococcus faecium bacteremia due to emergence of vanB E. faecium sequence type 203*. J Infect Dis 202:1278–86.

152. Rosvoll TCS, Lindstad BL, Lunde TM, Hegstad K, Aasnaes B, Hammerum AM, Lester CH, Simonsen GS, Sundsfjord A, Pedersen T. 2012. *Increased high-level gentamicin resistance in invasive Enterococcus faecium is associated with aac(6')Ie-aph(2")Ia-encoding transferable megaplasmids hosted by major hospital-adapted lineages.* FEMS Immunol Med Microbiol 66:166–76.

153. Panesso D, Montealegre MC, Rincón S, Mojica MF, Rice LB, Singh K V, Murray BE, Arias CA. 2011. *The hylEfm gene in pHylEfm of Enterococcus faecium is not required in pathogenesis of murine peritonitis*. BMC Microbiol 11:20.

154. Kim DS, Singh K V, Nallapareddy SR, Qin X, Panesso D, Arias CA, Murray BE. 2010. *The fms21 (pilA)-fms20 locus encoding one of four distinct pili of Enterococcus faecium is harboured on a large transferable plasmid associated with gut colonization and virulence*. J Med Microbiol 59:505–7.

155. Boon E, Meehan CJ, Whidden C, Wong DH-J, Langille MGI, Beiko RG. 2014. *Interactions in the microbiome: communities of organisms and communities of genes.* FEMS Microbiol Rev 38:90–118.

156. Smillie CS, Smith MB, Friedman J, Cordero OX, David LA, Alm EJ. 2011. *Ecology drives a global network of gene exchange connecting the human microbiome*. Nature 480:241–4.

157. Skippington E, Ragan MA. 2011. Within-species lateral genetic transfer and the evolution of transcriptional regulation in Escherichia coli and Shigella. BMC Genomics 12:532.

158. Baquero F, Coque TM, Cantón R. 2003. *Antibiotics, Complexity, and Evolution*. ASM News 69:547–553.

159. Baquero F, Tedim AP, Coque TM. 2013. *Antibiotic resistance shaping multi-level population biology of bacteria*. Front Microbiol 4:15.

160. Baquero F, Lanza VF, Cantón R, Coque TM. 2015. *Public health evolutionary biology of antimicrobial resistance: priorities for intervention*. Evol Appl 8:223–39.

161. Baquero F, Coque TM, Cantón R. 2014. *Counteracting antibiotic resistance: breaking barriers among antibacterial strategies.* Expert Opin Ther Targets 18:851–61.

162. Martínez JL. 2008. *Antibiotics and antibiotic resistance genes in natural environments*. Science (80-) 321:365–7.

163. Martínez JL. 2011. *Bottlenecks in the transferability of antibiotic resistance from natural ecosystems to human bacterial pathogens*. Front Microbiol 2:265.

164. Berryman DI, Rood JI. 1995. *The closely related ermB-ermAM genes from Clostridium perfringens, Enterococcus faecalis (pAM beta 1), and Streptococcus agalactiae (pIP501) are flanked by variants of a directly repeated sequence*. Antimicrob Agents Chemother 39:1830–4.

165. Howden BP, Holt KE, Lam MMC, Seemann T, Ballard S, Coombs GW, Tong SYC, Grayson ML, Johnson PDR, Stinear TP, Lindsay Grayson M, Johnson PDR, Stinear TP, Grayson ML, Johnson PDR, Stinear TP. 2013. *Genomic insights to control the emergence of vancomycin-resistant enterococci*. MBio 4:e00412–13.

166. Marvaud J-C, Mory F, Lambert T. 2011. *Clostridium clostridioforme and Atopobium minutum clinical isolates with vanB-type resistance in France*. J Clin Microbiol 49:3436–8.

167. Ballard SA, Grabsch EA, Johnson PDR, Grayson ML. 2005. *Comparison of three PCR primer sets for identification of vanB gene carriage in feces and correlation with carriage of vancomycin-resistant enterococci: interference by vanB-containing anaerobic bacilli*. Antimicrob Agents Chemother 49:77–81.

168. Domingo M-C, Huletsky A, Boissinot M, Hélie M-C, Bernal A, Bernard KA, Grayson ML, Picard FJ, Bergeron MG. 2009. *Clostridium lavalense sp. nov., a glycopeptide-resistant species isolated from human faeces*. Int J Syst Evol Microbiol 59:498–503.

169. Marshall BM, Ochieng D, Levy SB. 2009. *Commensals: underappreciated reservoir of antibiotic resistance.* Microbe Mag 4:231–238.

170. Lederberg J. 1998. *Plasmid* (1952-1997). Plasmid 39:1–9.

171. Maynard-Smith J. 1982. *The century since Darwin*. Nature 296:599–601.

172. Baquero F. 2014. *Genetic hyper-codes and multidimensional Darwinism: replication modes and codes in evolutionary individuals of the bacterial world*, p. 165–180. *In* Trueba, G (ed.), *Why does Evolution Matter?: The importance of understanding evolution*. Cambridge Scholars Publishing, Newcastle upon Tyne.

173. Baquero F. 2004. *From pieces to patterns: evolutionary engineering in bacterial pathogens*. Nat Rev Microbiol 2:510–8.

174. Okasha S. 2007. *Evolution and the Levels of Selection*. Clarendon Press, Oxford, UK.

175. Paulsson J. 2002. *Multileveled selection on plasmid replication*. Genetics 161:1373–84.

176. Cordero OX, Polz MF. 2014. *Explaining microbial genomic diversity in light of evolutionary ecology.* Nat Rev Microbiol 12:263–73.

177. Norton B. 1996. Change, Constancy, and Creativity: *The New Ecology and Some Old Problems*. Duke Environ Law Policy Forum.

178. Hiramatsu K, Ito T, Tsubakishita S, Sasaki T, Takeuchi F, Morimoto Y, Katayama Y, Matsuo M, Kuwahara-Arai K, Hishinuma T, Baba T. 2013. *Genomic Basis for Methicillin Resistance in Staphylococcus aureus*. Infect Chemother 45:117.

179. Haines AN, Gauthier DT, Nebergall EE, Cole SD, Nguyen KM, Rhodes MW, Vogelbein WK. 2013. *First report of Streptococcus parauberis in wild finfish from North America*. Vet Microbiol 166:270–5.

180. Richards VP, Palmer SR, Pavinski Bitar PD, Qin X, Weinstock GM, Highlander SK, Town CD, Burne RA, Stanhope MJ. 2014. *Phylogenomics and the dynamic genome evolution of the genus Streptococcus*. Genome Biol Evol 6:741–53.

181. Gao X-Y, Zhi X-Y, Li H-W, Klenk H-P, Li W-J. 2014. *Comparative Genomics of the Bacterial Genus Streptococcus Illuminates Evolutionary Implications of Species Groups*. PLoS One 9:e101229.

182. Jans C, Meile L, Lacroix C, Stevens MJA. 2014. *Genomics, evolution, and molecular epidemiology of the Streptococcus bovis/Streptococcus equinus complex (SBSEC)*. Infect Genet Evol.

183. Caufield PW, Saxena D, Fitch D, Li Y. 2007. *Population structure of plasmid-containing strains of Streptococcus mutans, a member of the human indigenous biota*. J Bacteriol 189:1238–43.

184. Wescombe PA, Burton JP, Cadieux PA, Klesse NA, Hyink O, Heng NCK, Chilcott CN, Reid G, Tagg JR. 2006. *Megaplasmids encode differing combinations of lantibiotics in Streptococcus salivarius*. Antonie Van Leeuwenhoek 90:269–280.

185. Pepper K, Le Bouguénec C, de Cespédès G, Horaud T. 1986. *Dispersal of a plasmid-borne chloramphenicol resistance gene in streptococcal and enterococcal plasmids*. Plasmid 16:195–203.

186. Woodbury RL, Klammer KA, Xiong Y, Bailiff T, Glennen A, Bartkus JM, Lynfield R, Van Beneden C, Beall BW. 2008. *Plasmid-Borne erm(T) from invasive, macrolide-resistant Streptococcus pyogenes strains*. Antimicrob Agents Chemother 52:1140–3.

187. Whitehead TR, Cotta MA. 2001. *Sequence Analyses of a Broad Host-Range Plasmid Containing erm T from a Tylosin-Resistant Lactobacillus sp. Isolated from Swine Feces*. Curr Microbiol 43:17–20.

188. DiPersio LP, DiPersio JR. 2006. *High rates of erythromycin and clindamycin resistance among OBGYN isolates of group B Streptococcus*. Diagn Microbiol Infect Dis 54:79–82.

189. Palmieri C, Magi G, Creti R, Baldassarri L, Imperi M, Gherardi G, Facinelli B. 2012. *Interspecies mobilization of an erm(T)-carrying plasmid of Streptococcus dysgalactiae subsp. equisimilis by a coresident ICE of the ICESa2603 family*. J Antimicrob Chemother 68:23–26.

190. Holden MTG, Hauser H, Sanders M, Ngo TH, Cherevach I, Cronin A, Goodhead I, Mungall K, Quail MA, Price C, Rabbinowitsch E, Sharp S, Croucher NJ, Chieu TB, Mai NTH, Diep TS, Chinh NT, Kehoe M, Leigh JA, Ward PN, Dowson CG, Whatmore AM, Chanter N, Iversen P, Gottschalk M, Slater JD, Smith HE, Spratt BG, Xu J, Ye C, Bentley S, Barrell BG, Schultsz C, Maskell DJ, Parkhill J. 2009. *Rapid evolution of virulence and drug resistance in the emerging zoonotic pathogen Streptococcus suis.* PLoS One 4:e6072.

191. Cantin M, Harel J, Higgins R, Gottschalk M. 1992. *Antimicrobial resistance patterns and plasmid profiles of Streptococcus suis isolates*. J Vet Diagn Invest 4:170–4.

192. Zhu X, Zheng B, Wang S, Willems RJL, Xue F, Cao X, Li Y, Bo S, Liu J. 2009. *Molecular characterisation of outbreak-related strains of vancomycin-resistant Enterococcus faecium from an intensive care unit in Beijing, China*. J Hosp Infect 72:147–54.

193. Chander Y, Oliveira SR, Goyal SM. 2011. *Identification of the tet(B) resistance gene in Streptococcus suis*. Vet J 189:359–360.

194. Lebreton F, Willems RJL, Gilmore MS. 2014. *Enterococcus Diversity, Origins in Nature, and Gut Colonization*. *In* Gilmore, MS, Clewell, DB, Ike, Y, Shankar, N (eds.), *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary, Boston: Massachusetts Eye and Ear Infirmary.

195. Willems RJL, Top J, van Schaik W, Leavis H, Bonten M, Sirén J, Hanage WP, Corander J. 2012. *Restricted gene flow among hospital subpopulations of Enterococcus faecium*. MBio 3:e00151–12.

196. Tedim AP, Ruiz-Garbajosa P, Corander J, Rodríguez CM, Cantón R, Willems RJ, Baquero F, Coque TM. 2015. *Population Biology of Intestinal Enterococcus Isolates from Hospitalized and Nonhospitalized Individuals in Different Age Groups*. Appl Environ Microbiol 81:1820–1831.

197. McBride SM, Fischetti VA, Leblanc DJ, Moellering RC, Gilmore MS. 2007. *Genetic diversity among Enterococcus faecalis*. PLoS One 2:e582.

198. Kuch A, Willems RJL, Werner G, Coque TM, Hammerum AM, Sundsfjord A, Klare I, Ruiz-Garbajosa P, Simonsen GS, van Luit-Asbroek M, Hryniewicz W, Sadowy E. 2012. *Insight into antimicrobial susceptibility and population structure of contemporary human Enterococcus faecalis isolates from Europe*. J Antimicrob Chemother 67:551–8.

199. López M, Tedim AP, Baquero F, Torres C, Coque TM. 2011. *Plasmid Diversity among Enterococcus spp from Humans and Animals (1991-2010).* 4th Congress of European Microbiologist, FEMS. Geneva, Switzerland.

200. LeBlanc DJ, Inamine JM, Lee LN. 1986. *Broad geographical distribution of homologous erythromycin, kanamycin, and streptomycin resistance determinants among group D streptococci of human and animal origin.* Antimicrob Agents Chemother 29:549–55.

201. Murray BE, An FY, Clewell DB. 1988. *Plasmids and pheromone response of the beta-lactamase producer Streptococcus (Enterococcus) faecalis HH22.* Antimicrob Agents Chemother 32:547–51.

202. Murray BE, Singh K V, Markowitz SM, Lopardo HA, Patterson JE, Zervos MJ, Rubeglio E, Eliopoulos GM, Rice LB, Goldstein FW. 1991. *Evidence for clonal spread of a single strain of beta-lactamase-producing Enterococcus (Streptococcus) faecalis to six hospitals in five states*. J Infect Dis 163:780–5.

203. Patterson JE, Singh K V, Murray BE. 1991. *Epidemiology of an endemic strain of beta-lactamase-producing Enterococcus faecalis.* J Clin Microbiol 29:2513–6.

204. Werner GDA, Strassmann JE, Ivens ABF, Engelmoer DJP, Verbruggen E, Queller DC, Noë R, Johnson NC, Hammerstein P, Kiers ET. 2014. *Evolution of microbial markets*. Proc Natl Acad Sci U S A 111:1237–44.

205. Datta N, Hughes VM. 1983. *Plasmids of the same Inc groups in Enterobacteria before and after the medical use of antibiotic*s. Nature 306:616–7.

206. Wang X-M, Li X-S, Wang Y-B, Wei F-S, Zhang S-M, Shang Y-H, Du X-D. 2015. *Characterization of a multi-drug resistance plasmid from Enterococcus faecium that harbours a mobilized bcrABDR locus*. J Antimicrob Chemother 70:609–11.

207. Devirgiliis C, Zinno P, Perozzi G. 2013. *Update on antibiotic resistance in foodborne Lactobacillus and Lactococcus species*. Front Microbiol 4:301.

208. Becker K, Heilmann C, Peters G. 2014. *Coagulase-negative staphylococci*. Clin Microbiol Rev 27:870–926.

209. Lindsay JA, Holden MTG. 2004. *Staphylococcus aureus: Superbug, super genome?* Trends Microbiol 8: 378-85.

210. Lindsay JA. 2010. *Genomic variation and evolution of Staphylococcus aureus*. Int J Med Microbiol 300:98–103.

211. Dethlefsen L, McFall-Ngai M, Relman DA. 2007. *An ecological and evolutionary perspective on human-microbe mutualism and disease*. Nature 449:811–8.

212. Lindsay JA, Knight GM, Budd EL, McCarthy AJ. 2012. *Shuffling of mobile genetic elements (MGEs) in successful healthcare-associated MRSA (HA-MRSA)*. Mob Genet Elements 2:239–243.

213. Livermore DM. 2000. *Antibiotic resistance in staphylococci*. Int J Antimicrob Agents 16:S3–10.

214. Sidhu MS, Heir E, Leegaard T, Wiger K, Holck A. 2002. *Frequency of disinfectant resistance genes and genetic linkage with beta-lactamase transposon Tn552 among clinical staphylococci*. Antimicrob Agents Chemother 46:2797–803.

215. Willems RJL, Hanage WP, Bessen DE, Feil EJ. 2011. *Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance.* FEMS Microbiol Rev 35:872–900.

216. Lindsay JA, Holden MTG. 2006. *Understanding the rise of the superbug: investigation of the evolution and genomic variation of Staphylococcus aureus*. Funct Integr Genomics 6:186–201.

217. Lutz C, Erken M, Noorian P, Sun S, McDougald D. 2013. *Environmental reservoirs and mechanisms of persistence of Vibrio cholerae*. Front Microbiol 4:375.

218. Lindsay JA. 2014. *Evolution of Staphylococcus aureus and MRSA during outbreaks*. Infect Genet Evol 21:548–53.

219. McDougal LK, Fosheim GE, Nicholson A, Bulens SN, Limbago BM, Shearer JES, Summers AO, Patel JB. 2010. *Emergence of resistance among USA300 methicillin-resistant Staphylococcus aureus isolates causing invasive disease in the United States*. Antimicrob Agents Chemother 54:3804–11.

220. McCarthy AJ, Witney AA, Gould KA, Moodley A, Guardabassi L, Voss A, Denis O, Broens EM, Hinds J, Lindsay JA. 2011. *The distribution of mobile genetic elements (MGEs) in MRSA CC398 is associated with both host and country*. Genome Biol Evol 3:1164–74.

221. Alibayov B, Baba-Moussa L, Sina H, Zdeňková K, Demnerová K. 2014. *Staphylococcus aureus mobile genetic elements*. Mol Biol Rep 41:5005–5018.

222. Uhlemann A-C, Dordel J, Knox JR, Raven KE, Parkhill J, Holden MTG, Peacock SJ, Lowy FD. 2014. *Molecular tracing of the emergence, diversification, and transmission of S. aureus sequence type 8 in a New York community.* Proc Natl Acad Sci U S A 111:6738–43.

223. Rossi F, Diaz L, Wollam A, Panesso D, Zhou Y, Rincon S, Narechania A, Xing G, Di Gioia TSR, Doi A, Tran TT, Reyes J, Munita JM, Carvajal LP, Hernandez-Roldan A, Brandão D, van der Heijden IM, Murray BE, Planet PJ, Weinstock GM, Arias CA. 2014. *Transferable vancomycin resistance in a community-associated MRSA lineage*. N Engl J Med 370:1524–31.

224. Strommenger B, Bartels MD, Kurt K, Layer F, Rohde SM, Boye K, Westh H, Witte W, De Lencastre H, Nübel U. 2014. *Evolution of methicillin-resistant Staphylococcus aureus towards increasing resistance.* J Antimicrob Chemother 69:616–22.

225. Locke JB, Zuill DE, Scharn CR, Deane J, Sahm DF, Goering R V, Jenkins SG, Shaw KJ. 2014. *Identification and characterization of linezolid-resistant cfr-positive Staphylococcus aureus USA300 isolates from a New York City medical center*. Antimicrob Agents Chemother 58:6949–52.

226. Sung JM-L, Lloyd DH, Lindsay JA. 2008. *Staphylococcus aureus host specificity: comparative genomics of human versus animal isolates by multi-strain microarray.* Microbiology 154:1949–59.

227. Holden MTG, Feil EJ, Lindsay JA, Peacock SJ, Day NPJ, Enright MC, Foster TJ, Moore CE, Hurst L, Atkin R, Barron A, Bason N, Bentley SD, Chillingworth C, Chillingworth T, Churcher C, Clark L, Corton C, Cronin A, Doggett J, Dowd L, Feltwell T, Hance Z, Harris B, Hauser H, Holroyd S, Jagels K, James KD, Lennard N, Line A, Mayes R, Moule S, Mungall K, Ormond D, Quail MA, Rabbinowitsch E, Rutherford K, Sanders M, Sharp S, Simmonds M, Stevens K, Whitehead S, Barrell BG, Spratt BG, Parkhill J. 2004. *Complete genomes of two clinical Staphylococcus aureus strains: evidence for the rapid evolution of virulence and drug resistance*. Proc Natl Acad Sci U S A 101:9786–91.

228. Robinson DA, Kearns AM, Holmes A, Morrison D, Grundmann H, Edwards G, O'Brien FG, Tenover FC, McDougal LK, Monk AB, Enright MC. 2005. *Re-emergence of early pandemic Staphylococcus aureus as a community-acquired meticillin-resistant clone*. Lancet 365:1256–1258.

229. Yutin N, Galperin MY. 2013. *A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia*. Environ Microbiol 15:n/a–n/a.

230. Magot M. *Physical characterization of the Clostridium perfringens tetracycline-chloramphenicol resistance plasmid pIP401*. Ann Microbiol (Paris) 135B:269–82.

231. Lyras D, Adams V, Ballard SA, Teng WL, Howarth PM, Crellin PK, Bannam TL, Songer JG, Rood JI. 2009. *tISCpe8, an IS1595-family lincomycin resistance element located on a conjugative plasmid in Clostridium perfringens*. J Bacteriol 191:6345–51.

232. Bannam TL, Teng WL, Bulach D, Lyras D, Rood JI. 2006. *Functional identification of conjugation and replication regions of the tetracycline resistance plasmid pCW3 from Clostridium perfringens.* J Bacteriol 188:4942–51.

233. Gurjar A, Li J, McClane BA. 2010. *Characterization of toxin plasmids in Clostridium perfringens type C isolates*. Infect Immun 78:4860–9.

234. Nowell VJ, Kropinski AM, Songer JG, MacInnes JI, Parreira VR, Prescott JF. 2012. *Genome sequencing and analysis of a type A Clostridium perfringens isolate from a case of bovine clostridial abomasitis*. PLoS One 7:e32271.

235. Sasaki Y, Yamamoto K, Tamura Y, Takahashi T. 2001. *Tetracycline-resistance genes of Clostridium perfringens, Clostridium septicum and Clostridium sordellii isolated from cattle affected with malignant edema*. Vet Microbiol 83:61–9.

236. Franciosa G, Scalfaro C, Di Bonito P, Vitale M, Aureli P. 2011. *Identification of novel linear megaplasmids carrying a ß-lactamase gene in neurotoxigenic Clostridium butyricum type E strains*. PLoS One 6:e21706.

237. Slavić D, Boerlin P, Fabri M, Klotins KC, Zoethout JK, Weir PE, Bateman D. 2011. *Antimicrobial susceptibility of Clostridium perfringens isolates of bovine, chicken, porcine, and turkey origin from Ontario*. Can J Vet Res 75:89–97.

238. Farrow KA, Lyras D, Rood JI. 2000. *The macrolide-lincosamide-streptogramin B resistance determinant from Clostridium difficile 630 contains two erm(B) genes.* Antimicrob Agents Chemother 44:411–3.

239. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs*. Nucleic Acids Res 25:3389–402.

SUPPLEMENTARY MATERIAL

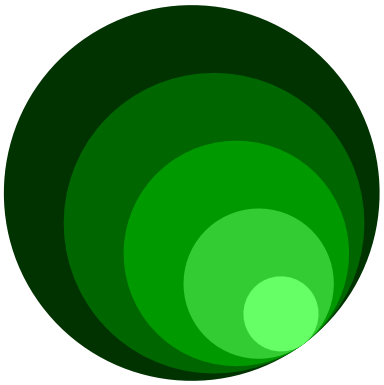
Table S1. List of all plasmids analysed.

Accesion	Strain	Length	Plasmid Name	Taxonomy	Metals and Biocides	AbR	Typing
NC_000906	Lactococcus lactis IL964 plasmid pIL105, complete sequence.	8506	pIL105	Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Lactococcus.	-	-	-
NC_000937	Streptococcus thermophilus ST135 plasmid pER35, complete sequence.	9531	pER35	Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Streptococcus.	-	-	-
NC_000938	Streptococcus thermophilus ST136 plasmid pER36, complete sequence.	3498	pER36	Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Streptococcus.	-	-	-
NC_001272	Bacillus thuringiensis serovar kurstaki str. YBT-1520 plasmidpBMB9741, complete sequence.	6578	pBMB9741	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;Bacilluscereusgroup.	-	-	-
NC_001277	Pediococcus pentosaceus ATCC43200 plasmid pMD136, completesequence.	19515	pMD136	Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Pediococcus.	-	-	-
NC_001370	Lactobacillus plantarum plasmid pC30il, complete sequence.	2140	pC30il	Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Lactobacillus.	-	-	-
NC_001376	Bacillus subtilis plasmid pIM13, complete sequence.	2246	pIM13	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus.	-	*ErmC, AadC*	10
NC_001379	Lactobacillus helveticus subsp. jugurti plasmid pLJ1, completesequence.	3292	pLJ1	Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Lactobacillus.	-	-	-
NC_001380	Streptococcus agalactiae plasmid pLS1, complete sequence.	4408	pLS1	Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Streptococcus.	-	*TetL*	-
NC_001381	Mycobacterium fortuitum plasmid pAL5000, complete sequence.	4837	pAL5000	Bacteria;Actinobacteria;Actinobacteridae;Actinomycetales;Corynebacterineae;Mycobacteriaceae;Mycobacterium.	-	-	-
NC_001384	Staphylococcus aureus plasmid pUB110, complete sequence.	4548	pUB110	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcus.	-	*AadD*	22
NC_001385	Corynebacterium glutamicum plasmid pAM330 DNA, complete sequence.	4448	plasmid pAM330	Bacteria;Actinobacteria;Actinobacteridae;Actinomycetales;Corynebacterineae;Corynebacteriaceae;Corynebacterium.	-	-	-
NC_001387	Streptomyces lividans plasmid pIJ101, complete sequence.	8830	pIJ101	Bacteria;Actinobacteria;Actinobacteridae;Actinomycetales;Streptomycineae;Streptomycetaceae;Streptomyces.	-	-	-
NC_001388	Clostridium perfringens plasmid pIP404, complete sequence.	10206	pIP404	Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Clostridium.	-	-	-
NC_001390	Staphylococcus epidermidis plasmid pNE131, complete sequence.	2355	pNE131	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcus.	-	*AadC, ErmC*	-
NC_001391	Staphylococcus aureus plasmid pNS1, complete sequence.	3879	pNS1	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcus.	-	*TetK*	7
NC_001393	Staphylococcus aureus plasmid pT181, complete sequence.	4439	pT181	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcus.	-	*TetK, TetK*	7
NC_001395	Staphylococcus aureus strain T48 plasmid pT48, complete sequence.	2475	pT48	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcus.	-	*AadC, ErmC*	10
NC_001415	Corynebacterium glutamicum strain 22243 R-plasmid pAGl, completesequence.	19751	R-plasmid pAGl	Bacteria;Actinobacteria;Actinobacteridae;Actinomycetales;Corynebacterineae;Corynebacteriaceae;Corynebacterium.	-	*TetZ*	-
NC_001425	Streptomyces flavovirens plasmid pSN22 DNA, complete sequence.	11046	pSN22	Bacteria;Actinobacteria;Actinobacteridae;Actinomycetales;Streptomycineae;Streptomycetaceae;Streptomyces.	-	-	-
NC_001446	Bacillus thuringiensis sv israelensis HI4 plasmid pTX14-3, completesequence.	7649	pTX14-3	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;Bacilluscereusgroup.	-	-	-
NC_001456	Corynebacterium glutamicum ATCC 19223 plasmid pSR1, completesequence.	3054	pSR1	Bacteria;Actinobacteria;Actinobacteridae;Actinomycetales;Corynebacterineae;Corynebacteriaceae;Corynebacterium.	-	-	-
NC_001496	Bacillus anthracis virulence plasmid PX01, complete sequence.	181654	virulence plasmid PX01	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;Bacilluscereusgroup.	-	-	-
NC_001670	Lactobacillus delbrueckii plasmid pWS58, complete sequence.	7921	pWS58	Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Lactobacillus.	-	-	-
NC_001705	Bacillus cereus plasmid pBC16, complete sequence.	4630	pBC16	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;Bacilluscereusgroup.	-	*TetL*	22
NC_001738	Streptomyces clavuligerus ATCC 27064 plasmid pSCL, completesequence.	11696	pSCL	Bacteria;Actinobacteria;Actinobacteridae;Actinomycetales;Streptomycineae;Streptomycetaceae;Streptomyces.	-	-	-
NC_001757	Lactobacillus reuteri 100-23 plasmid pGT232, complete sequence.	5113	pGT232	Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Lactobacillus.	-	-	-
NC_001758	Ruminococcus flavefaciens R13e2 cryptic plasmid pBAW301, completesequence.	1768	pBAW301	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Ruminococcus.	-	-	-
NC_001759	Streptomyces phaeochromogenes plasmid pJV1, complete sequence.	11143	pJV1	Bacteria;Actinobacteria;Actinobacteridae;Actinomycetales;Streptomycineae;Streptomycetaceae;Streptomyces.	-	-	-
NC_001763	Staphylococcus aureus plasmid J3358, complete sequence.	6024	pT181	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcus.	-	*TetK*	7
NC_001764	Bacillus subtilis plasmid pTA1040, complete sequence.	7837	pTA1040	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus.	-	-	-
NC_001765	Bacillus subtilis plasmid pTA1015, complete sequence.	5807	pTA1015	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus.	-	-	-
NC_001766	Bacillus subtilis plasmid pTA1060, complete sequence.	8737	pTA1060	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus.	-	-	-
NC_001767	Staphylococcus aureus plasmid pKH6, complete sequence.	4439	pKH6	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcus.	-	*TetK*	7
NC_001772	Clostridium sp. MCF-1 indigenous plasmid, complete sequence.	2450	indigenous plasmid	Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Clostridium.	-	-	-
NC_001787	Trueperella pyogenes plasmid pAP1, complete sequence.	2439	pAP1	Bacteria;Actinobacteria;Actinobacteridae;Actinomycetales;Actinomycineae;Actinomycetaceae;Trueperella.	-	-	-
NC_001791	Corynebacterium glutamicum strain 1014 plasmid pXZ10145.1, completesequence.	4885	pXZ10145.1	Bacteria;Actinobacteria;Actinobacteridae;Actinomycetales;Corynebacterineae;Corynebacteriaceae;Corynebacterium.	-	*Cmr*	-
NC_001797	Streptococcus agalactiae plasmid pGB354, complete plasmid sequence.	6437	pGB354	Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Streptococcus.	-	*Cat-pC221*	1, 7
NC_001858	Bacillus pumilus plasmid pPL10, complete plasmid sequence.	7028	pPL10	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus.	-	-	-
NC_001949	Lactococcus lactis DPC3147 plasmid pMRC01, complete sequence.	60232	pMRC01	Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Lactococcus.	-	-	-
NC_001988	Clostridium acetobutylicum ATCC 824 plasmid pSOL1, completesequence.	192000	pSOL1	Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Clostridium.	*arsB*	-	-
NC_001994	Staphylococcus aureus plasmid pSK3, complete sequence.	1658	pSK3	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcus.	-	-	10b
NC_001995	Staphylococcus aureus plasmid pSK6, complete sequence.	1551	pSK6	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcus.	-	-	10b
NC_002013	Staphylococcus aureus plasmid pC194, complete sequence.	2910	pC194	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcus.	-	*CatA9*	13
NC_002059	Butyrivibrio fibrisolvens plasmid pOM1, complete genome.	2804	pOM1	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Butyrivibrio.	-	-	-
NC_002062	Geobacillus stearothermophilus plasmid pSTK1 DNA, completesequence.	1883	pSTK1	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Geobacillus.	-	-	-
NC_002075	Bacillus subtilis plasmid p1414, complete plasmid sequence.	7949	p1414	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus.	-	-	-
NC_002091	Bacillus thuringiensis sv israelensis plasmid pTX14-1, completesequence.	5415	pTX14-1	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;Bacilluscereusgroup.	-	-	-
NC_002093	Staphylococcus lugdunensis strain 995 cadmium resistance plasmidpLUG10, complete sequence.	3117	pLUG10	Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcus.	*cadD*	-	-

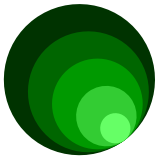
Due to the size of the table it will be provided only in digital format.

I did then what I knew how to do. Now that I know better, I do better.

Maya Angelou



Chapter 4



Multi-Level Population Genetic Analysis of *vanA* and *vanB* *Enterococcus faecium* Causing Nosocomial Outbreaks in 27 Countries (1986-2012)

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ABSTRACT

Objectives. Vancomycin-resistant *Enterococcus faecium* (VREfm) have been increasingly reported since the 1980s. Despite the high number of published studies about VRE epidemiology, the dynamics and evolvability of these microorganisms is still not fully understood. A multilevel population genetic analysis of VREfm outbreak strains since 1986, representing the first comprehensive characterization of plasmid content in *E. faecium,* was performed to provide a detailed view of potential transmissible units.

Methods. From a comprehensive MeSH search, we identified VREfm strains causing hospital outbreaks (1986-2012). In total, 53 VanA and 18 VanB isolates (27 countries, 5 continents) were analysed and 82 vancomycin-susceptible *E. faecium* (VSEfm) were included for comparison. Clonal relatedness was established by PFGE and MLST (goeBURST/Bayesian Analysis of Population Structure, BAPS). Characterization of *van* transposons (PCR mapping, RFLP, sequencing), plasmids (transfer, *Cla*I-RFLP, PCR-typing of relaxases, replication-initiation proteins and toxin-antitoxin systems, hybridization, sequencing), bacteriocins and virulence determinants (PCR, sequencing) was performed.

Results. VREfm were mainly associated with major human lineages ST17, ST18 and ST78. VREfm and VSEfm harboured plasmids of different families [RCR, small-theta plasmids, RepA_N (pRUM/pLG1) and Inc18] able to yield mosaic elements. Tn*1546*-*vanA* was mainly located on pRUM/Axe-Txe (USA), and Inc18-pIP186 (Europe) plasmids. VanB2 (Tn*5382*/Tn*1549*) was predominant (chromosome and plasmids).

Conclusions. Both strains and plasmids contributed to the spread and persistence of vancomycin resistance among *E. faecium.* Horizontal gene transfer events among genetic elements from different clonal lineages (same or different species) result in chimeras with different stability and host range complicating the surveillance of epidemic plasmids.

INTRODUCTION

VRE have increasingly been reported since their first description in 1986, with remarkable epidemiological differences in diverse geographical areas.[1,2] Acquired vancomycin resistance has mostly been detected in *Enterococcus faecium*, even though *Enterococcus faecalis* is the main species recovered from human infections.

Different acquired operons (*vanA*/*B/D/E/G/L/M/N*) confer resistance to vancomycin although *vanA* and *vanB* gene clusters remain predominant.[2,3] The *vanA* cluster is often part of a Tn*3* element named Tn*1546* which may be located in plasmids of different families.[4] Of the three *vanB* operons (*vanB*$_1$, *vanB*$_2$, *vanB*$_3$), *vanB*$_2$ (CTn*5382/1549*) is the most commonly found. It can be located in a transferable chromosomal region, eventually linked to *pbp5* conferring resistance to ampicillin, or in plasmids.[5–10] Other *van* operons were sporadically observed in *E. faecium* (*vanM, vanN*), *E. faecalis* (*vanE, vanG, vanL*) or several enterococcal species (*vanD*).[3]

To date, only few studies provided information of the plasmids involved in the transmission of vancomycin resistance, most of them confined to the analysis of local collections or to the comparison of isolates of particular clones collected in Europe.[9,11–16] Additionally, the short length fragments provided by most available next generation sequencing (NGS) methods and the high cost of those that offer long reads as PacBio, makes extremely difficult to analyse the plasmid content of a large set of enterococcal isolates.[17–20] Thus, the fractionated information about transmission of vancomycin resistance between isolates from different locations, and the lack of knowledge about early VRE isolates, hinder the full comprehension of its epidemiology and evolution at global level.

This work provides a comprehensive multilevel molecular analysis of vancomycin-resistant *E. faecium* (VREfm) by characterizing for the first time representative strains causing hospital outbreaks in different continents since 1986, mainly America, Europe and Australia from where most studies have been published.

MATERIAL AND METHODS

Selection of VREfm outbreak strains. We searched PubMed indexed records in MEDLINE for VREfm outbreaks using the medical subject headings (MeSH) terms "*Enterococcus faecium*", "disease outbreaks" and "vancomycin resistance". We identified 171 entries, which resulted in 124 publications that documented outbreaks due to strains carrying *vanA*, *vanB* or *vanA*+*vanB*. The identification of *van* genes was not available in 19 cases. An additional refined search for interhospital disseminated strains with the MeSH terms "*Enterococcus faecium*" and "vancomycin resistance" plus the words "interhospital" or "inter-hospital" gave 10 additional entries (Table 1). Only full-text articles with an English abstract were suitable for inclusion. Strains reported in these publications were requested (n=86). In some cases, strains were not available or no response was obtained from the authors.

Table 1. Systematic search of studies documenting vancomycin-resistant *Enterococcus faecium* outbreaks or interhospital disseminated VREfm strains

Van genotype (no. of isolates)	Country	Year	Epidemiology	Number of VRE patient (isolates)	Number of colonizations	Number of persons infected	Method for strain typing	MLST	Plasmid typing	VREfm included in this study[a]	Ref(s)
VanA	Turkey	2013	Hospital **outbreak**	17 (24)	15	5	PFGE	ND	ND	-	64
VanA	Denmark (18 hospitals)	2012-2013	Multiple hospital **outbreaks**	(132)	NE	NE	PFGE, MLST WGS	Mostly ST80, ST117, ST192	ND	V207	20
VanA+VanB (n=7) VanA (n=1)	France (Paris)	2012-2013	Hospital **outbreak**	8	7	1	PFGE	ND	ND	-	65
VanA	China (Beijing)	2011-2013	Hospital **outbreak**	69 (15)	NE	NE	MLST	ST571	ND	-	66
VanA	Brazil (Uberlândia)	2010-2012	Hospital **outbreak**	158	NE	22	none	ND	ND	-	67
VanA	Brazil (Porto Alegre)	2010-2011	Hospital **outbreak**	29	11	18	PFGE	ND	ND	-	68
VanA (VSE)	Canada	2009-2011	Hospital **outbreak**	52	44	0	PFGE/MLST	ST18	pF856 (31kb)	-	69
VanA	Taiwan	2009-2011	Hospital **outbreak**	54	22	32	PFGE/MLST	Mostly ST414	ND	-	70
VanB	Sweden (3 hospitals)	2007-2011	Multicentre hospital **outbreak**	(872)	NE	<10%	PFGE, MLST, WGS	Mostly ST192	pRUM-like (70kb)	-	9
VanB (n=15); VanA (n=1) Unknown (n=1)	Switzerland (Zurich)	2009-2010	Hospital **outbreak**	17	13	4	PFGE	ND	ND	-	71
VanA (n=41) VanB (n=4)	France	2004-2010	Hospital **outbreaks** (45 VREfm outbreaks)	(533)	NE	ratio 1:6 (infected: colonized)	PFGE	ND	ND	-	72
Not determined	USA (Dallas, Texas)	2010	Hospital **outbreak**	(20)	13	0	rep-PCR	ND	ND	-	73
VanB (n=33) VanA (n=1)	Australia (Austin)	1998-2009	Sustained hospital outbreak (bacteremia)	(34)	NE	NE	PFGE, MLST, WGS	ST203	ND	-	30
VanA	Brazil (Curitiba)	2009	Hospital **outbreak**	9	8	1	PFGE	ND	ND	-	74
VanA	Japan (Hong Kong)	2009	Hospital **outbreak**	9	9	0	PFGE	ND	ND	-	75
VanA	Poland (Warsaw)	2009	Hospital **outbreaks** (2 hospitals)	42 (44)	NE	NE	PFGE/MLST	Mostly ST18	pRUM-like	-	14
VanB	Germany (South-Western)	2008-2009	Hospital **outbreak**	80 (56)	NE	NE	PFGE/MLST	ST192	ND	-	76
VanA	Spain (Granada)	2008-2009	Hospital **outbreak**	(13)	6	7	PFGE, MLVA, MLST	ST17	Inc18-like (30kb)	232/09	16
VanA	China (Beijing)	2008-2009	Hospital **outbreak**	32	11	21	PFGE/MLST	CC17	ND	-	77
VanA	France (>40 facilities)	2004-2009	Regional hospital **outbreak**	(1077)	NE	5.4%	none	ND	ND	-	78
VanA	China (Beijing)	2008	Hospital **outbreak**	8 (37)	15	NE	PFGE/MLST	Mostly ST17	ND	-	79
VanA	Greece (Thessaloniki)	2008	Hospital **outbreak**	108	106	2	PFGE	ND	ND	-	80
VanB	Spain (Burgos)	2006-2008	Hospital **outbreak**	50	40	10	PFGE/MLST	ST17	ND	604/06	81
VanA	South America (4 countries)	2006-2008	Epidemiological study of clinical strains (32 hospitals)	(35)	NE	NE	PFGE/MLST	Mostly ST18/ST412	*pilA-hyl*-plasmids	E417, E422, P575, V689, C497, P1123	82

Table 1. Systematic search of studies documenting vancomycin-resistant *Enterococcus faecium* outbreaks or interhospital disseminated VREfm strains (cont)

Van genotype (no. of isolates)	Country	Year	Epidemiology	Number of VRE patient (isolates)	Number of colonizations	Number of persons infected	Method for strain typing	MLST	Plasmid typing	VREfm included in this study[a]	Ref(s)
VanA	Korea (Incheon)	2007	Hospital **outbreak**	30	12	NE	PFGE	ND	ND	-	83
VanB	France (Paris)	2007	Hospital **outbreak**	14	14	0	PFGE	ND	ND	-	84
VanB	Japan (Kitakyushu)	2007	Hospital **outbreak**	15 (22)	NE	NE	PFGE	ND	ND	-	85
VanA+VanB (n=6) **VanB (n=37)**	Australia (Melbourne)	2006	Hospital **outbreak**	(43)	NE NE	0 NE	PFGE	ND	ND	-	86
VanB	Canada (Toronto)	2006	Hospital **outbreak**	15	15	0	none	ND	ND	-	87
VanA	Turkey (Izmir)	2006	Hospital **outbreak**	12	7	NE	PFGE/MLST	ST17, ST78	ND	-	88
VanA	Brazil (São Paulo)	2007-2008	Hospital **outbreak**	(64)	45	8	PFGE	ND	ND	-	89
VanA (n=33) VanB (n=18)	Denmark	2005-2008	Epidemiological study of clinical strains (7 departments)	(51)	NE	NE	PFGE/MLST	Mostly ST18/ST203	ND	VRE2, VRE101	90
VanA	Korea	2006-2007	Hospital **outbreak**	46	NE	NE	PFGE	ND	ND	-	91
VanA	China (Beijing)	2006-2007	Hospital **outbreak**	14 (18)	5	NE	PFGE/MLST	Mostly ST78/ST203	ND	-	92
VanA	Paraguay (Asunción)	2005-2007	Epidemiological study of infection-associated strains	(40)	NE	40	PFGE/MLST	Mostly ST78	ND	P1, P118	93
VanA	Poland (Krakow)	2005	Hospital **outbreak**	27 (14 VREfm)	NE	NE	PFGE	ND	ND	2227/05	94
VanA	Germany (31 hospitals)	1996-2006	Hospital **outbreaks**	NE	NE	NE	PFGE, MLVA, MLST	Several STs	ND	UW1806, 5905, 6511, UW6337	95
VanA (n=30) **VanB (n=3)**	Germany	2005	Hospital **outbreak**	33 (33)	28	5	MLVA	Mostly MLVA-7	ND	-	96
VanA	Turkey (Antalya)	2005	Hospital **outbreak**	10 (36)	6	4	PFGE, MLST	ST18, ST31	ND	-	97
VanA	Turkey (Zonguldak)	2005	Hospital **outbreak**	4 (6)	4	0	PFGE	ND	ND	-	98
VanA	Spain (Canarias)	2005	Hospital **outbreak**	20	17	8	PFGE, MLST	ST18	ND	-	99
VanA	Greece (Athens)	2005	Hospital **outbreak**	53	53	NE	PFGE	ND	ND	-	100
VanA	Israel (Jerusalem)	2005	Hospital **outbreak**	18	11	3	PFGE	ND	ND	-	101
VanA	Canada (Toronto)	2005	Hospital **outbreak**	34	NE	NE	PFGE	ND	ND	Efm-Canada	102
VanA	France (Paris)	2005	Hospital **outbreak**	39	37	2	RAPD	ND	ND	-	103
VanB	Singapore	2005	Hospital **outbreak**	84 (82 VREfm)	79	3	PFGE	ND	ND	-	104
VanB	Chile (16 hospitals)	2003-2005	Interhospital dissemination of clinical strains	(60)	34	NE	PFGE/MLST	ST64	ND	E76, E94	105
VanB	Australia (Melbourne)	2003-2005	Hospital **outbreak**	41	27	14	none	ND	ND	-	106
VanA	France (Clermont-Ferrand)	2004-2007	Hospital **outbreak**	(220)	163	57	PFGE	ND	ND	-	107

Table 1. Systematic search of studies documenting vancomycin-resistant *Enterococcus faecium* outbreaks or interhospital disseminated VREfm strains (cont)

Van genotype (no. of isolates)	Country	Year	Epidemiology	Number of VRE patient (isolates)	Number of colonizations	Number of persons infected	Method for strain typing	MLST	Plasmid typing	VREfm included in this study[a]	Ref(s)
VanB	Spain (Soria)	2004-2006	Hospital **outbreak**	34 (34)	34	NE	PFGE/MLST	ST17	ND	Ent35	108
VanA	France (Paris)	2004-2005	Hospital **outbreak**	48	NE	1	PFGE	ND	ND	-	109
VanA	Germany (Freiburg)	2004-2005	Hospital **outbreak**	167	NE	25	none	ND	ND	-	110
VanA (predominant)	Germany (south-west)	2004-2005	Hospital **outbreak**	248	154	94	PFGE, MLVA, MLST	ST203 (predominant)	ND	-	111
VanA	Belgium (Brussels)	2004-2005	Hospital **outbreak**	13 (20)	12	1	PFGE/MLST	ST16	ND	-	112
VanA	USA (Alabama)	2004-2005	Hospital **outbreak** (Linezolid-resistant)	40	NE	22	PFGE	ND	ND	Lin1	113
VanB	Australia	2004 2005	Hospital **outbreak**	(110)	NE	NE		ST280 (n=6) ST17 (n=104)	ND	OB19275, DB20546	114 115
VanA	Serbia (Belgrade)	2003-2005	Epidemiological study of clinical strains	7 (9)	NE	NE	PFGE, MLVA MLST	ST17, ST18 ST78	ND	E2373	116
VanA (VanD phenotype)	France (Bicêtre-Paris)	2004	Hospital **outbreak**	24 (26)	18	7	PFGE	Not determined	ND	-	117
VanB	Hungary (Budapest)	2004	Hospital **outbreak**	21	7	14	PFGE	ND	ND	E2365	118
VanB	Australia (Westmead)	2004	Hospital **outbreak**	13	7	6	PFGE	ND	ND	-	119
VanA	Tunisia (Sfax)	2002-2003	Epidemiological study (clinical specimens)	2	NE	2	PFGE	ND	ND	A437	120
VanA	Argentina	2003	Hospital **outbreak**	8 (24)	24	0	PFGE	ND	ND	-	121
VanA	Portugal (3 hospitals)	2001-2003	Interhospital dissemination of clinical strains	(101)	NE	NE	PFGE	ND	ND	H74, H182, H305, H311, H358, 48311	122
VanA	Saudi Arabia (2 hospitals)	2000-2003	Epidemiological study (clinical specimens)	(34)	NE	NE	PFGE/MLST	Mostly ST17	ND	S1, S13, S30	123
VanA (n=28) VanB (n=1)	Italy (18 hospitals) Italy (1 hospital)	2001-2003 2002	Interhospital spread of bloodstream-infecting isolates	(28) (1)	NE NE	28 1	PFGE/MLST	ST78 ST209	ND	AE01 AE12	124
VanA	Korea (Seoul)	2002	Hospital **outbreak**	17	NE	NE	PFGE	ND	ND	-	125
VanA	Italy (Pavia)	2001-2002	Hospital **outbreak**	56	56	2	Ribotyping/ MLST	ST18, ST78	ND	-	126
VanA	Germany (south-west)	2001	Hospital **outbreak**	24	24	1	PFGE	ND	ND	-	111
VanB	Australia (Perth)	2001	Hospital **outbreak**	172	172	4	PFGE/ Ribotyping	ND	ND	VRE100	127,128
VanA	The Netherlands (Utrecht)	2000-2001	Hospital **outbreak**	89	89	0	PFGE	ND	ND	E0805	129,13
VanA	Brazil	1998-2003	Epidemiological study (infection and faecal isolates)	(23)	13	10	PFGE/MLST	ST17/ST50 ST1114	ND	Vri16, Vri22	131
VanA	Germany	1999-2001	Hospital **outbreak**	44 (159)	NE	NE	PFGE	ND	ND	-	132
VanA	Greece (Athens)	1999-2001	Hospital **outbreak**	21	NE	21	AFLP	ND	ND	E1438	133
VanA	Argentina (30 hospitals)	1997-2000	Interhospital spread of clinical strains	189 (189)	152	28	PFGE	ND	ND	2348, 2391, 2664	134

Table 1. Systematic search of studies documenting vancomycin-resistant *Enterococcus faecium* outbreaks or interhospital disseminated VREfm strains (cont)

Van genotype (no. of isolates)	Country	Year	Epidemiology	Number of VRE patient (isolates)	Number of colonizations	Number of persons infected	Method for strain typing	MLST	Plasmid typing	VREfm included in this study[a]	Ref(s)
VanB	Spain (Soria)	2004-2006	Hospital **outbreak**	34 (34)	34	NE	PFGE/MLST	ST17	ND	Ent35	108
VanA	France (Paris)	2004-2005	Hospital **outbreak**	48	NE	1	PFGE	ND	ND	-	109
VanA	Germany (Freiburg)	2004-2005	Hospital **outbreak**	167	NE	25	none	ND	ND	-	110
VanA (predominant)	Germany (south-west)	2004-2005	Hospital **outbreak**	248	154	94	PFGE, MLVA, MLST	ST203 (predominant)	ND	-	111
VanA	Belgium (Brussels)	2004-2005	Hospital **outbreak**	13 (20)	12	1	PFGE/MLST	ST16	ND	-	112
VanA	USA (Alabama)	2004-2005	Hospital **outbreak** (Linezolid-resistant)	40	NE	22	PFGE	ND	ND	Lin1	113
VanB	Australia	2004 2005	Hospital **outbreak**	(110)	NE	NE		ST280 (n=6) ST17 (n=104)	ND	OB19275, DB20546	114 115
VanA	Serbia (Belgrade)	2003-2005	Epidemiological study of clinical strains	7 (9)	NE	NE	PFGE, MLVA MLST	ST17, ST18 ST78	ND	E2373	116
VanA (VanD phenotype)	France (Bicêtre-Paris)	2004	Hospital **outbreak**	24 (26)	18	7	PFGE	Not determined	ND	-	117
VanB	Hungary (Budapest)	2004	Hospital **outbreak**	21	7	14	PFGE	ND	ND	E2365	118
VanB	Australia (Westmead)	2004	Hospital **outbreak**	13	7	6	PFGE	ND	ND	-	119
VanA	Tunisia (Sfax)	2002-2003	Epidemiological study (clinical specimens)	2	NE	2	PFGE	ND	ND	A437	120
VanA	Argentina	2003	Hospital **outbreak**	8 (24)	24	0	PFGE	ND	ND	-	121
VanA	Portugal (3 hospitals)	2001-2003	Interhospital dissemination of clinical strains	(101)	NE	NE	PFGE	ND	ND	H74, H182, H305, H311, H358, 48311	122
VanA	Saudi Arabia (2 hospitals)	2000-2003	Epidemiological study (clinical specimens)	(34)	NE	NE	PFGE/MLST	Mostly ST17	ND	S1, S13, S30	123
VanA (n=28) VanB (n=1)	Italy (18 hospitals) Italy (1 hospital)	2001-2003 2002	Interhospital spread of bloodstream-infecting isolates	(28) (1)	NE NE	28 1	PFGE/MLST	ST78 ST209	ND	AE01 AE12	124
VanA	Korea (Seoul)	2002	Hospital **outbreak**	17	NE	NE	PFGE	ND	ND	-	125
VanA	Italy (Pavia)	2001-2002	Hospital **outbreak**	56	56	2	Ribotyping/ MLST	ST18, ST78	ND	-	126
VanA	Germany (south-west)	2001	Hospital **outbreak**	24	24	1	PFGE	ND	ND	-	111
VanB	Australia (Perth)	2001	Hospital **outbreak**	172	172	4	PFGE/ Ribotyping	ND	ND	VRE100	127,128
VanA	The Netherlands (Utrecht)	2000-2001	Hospital **outbreak**	89	89	0	PFGE	ND	ND	E0805	129,13
VanA	Brazil	1998-2003	Epidemiological study (infection and faecal isolates)	(23)	13	10	PFGE/MLST	ST17/ST50 ST1114	ND	Vri16, Vri22	131
VanA	Germany	1999-2001	Hospital **outbreak**	44 (159)	NE	NE	PFGE	ND	ND	-	132
VanA	Greece (Athens)	1999-2001	Hospital **outbreak**	21	NE	21	AFLP	ND	ND	E1438	133
VanA	Argentina (30 hospitals)	1997-2000	Interhospital spread of clinical strains	189 (189)	152	28	PFGE	ND	ND	2348, 2391, 2664	134

Table 1. Systematic search of studies documenting vancomycin-resistant *Enterococcus faecium* outbreaks or interhospital disseminated VREfm strains (cont)

Van genotype (no. of isolates)	Country	Year	Epidemiology	Number of VRE patient (isolates)	Number of colonizations	Number of persons infected	Method for strain typing	MLST	Plasmid typing	VREfm included in this study[a]	Ref(s)
VanA	Poland (Gdask)	1997-1999	Hospital **outbreak**	128 (21)	NE	NE	PFGE	ND	ND	2219/05	135
VanA	Pakistan (Karachi)	2000	Hospital **outbreak**	10	4	6	PFGE	ND	ND	-	136
VanA	The Netherlands (Amersfoort)	2000	Hospital **outbreak**	12	12	NE	PFGE	ND	ND	-	137
VanB	Poland (Cracow)	2000	Hospital **outbreak**	(7)	2	5	PFGE	ND	ND	-	138
VanB	Poland (Warsaw)	1999-2000	Hospital **outbreak**	20	14	6	PFGE	ND	ND	-	138,139
VanB	Taiwan (Southern)	1999-2000	Epidemiological study	(10)	NE	NE	PFGE	ND	ND	-	140
VanB	Sweden (Umeå)	1997-2000	Hospital **outbreak**	51	NE	5	PFGE	ND	ND	-	141
VanA	South Africa (Johannesburg)	1998-1999	Hospital **outbreak**	31	31	5	PFGE	ND	ND	-	142
VanA	Turkey (Antalya)	1998-1999	Hospital **outbreak**	5 (20)	3	2	PFGE	ND	ND	-	143
VanA	Colombia (Medellín)	1998-1999	Hospital **outbreak**	23	NE	23	PFGE	ND	ND	-	144
VanA	Canada (Quebec)	1998-1999	Hospital **outbreak**	109	NE	NE	PFGE	ND	ND	-	145
Not determined (VanA phenotype)	The Netherlands (Amsterdam)	1998-1999	Hospital **outbreak**	24	19	5	AFLP	ND	ND	E0481	146
VanA	Australia (Brisbane)	1996-1999	Hospital **outbreak**	47	38	4	PFGE	ND	ND	PAO2	147
VanB (n=114); VanA (n=36) VanA+VanB (n=5)	Finland (Helsinki)	1996-1998	Hospital **outbreak**	(155)	155	NE	PFGE	ND	ND	VREI, VREII	28
VanA	Brazil (São Paulo)	1998	Hospital **outbreak**	62	51	11	PFGE/MLST	ST114 (predominant)	ND	E1686	148,149
VanB	Australia (Melbourne)	1998	Hospital **outbreak**	19 (85)	NE	NE	none	ND	ND	E0532	150
VanA	Korea	1997	Hospital **outbreak**	4	4	0	rep-PCR	ND	ND	-	151
VanB	USA (Baltimore)	1997	Hospital **outbreak**	5	5	1	PFGE	ND	ND	-	152
Not determined (VanA phenotype)	USA (Texas)	1996-1997	Hospital **outbreak**	21	21	4	PFGE	ND	ND	-	153
Not determined	USA (Houston, Texas)	1996-1997	Hospital **outbreak**	17	8	9	PFGE	ND	ND	-	154
Not determined	Germany (Munich)	1996-1997	Epidmiological study of colonisation	24	24	0	none	ND	ND	-	155
VanA	Germany (Hamburg)	1993-1997	Hospital **outbreak**	32 (29)	NE	4	PFGE	ND	ND	-	156
VanB	USA (Cleveland) (13 hospitals)	1996	Hospital **outbreak** Interhospital dissemination	(198)	NE	NE	PFGE	ND	ND	E2480 (C68)	157
VanA phenotype	Ireland (Dublin)	1996	Hospital **outbreak**	14 (12 Efm)	12	2	PFGE	ND	ND	-	158
VanA phenotype	Ireland (Belfast)	1996	Hospital **outbreak**	8 (11)	7	1	RAPD	ND	ND	-	159
VanB	USA (Salt Lake City)	1995-1996	Hospital **outbreak**	10	NE	NE	PFGE	ND	ND	-	160
VanA	Italy	1993-1996	Hospital **outbreak**	(29, an (outbreak strain)	51*	33*	PFGE	ND	ND	-	161

Table 1. Systematic search of studies documenting vancomycin-resistant *Enterococcus faecium* outbreaks or interhospital disseminated VREfm strains (cont)

Van genotype (no. of isolates)	Country	Year	Epidemiology	Number of VRE patient (isolates)	Number of colonizations	Number of persons infected	Method for strain typing	MLST	Plasmid typing	VREfm included in this study[a]	Ref(s)
VanA	USA (Chicago)	1995	Epidemiological study of colonisation	92 (43)	92	0	PFGE	ND	ND	E0161	162
VanA (n=20), VanB (n=3)	England (Edinburgh)	1995	Hospital **outbreak**	23	17*	8*	PFGE	ND	ND	-	163
VanA (n=3)	Sweden (Örebro)	1995	Hospital **outbreak**	4	2	2	PFGE	ND	ND	-	164
VanA	USA (Boston)	1994-1995	Hospital **outbreak**	39	23	16	PFGE	ND	ND	-	165
Not determined	USA (Washington)	1994-1995	Hospital **outbreak**	23 (27)	NE	13	Ribotyping	ND	ND	-	166
VanB (predominant)	USA (Texas) (6 hospitals)	1993-1994	Hospital **outbreak** Interhospital dissemination	(41)	NE	NE	PFGE	ND	ND	Texas	33
Not determined (VanA/VanB phenotypes)	UK (Manchester)	1993-1995	Hospital **outbreak**	44	NE	6	PFGE	ND	ND	-	167
Not determined	Ireland (Dublin)	1994	Hospital **outbreak**	18 (42)	NE	6	PFGE	ND	ND	-	168
Not determined	USA (Virginia)	1994	Hospital **outbreak**	8 (14)	NE	7	none	ND	ND	-	169
Not determined	USA (Minneapolis)	1993-1994	Hospital **outbreak**	61 (187* , 179 Efm)	59	32	none	ND	ND	-	170
VanA	USA (Detroit)	1992-1994	Interhospital spread of a clinical strain in 6 hospitals	25 (32)	NE	NE	rep-PCR	ND	ND	E0292	32
Not determined	USA (New York)	1991-1994	Hospital **outbreak**	159	50%	NE	PFGE	ND	ND	-	171
Not determined (VanA phenotype)	GRB (London)	1990-1994	Hospital **outbreak**	103 (122)	NE	NE	RAPD	ND	ND	-	172
Not determined	USA (Nebraska)	1993	Hospital **outbreak**	9	NE	8	PFGE	ND	ND	-	173
Not determined (VanA phenotype)	USA (East Coast)	1993	Hospital **outbreak**	11 (20)	11	11	PFGE	ND	ND	-	174
VanA	UK (Oxford)	1992	Hospital **outbreak**	23	8	1	Ribotyping	ND	ND	E0013	175
VanA	USA (New York)	1991-1992	Hospital **outbreak**	29	22	7	PFGE	ND	ND	-	176
VanB	USA (Rhode Island)	1991-1992	Hospital **outbreak**	37	15	NE	PFGE	ND	ND	-	177
VanA (n=157), VanB (n=25)	USA (New York)	1990-1992	Hospital **outbreak**	(182)	NE	NE	PFGE	ND	ND	Ef80, Ef82	43
Not determined	USA (New York)	1990-1992	Hospital **outbreak**	(989)	NE	213*	REA	ND	ND	-	178
Not determined	USA (Pennsylvania)	1991	Hospital **outbreak**	9	4	5	ND	ND	ND	-	179
Not determined	USA (New York)	1990-1991	Hospital **outbreak**	16	8	8	PFGE	ND	ND	-	180

Abbreviations: VREfm, Vancomycin-resistant Enterococcus faecium; VRE, vancomycin-resistant enterococci; NE, Not specified; ND, not determined; PFGE, pulsed-field gel electrophoresis, MLST, multilocus sequence typing; MLVA, Multiple-Locus Variable number tandem repeat Analysis. *Cases for which the number of VRE cases also include *Enterococcus faecalis* isolates. [a]Some of the isolates included in this study could not be corresponded to a specific publication (unpublished or unknown results for isolates E1644, E1651, E0805, E1132, Vnr16, C853, 71689, 265859, 135487 and 361400) or were not involved in VREfm outbreaks (BM4147, BM4165).

Bacterial strains and epidemiological background. Table S1 and Fig. S1 shows the 71 VREfm isolates (53 *vanA,* 18 *vanB*) studied, which were responsible for documented hospital outbreaks (one single isolate per outbreak and phenotype), or disseminated in more than one healthcare institution (1986-2012, 27 countries). Vancomycin-susceptible blood isolates (VSEfm, n=82) from Spain, Norway and Denmark, countries with low VREfm prevalence rates, were analysed for comparison (Table S1). Antimicrobial susceptibility testing was performed for 14 antibiotics by the disk diffusion method (Oxoid) following CLSI guidelines.[21] Clonal relatedness was established by PFGE and MLST following standard procedures (http://pubmlst.org/efaecium/).[11] Sequence types (ST) were partitioned into BAPS (Bayesian Analysis Population Structure) (sub)groups as described.[22,23] The presence of genes specifying enterococcal surface protein (*esp*), glycosidase (hyl_{Efm}), and bacteriocins (Bac) previously identified on enterococci at chromosome (*entA*, *entB*) or plasmids (*entP*, *entQ*, L50A, L50B, Bac32 and Bac43) were assessed by PCR (Table S2). Bacterial strains and plasmids used as controls are described in Tables S3 and S4.

Characterization of transposons encoding glycopeptide resistance. The backbone structure of Tn*1546* (*vanA*) was determined by PCR mapping and further sequencing of fragments with unusual size.[11] Transposons containing *vanB* alleles were discriminated by comparing *Bsp*HI/*Dra*I digested DNA profiles of the 5,959-bp *van*RSYWHBX amplicons and analysing sequences corresponding to the right junction of Tn*5382*, the $vanS_B$-$vanY_B$ intergenic region and the relaxase of Tn*1549* (this study; Figure S2).[6,7] The link between *pbp5* and Tn*5382* was investigated according to Carias *et al*.[5] Transferability of *vanA/vanB* genes was analysed by filter mating using *E. faecium* strains GE-1 and BM4105RF as recipients (Table S3). Genomic location of *van* elements was assessed by hybridization of I-*Ceu*I-digested genomic DNA hybridization with *vanA/vanB* and 23S rRNA probes.[11,15]

Plasmid characterization. The plasmid content (number and size) of both wild-type strains and transconjugants was determined using the methods described by Kado & Liu (<10 kb) and Barton *et al.* (10 kb->300 kb).[11,24] Plasmids were classified according to the presence of sequences associated with replication (replication initiator proteins, RIPs), mobilization (relaxases, REL) and stability (toxin-antitoxin systems, TA), which were detected by PCR typing schemes[4,11,25–27] (Table S2) and further hybridization of plasmid DNA (Kado gels), *S1*- and/or I-*Ceu*I-digested genomic DNA with specific probes. Southern blot DNA transfer and hybridization were performed by using the Gene Images AlkPhos Direct labelling and detection system (Amersham GB/GE Healthcare Life Sciences UK Ltd).[11] Relationship among vancomycin-resistant plasmids of similar size and RIP-REL content was established by RFLP using *Eco*RI and *Cla*I (Promega, Woods Hollow Road-Madison, WI, USA).[11] Cluster analysis of RFLP patterns was performed with fingerprinting II Informatix version 3.0 software (Bio-Rad Laboratories, Hercules, CA, USA) using the unweighted pair group method with arithmetic averages (UPGMA clustering; optimization 0.5%, tolerance 1.00%).

Nucleotide sequence accession number. The new *vanB* transposon sequence has been deposited in the GenBank database under the accession number KT20162.

RESULTS

VREfm outbreak strains correspond to major human lineages ST17, ST18 and ST78. The 71 VREfm analysed represented 26 STs that were mainly classified into the BAPS groups 3 and 2 (62.0% versus 33.8%), followed by BAPS groups 5 and 7 (1.4% versus 2.8%). The 31 STs of the 82 VSEfm mainly clustered into BAPS groups 3, 2 and also 1 (73.2%, 15.9%, and 8.5%, respectively), followed by BAPS 6 and 7 (1.2% each) (Table S1). A highly similar or identical PFGE type was observed between VREfm isolates collected in either distant areas or the same country over long periods (2-6 years); VREfm and VSEfm isolates recovered during outbreaks in single hospitals in Finland,[28] Portugal,[11] and Spain;[24] and VREfm outbreak strains (this study) and VSEfm strains of different countries (outbreak or predominant strains where VREfm outbreak strains analysed here were described, or strains typed by the authors) (Table S1).

Within BAPS 3, most VREfm isolates corresponded to subgroup 3.3a that further split in 3.3a1 (n=13, 4 STs, mainly ST18) and 3.3a2 (n=27, 5 STs, mainly ST16 and ST17), both comprising strains collected since 1992. Early large VREfm outbreaks in the USA were caused by isolates of BAPS 3.3a2. Most isolates within BAPS 3.3a were resistant to ciprofloxacin (100% each), erythromycin (96-100%), ampicillin (92-100%), and contained *entA* (85%). The presence of *esp* and *hyl* was differently observed in the 3.3a1 and 3.3a2 subgroups (38% versus 85% and 37 versus 38%, respectively). VSEfm isolates of groups BAPS 3.3a1 (n=36, ST18) and 3.3a2 (n=12, mainly ST16 and ST17) were also frequently detected, with a similar occurrence of virulence traits and antibiotic resistance. VREfm and VSEfm often harboured *entB* (25-100%).

Within BAPS 2, BAPS 2.3a included the first two VREfm strains (both ampicillin-susceptible) described in France in 1986-87 (ST25), and the first VREfm outbreak strain from Brazil in 1998 (ST114). The ST25 isolates were isolated from colonized patients in two French hospitals and differed in the number of plasmids and the presence of *entB*. Although their PFGE patterns exhibited a high number of common bands, they were classified as clonally unrelated following standard criteria, confirming the difficulty in discriminate VREfm by traditional molecular methods.[29] They were classified as ampicillin susceptible (AmpS) according to CLSI criteria, but their MIC values to ampicillin were slightly increased in comparison with those of AmpS isolates of the VSEfm group (MIC of 8 mg/L and 4 mg/L for BM4147 and BM4165 strains, respectively).

BAPS subgroup 2.1a, comprising the major ST78 *E. faecium* lineage, was predominant in the strains recovered since 1998 (n=18 VREfm, 8 STs, including ST78, ST203, ST117, and ST412, all widespread in different countries nowadays).[20,30,31] Most strains were resistant to ciprofloxacin (100%), ampicillin and

erythromycin (94% each) and carried *esp* and *entA* (89% each), as well as other analysed markers at variable rates.

BAPS 5 was represented by a *vanB*-type VREfm (ST484) strain from Finland in early 90s, which was involved in one of the largest VRE outbreaks described to date. Besides the *vanB* ST484 strain, a *vanA*-type VREfm ST17 was detected (103 persons carried ST484 and 34 persons carried ST17 which were designated as types I and II in the original publication, respectively). Some ST484 isolates carried both *vanB* and *vanA* transposons.[28] BAPS 7 comprised two VREfm strains described in the USA in early 1990s (ampicillin-resistant ST20 and ST182, both *esp*$^{+}$/*entA*$^{+}$). Both strains caused large outbreaks involving interhospital clonal spread in Detroit and Texas (6 hospitals each).[32,33]

vanA (Tn*1546*) is mainly located on RepA_N (pRUM/pLG1) and Inc18 plasmid families.

***Diversity of Tn1546*.** Twenty-five *vanA* elements were identified according to indels and mutations in relation to the Tn*1546* prototype (Fig. S3). In order to simplify the reading and to be consistent with the literature, they will be designated here as "Tn*1546* variants" although most of *vanA* elements lack key components of the transposon.[34–36] The "Tn*1546* variants" that contains IS*1251* within the *vanS-vanH* region were predominant (n=21/53 isolates; from Europe and America). They were designated as "F" (F_{1-6}) in agreement with previous reports that designed Tn*1546* carrying this IS as "type F" or "US hospital type".[1,35] "Tn*1546* variants" containing IS*1216* within *vanX-vanY* (n=9), in both *vanX-vanY* and *vanS-vanH* (n=1), in both *vanX-vanY* and upstream *vanR* (n=1) or within *vanS-vanH* (n=2) regions were also common (n=13/53), and mainly found in VREfm from Europe. "Tn*1546* variants" carrying both IS*1251* and IS*1216* within *vanS-vanH* and *vanX-vanY* regions, respectively, corresponded to two isolates from South America. Variants carrying IS*Ef1* within *vanX-vanY* (n=5/53; from Germany, Portugal and Spain) or IS*1485* upstream *vanR* (one isolate from Saudi Arabia) were also observed. Other Tn*1546* with/without indels upstream *vanR* (types A, n=6/53; "D", n=5/53; and "M", n=1/53) corresponded to isolates from Europe, Brazil or Australia. Tn*1546-vanA* was located on plasmids in all but one ST117 isolate from Germany for which *vanA* and rel_6 probes hybridized in the chromosome.

Plasmids carrying* Tn*1546 belonged to either RepA_N (pRUM or pLG1) or Inc18 families or were mosaics containing RIPs of these two plasmid families (see below, Fig. S4). They were transferred by conjugation in most cases (79%), the plasmid size being increased after *in vitro* transfer in three cases (1 Inc18, 1 pRUM and 1 Inc18-pRUM chimera).

Inc18::Tn*1546* plasmids (rep_1 and/or rep_2, n=12; 30-145 kb) included the 30kb plasmid isolated from one of the very first VanA-VREfm strain (ST25) reported in France in 1986 and designed as pIP186 years later.[37,38] Rep_1/Rep_2 plasmids were commonly detected in different clonal lineages (BAPS 2.1a, BAPS 3.3a2) and different continents since 1992. Tn*1546* variants in these isolates were often truncated by IS*1216* or IS*1485.*

pRUM-like plasmids (rep_{17}; n=17) differed in the *rep* sequence and the presence of the $TA_{Axe\text{-}Txe}$. Those with $TA_{Axe\text{-}Txe}$ (30-60kb, n=11) mostly carried Tn*1546* variants "F-like", and were isolated from clonally unrelated isolates (9 STs of 4 BAPS subgroups) of America, Australia, and Europe since 1992 (cluster I, Fig. S5). pRUM-like plasmids ($rep_{17.1}$) lacking the Axe-Txe system (80-90 kb, n=3) contained Tn*1546* variants truncated by IS*Ef1*, and were recovered in Portugal[11] and Poland.[14]

Mosaic *vanA* plasmids appeared in isolates collected since 1988. The second VREfm isolated in France had three plasmids, a mosaic Inc18-pRUM::Tn*1546,* a pLG1 and a pHTß-like (see below). Inc18-pRUM chimeras (30-60 kb; n=12) with several RIPs (rep_{17}+rep_1+rep_2), RELs (rel_6+rel_7) and TAs ($TA_{Axe\text{-}Txe}$±TAε-ζ) contained either Tn*1546* type "F" (Europe and America since 1995), IS*1216*-Tn*1546* variants (Europe, 2001-2006) or Tn*1546* type "A" (France, 1988). It is of note that mosaic plasmids with rep_1+rep_2+rep_{17}+rel_6+$TA_{Axe\text{-}Txe}$ from different countries exhibited similar RFLP patterns. Among them, the first strains identified in France in 1986-1988 (ST25), and the first outbreak strains reported in Brazil (ST114) and Argentina (ST17) in 1998 (cluster IIa; Fig. S5).

Inc18-pHTβ mosaic plasmids (n=2; 50-85 kb; rep_{22}+rep_1+rel_8, one of them also containing rep_2+$TA_{\varepsilon\text{-}\zeta}$) carried IS*1216*-Tn*1546* and were identified in a ST18 isolate from the UK (1992), a ST16 isolate from the Netherlands (2002) and a transconjugant obtained from a ST132 isolate from Portugal that harboured these two plasmids (Fig. S4). Their RFLP patterns were closely related to the prototype pMG1 (cluster IIb; Fig. S5).

Finally, *vanA* large mosaic plasmids (n=11; 150-300 kb; rep_{20}±rep_1±rep_2±rel_6) were present in clonally unrelated strains from America and Europe since 1992. They contained either Tn*1546* variants without ISs (n=7) or Tn*1546* type "F" (n=4).

***vanB* transposons are chromosomally and plasmid located.** The backbone of *vanB* operons is shown in Fig. S2. Most were Tn*5382/1549* (*vanB2*) with an additional *Bsp*HI site within the 1,086-bp $vanS_B$-$vanY_B$ region (RFLP-2, n=15). They were categorized as variants with ISEnfa*110* insertions downstream the left inverted repeat of the transposon (Chile) or with IS*Enfa200* insertions within $vanS_B$-$vanY_B$ and a *pbp5* region upstream Tn*5382* (Finland, Australia and the USA).

Two of the three *vanB1* isolates detected were similar to that found in strain BM4281 (RFLP-1, n=2) and corresponded to strains causing large outbreaks in New York and Texas in early 90s. A *vanB* transposon showing an RFLP-3 was a hybrid resulting from recombination between the *vanB1* (BM4281) cluster and *vanB2* (Tn*1549)* transposon (this study, GenBank accession number KT201628) and corresponded to a ST209 isolate recovered in Italy in 2002. Mosaic structures in elements with RFLP-1 cannot be discarded as only the left arm of the transposon is usually analysed (this study).[6]

All but four *vanB* operons were chromosomally located. The four *vanB2* plasmids (50-60 kb) had variable *rep* content, similar RFLP patterns (data not shown) and correspond to two clonally related ST17 isolates from Spain (rep_1+rel_6 and rep_{17}+rel_6 plasmids); a ST265 isolate also from Spain (rep_{17}+$TA_{Axe\text{-}Txe}$+rep_1), and

a ST280 from Singapore (rep_{17}+rep_2) (Fig. S4). It is of note that *vanB2* located on pRUM plasmids of variable sizes (70-130 kb) have been recently detected in Sweden associated with hospital outbreaks.[9] The 70-kb pRUM plasmids were chimeras with rep_2 or an unknown replicon.[9]

Plasmid content of VREfm and VSEfm isolates. A variable number of plasmid bands (n=1-8) was observed for VREfm isolates (Fig. S4 and Fig. S6). Most isolates contained RCR, small theta, and megaplasmids while pRUM, Inc18 or pHTß were variably detected (Fig. 1 and Fig. S4). Similar plasmid content was detected in VSEfm (Fig. S7).

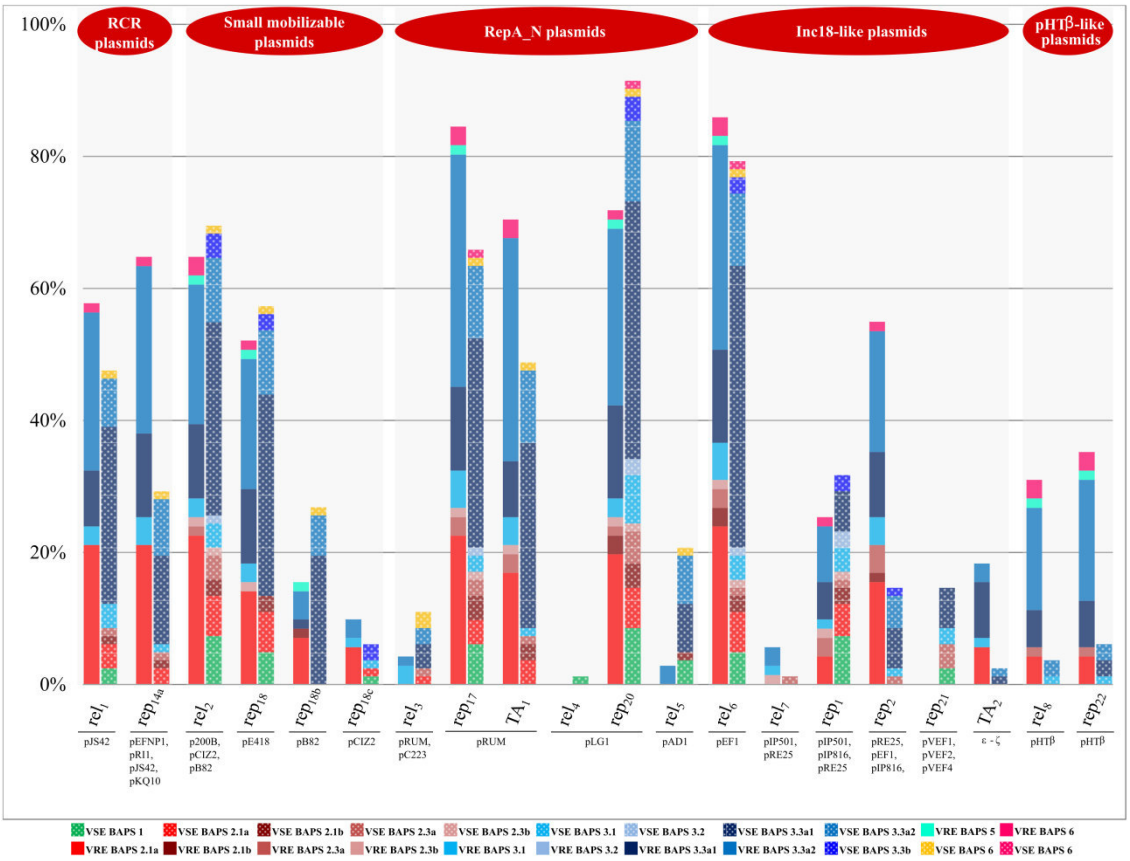

Figure 1. Plasmid content (rep/rel/TA) of VREfm and VSEfm isolates.

Percentages of rep (replication), rel (relaxase), and TA (toxin-antitoxin) genes for isolates of each BAPS subgroup are represented. The prototype or main plasmids associated with a given gene are indicated above.

RCR plasmids (2-4 kb) were present in VREfm and VSEfm isolates (65% versus 29%). Most were positive for rep_{14a}, the presence of REL_{MOBV4} (pRI1/pJS42) being variable.

Small mobilizable theta-replicating plasmids were detected in VREfm and VSEfm isolates (65% versus 61%). Rep_{18a} or rep_{18c} (8-18kb) occasionally contained the *bac32* (5-14%) or *entQ* (2% in VSEfm) while rep_{18b} plasmids (5-7 kb) often harboured *bac43* (5/7 plasmids, 71% VREfm, all collected after 2005). Most of them had a REL of the MOB_{P7} family (65%-72%). Both RCR and small theta-replicating plasmids were often co-transferred with vancomycin-resistance plasmids.

Megaplasmids (135-295kb) were present in all but the first VREfm isolates described in France (1986-1988) and the UK (1992). They were often positive for rep_{20} (present in 72% of VREfm and 93% of VSEfm) while the rel_{pLG1} was only identified in one ST74 VSEfm isolate. Some of them also hybridized with probes specific for rel_6 and/or rel_5. Megaplasmids often carried hyl_{Efm} (18-30%) and *entP* (7- 21%).

Finally, pRUM, Inc18 or pHTß were present at different rates in both VREfm and VSEfm. The pRUM-like plasmids that do not carry *van* genes had similar size (20-25 kb) and identical rep_{17} and $TA_{Axe\text{-}Txe}$ genes to that of the original pRUM sequence.[39] The rel_3, detected in the original pRUM, is the *rel* of pC223 of *Staphylococcus aureus*, and was only identified in a few VREfm (4%) and VSEfm (7%). All early US strains contained pRUM with or without Tn*1546*. Plasmids carrying RIP and TA sequences of the Inc18 plasmid family (rep_1, rep_2, rep_{21}, ε-ζ, ω-δ; 20-280 kb) in VREfm and VSEfm usually had a rel_6 of the MOB_{P7} family (86% versus 78%, respectively) instead the REL prototype of Inc18 plasmids (rel_7 of the MOB_{Q3} family, <5%). Rep_{22} plasmids (50-90 kb) were detected in isolates since 1988 although at lower rates than other plasmid families (32% versus 5% of VREfm and VSEfm, respectively). They may also contain other RIPs (rep_1 or rep_2) and different RELs (rel_8 or rel_6).

Some large and small plasmids could not be classified. A recent search also demonstrated that the plasmid pool in fully sequenced enterococci available at gene databases is larger than that detectable by the available PCR typing schemes used here.27

DISCUSSION

This study showed the influence of both clonal lineages and plasmids in the spread and persistence of vancomycin resistance among *E. faecium,* by characterizing for the first time available strains causing outbreaks in different continents. Predominant ampicillin-resistant strains of major *E. faecium* human lineages ST17, ST18 and ST78 have contributed to reach high rates of vancomycin resistance after acquisition of *van* transposons.[40] This is reflected by the emblematic clonal VREfm outbreaks in the US (NY 1991-1992, Detroit, Texas-San Antonio 1993-1994, Cleveland 1996, Chicago 1995 or Alabama 2004-2005 mostly caused by either ST16 or ST17, all included in this study), Australia (2007-2009, ST203), Sweden (2007-2011, ST192), Portugal (2001-2003, ST18/ST132), Brazil and Canada (2006-2008, ST412) or Netherlands (ST117) (Table 1).[9,11,33,40–43] However, horizontal gene transfer (HGT) events often occurred when the prevalence of VRE rapidly increase or when they became endemic[2,11] as reflect the examples of polyclonal and plasmid outbreaks (New York, 1991-92;[43] Texas-Houston, 1993-94;[33] Korea, 1997-2001[44]) or the surveillance studies of colonized hospitalized patients (Finland, 1996-1998,[28] Belgium, 1993,[42] The Netherlands, 1995-1996,[45] and China, 2001-2005[46]).

The diversity of plasmids able to acquire *van* operons and their ability to recombine and yield plasmid mosaics played a critical role in the spread of *vanA*. Frequent HGT events between clonally related populations of *S. aureus* and their mobile genetic elements generated local "clonal clouds" that result in genetic variants with variable levels of fitness confronting local selective forces.[47,48] It is tempting to

suggest a similar scenario in *E. faecium*, where HGT not only plays a relevant role in shaping the transmission dynamics of VREfm, but also may influence the global presence of *E. faecium* lineages that have already been circulating for decades;[41,49] and also the diversity of plasmid and chimeras of narrow host *E. faecium* plasmids even for isolates of the same clone.[11] The recovery of pRUM::Tn*1546* highly related to the original pRUM[39] among VREfm from the USA and Inc18::Tn*1546* similar to the first *vanA* plasmid pIP816[37,38] among VREfm from humans and animals in Europe since late 80s to date,[11–13,50] revealed the outstanding contribution of pRUM and pIP186 to the evolvability of Tn*1546*. However, the lack of isolates from Asian and African countries in the study precludes a real global vision of VREfm dynamics.

The high mosaicism of enterococcal plasmids linked to antibiotic resistance was also observed after the sequencing of few strains[18,51,52] and *van* plasmids.[37,39,50,53] Although the similarity of plasmid chimeras in strains causing early outbreaks in different countries[9,11,13,14] could suggest selection of some hybrids, independent recombination events between plasmids cannot be discarded taking in account the frequent *in vivo* transfer of *vanA* between *E. faecium* strains in food and gut flora.[54] Besides the dominant impact of these pRUM and Inc18 plasmids, we showed how any enterococcal plasmid may be a vehicle of Tn*1546* or CTn*5382*, eventually causing self-limited outbreaks. An example of this is the pLG1-like megaplasmids that often carry *vanA* and other genes linked to persistence or colonization (e.g. hyl_{Efm} or *pilA*) or antibiotic resistance [*aac(6')-Ie-aph(2'')-Ia*].[14,24,41,55–57] Although pHTß plasmids were significantly involved in the early spread of resistance to high levels of glycopeptides or gentamicin in Japan and the USA,[58] they are less frequently recovered in most contemporary collections including that analysed in this study although this might be caused by the absence of strains from Asian countries. Finally, mosaics carrying sequences of *E. faecium* plasmids and pheromone responsive plasmids of *E. faecalis* (this study)[11,59] or *S. aureus,*[39] indicate close HGT events between *E. faecium* and species of *Enterococcus*, *Streptococcus* and *Staphylococcus*. Besides the antibiotic resistance, plasmids carry a diversity of genes that confer adaptive traits (metabolic, bacteriocins, RM-systems) that would have facilitated the persistence of different hierarchical units.

In contrast with what has been observed for *vanA*, some *vanB2*-Tn*5382*/Tn*1549* types are widely spread.[9,60] Studies in areas with high prevalence of *vanB* as Australia, demonstrated that *vanB* transposon is repeatedly acquired by *E. faecium* from anaerobes of the gut flora in hospitalized patients treated with antibiotics.[61] Gut flora may also be the origin of *vanD* and *vanG* transposons, each independently acquired by different species of enterococci.[62]

In conclusion, we document the clonal and plasmid backgrounds responsible for the acquisition, spread and persistence of vancomycin resistance in different areas by studying available outbreak *E. faecium* strains from 1986. The high content of plasmid and mobile genetic elements in *E. faecium* human lineages seems to be relevant in the emergence and persistence of antibiotic resistance. The narrow

host range of the plasmids carrying Tn*1546* might partially explain why this resistance has not yet been widely disseminated to other species.[63]

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REFERENCES

1. Bonten MJ, Willems R, Weinstein RA. *Vancomycin-resistant enterococci: why are they here, and where do they come from?* Lancet Infect Dis 2001; 1: 314–25.

2. Werner G, Coque TM, Hammerum AM et al. *Emergence and spread of vancomycin resistance among enterococci in Europe*. Euro Surveill 2008; 13: pii: 19046.

3. Miller WR, Munita JM, Arias CA. *Mechanisms of antibiotic resistance in enterococci*. Expert Rev Anti Infect Ther 2014; 12: 1221–36.

4. Clewell DB, Weaver KE, Coque TM et al. *Extrachromosomal and Mobile Elements in Enterococci: Transmission, Maintenance, and Epidemiology*. *In*: Gilmore MS, Clewell DB, Ike Y, Shankar N, eds. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Boston: Massachusetts Eye and Ear Infirmary, 2014.

5. Carias LL, Rudin SD, Donskey CJ et al. *Genetic linkage and cotransfer of a novel, vanB-containing transposon (Tn5382) and a low-affinity penicillin-binding protein 5 gene in a clinical vancomycin-resistant Enterococcus faecium isolate*. J Bacteriol 1998; 180: 4426–34.

6. Dahl KH, Simonsen GS, Olsvik O et al. *Heterogeneity in the vanB gene cluster of genomically diverse clinical strains of vancomycin-resistant enterococci*. Antimicrob Agents Chemother 1999; 43: 1105–10.

7. Dahl KH, Lundblad EW, Rokenes TP et al. *Genetic linkage of the vanB2 gene cluster to Tn5382 in vancomycin-resistant enterococci and characterization of two novel insertion sequences*. Microbiology 2000; 146: 1469–79.

8. Dahl KH, Mater DDG, Flores MJ et al. *Transfer of plasmid and chromosomal glycopeptide resistance determinants occurs more readily in the digestive tract of mice than in vitro and exconjugants can persist stably in vivo in the absence of glycopeptide selection*. J Antimicrob Chemother 2007; 59: 478–86.

9. Sivertsen A, Billström H, Melefors Ö et al. *A multicentre hospital outbreak in Sweden caused by introduction of a vanB2 transposon into a stably maintained pRUM-plasmid in an Enterococcus faecium ST192 clone*. PLoS One 2014; 9: e103274.

10. Umeda A, Garnier F, Courvalin P et al. *Association between the vanB2 glycopeptide resistance operon and Tn1549 in enterococci from France*. J Antimicrob Chemother 2002; 50: 253–6.

11. Freitas AR, Novais C, Tedim AP et al. *Microevolutionary Events Involving Narrow Host Plasmid Influences Local Fixation of Vancomycin-Resistance in Enterococcus*. PLoS One 2013: e60589.

12. Garcia-Migura L, Sanchez-Valenzuela AJ, Jensen LB. *Presence of glycopeptide-encoding plasmids in enterococcal isolates from food and humans in Denmark.* Foodborne Pathog Dis 2011; 8: 1191–7.

13. Rosvoll TCS, Pedersen T, Sletvold H et al. *PCR-based plasmid typing in Enterococcus faecium strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHTbeta-related replicons associated with glycopeptide resistance and stabilizing toxin-antitoxin systems*. FEMS Immunol Med Microbiol 2010; 58: 254–68.

14. Wardal E, Markowska K, Zabicka D et al. *Molecular analysis of vanA outbreak of Enterococcus faecium in two Warsaw hospitals: the importance of mobile genetic elements.* Biomed Res Int 2014; 2014: 575367.

15. Freitas AR, Coque TM, Novais C et al. *Human and swine hosts share vancomycin-resistant Enterococcus faecium CC17 and CC5 and Enterococcus faecalis CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids*. J Clin Microbiol 2011; 49: 925–31.

16. Valdezate S, Miranda C, Navarro A et al. *Clonal outbreak of ST17 multidrug-resistant Enterococcus faecium harbouring an Inc18-like::Tn1546 plasmid in a haemo-oncology ward of a Spanish hospital*. J Antimicrob Chemother 2012; 67: 832–6.

17. de Been M, Pinholt M, Top J et al. *A core genome MLST scheme for high-resolution typing of Enterococcus faecium*. J Clin Microbiol 2015.

18. Lam MMC, Seemann T, Tobias NJ et al. *Comparative analysis of the complete genome of an epidemic hospital sequence type 203 clone of vancomycin-resistant Enterococcus faecium*. BMC Genomics 2013; 14: 595.

19. Lebreton F, van Schaik W, McGuire AM et al. *Emergence of epidemic multidrug-resistant Enterococcus faecium from animal and commensal strains*. MBio 2013; 4.

20. Pinholt M, Larner-Svensson H, Littauer P et al. *Multiple hospital outbreaks of vanA Enterococcus faecium in Denmark, 2012-13, investigated by WGS, MLST and PFGE*. J Antimicrob Chemother 2015; 70: 2474–82.

21. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement M100-S22*. CLSI, Wayne, PA, USA; 2012.

22. Tedim AP, Ruiz-Garbajosa P, Corander J et al. *Population Biology of Intestinal Enterococcus Isolates from Hospitalized and Nonhospitalized Individuals in Different Age Groups*. Appl Environ Microbiol 2015; 81: 1820–31.

23. Willems RJL, Top J, van Schaik W et al. *Restricted gene flow among hospital subpopulations of Enterococcus faecium*. MBio 2012; 3: e00151–12.

24. Freitas AR, Tedim AP, Novais C et al. *Global spread of the hyl(Efm) colonization-virulence gene in megaplasmids of the Enterococcus faecium CC17 polyclonal subcluster.* Antimicrob Agents Chemother 2010; 54: 2660–5.

25. Goicoechea P, Romo M, Coque TM et al. *Identification of enterococcal plasmids by multiplex-PCR-based relaxase typing* (Abstract number: P1669). In: 18th European Congress of Clinical Microbiology and Infectious Diseases. Barcelona, Spain, 2008.

26. Jensen LB, Garcia-Migura L, Valenzuela AJS et al. *A classification system for plasmids from enterococci and other Gram-positive bacteria*. J Microbiol Methods 2010; 80: 25–43.

27. Lanza VF, Tedim AP, Martínez JL et al. *The Plasmidome of Firmicutes: Impact on the Emergence and the Spread of Resistance to Antimicrobials*. Microbiol Spectr 2015; 3: PLAS – 0039–2014.

28. Suppola JP, Kolho E, Salmenlinna S et al. *vanA and vanB incorporate into an endemic ampicillin-resistant vancomycin-sensitive Enterococcus faecium strain: effect on interpretation of clonality.* J Clin Microbiol 1999; 37: 3934–9.

29. Morrison D, Woodford N, Barrett SP et al. *DNA banding pattern polymorphism in vancomycin-resistant Enterococcus faecium and criteria for defining strains.* J Clin Microbiol 1999; 37: 1084–91.

30. Johnson PDR, Ballard SA, Grabsch EA et al. *A sustained hospital outbreak of vancomycin-resistant Enterococcus faecium bacteremia due to emergence of vanB E. faecium sequence type 203*. J Infect Dis 2010; 202: 1278–86.

31. Coombs GW, Pearson JC, Daley DA et al. *Molecular epidemiology of enterococcal bacteremia in Australia*. J Clin Microbiol 2014; 52: 897–905.

32. Dunne WM, Wang W. *Clonal dissemination and colony morphotype variation of vancomycin-resistant Enterococcus faecium isolates in metropolitan Detroit, Michigan*. J Clin Microbiol 1997; 35: 388–92.

33. Moreno F, Grota P, Crisp C et al. *Clinical and molecular epidemiology of vancomycin-resistant Enterococcus faecium during its emergence in a city in southern Texas*. Clin Infect Dis 1995; 21: 1234–7.

34. Novais C, Freitas AR, Sousa JC et al. *Diversity of Tn1546 and its role in the dissemination of vancomycin-resistant enterococci in Portugal*. Antimicrob Agents Chemother 2008; 52: 1001–8.

35. Willems RJ, Top J, van den Braak N et al. *Molecular diversity and evolutionary relationships of Tn1546-like elements in enterococci from humans and animals.* Antimicrob Agents Chemother 1999; 43: 483–91.

36. Woodford N, Adebiyi AM, Palepou MF et al. *Diversity of VanA glycopeptide resistance elements in enterococci from humans and nonhuman sources*. Antimicrob Agents Chemother 1998; 42: 502–8.

37. Sletvold H, Johnsen PJ, Wikmark O-G et al. *Tn1546 is part of a larger plasmid-encoded genetic unit horizontally disseminated among clonal Enterococcus faecium lineages.* J Antimicrob Chemother 2010; 65: 1894–906.

38. Leclercq R, Derlot E, Duval J et al. *Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium*. N Engl J Med 1988; 319: 157–61.

39. Grady R, Hayes F. *Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multidrug-resistant, clinical isolate of Enterococcus faecium*. Mol Microbiol 2003; 47: 1419–32.

40. Willems RJL. *Genetic Evolution of E. faecium and E. faecalis: Similarities and Differences. In: 4th ASM Conference on Enterococci.* Cartagena, Colombia: American Society for Microbiology, 2014.

41. Galloway-Peña JR, Nallapareddy SR, Arias CA et al. *Analysis of clonality and antibiotic resistance among early clinical isolates of Enterococcus faecium in the United States*. J Infect Dis 2009; 200: 1566–73.

42. Gordts B, Van Landuyt H, Ieven M et al. *Vancomycin-resistant enterococci colonizing the intestinal tracts of hospitalized patients*. J Clin Microbiol 1995; 33: 2842–6.

43. Mato R, de Lencastre H, Roberts RB et al. *Multiplicity of genetic backgrounds among vancomycin-resistant Enterococcus faecium isolates recovered from an outbreak in a New York City hospital*. Microb Drug Resist 1996; 2: 309–17.

44. Yoo SJ, Sung H, Cho Y-U et al. *Role of horizontal transfer of the transposon Tn1546 in the nosocomial spread of vanA vancomycin-resistant enterococci at a tertiary care hospital in Korea.* Infect Control Hosp Epidemiol 2006; 27: 1081–7.

45. Endtz HP, van den Braak N, van Belkum A et al. *Fecal carriage of vancomycin-resistant enterococci in hospitalized patients and those living in the community in The Netherlands*. J Clin Microbiol 1997; 35: 3026–31.

46. Zheng B, Tomita H, Xiao YH et al. *Molecular characterization of vancomycin-resistant enterococcus faecium isolates from mainland China.* J Clin Microbiol 2007; 45: 2813–8.

47. Chambers HF, Deleo FR. *Waves of resistance: Staphylococcus aureus in the antibiotic era*. Nat Rev Microbiol 2009; 7: 629–41.

48. Stanczak-Mrozek KI, Manne A, Knight GM et al. *Within-host diversity of MRSA antimicrobial resistances*. J Antimicrob Chemother 2015; 70: 2191–8.

49. Willems RJL, Top J, van Santen M et al. *Global spread of vancomycin-resistant Enterococcus faecium from distinct nosocomial genetic complex*. Emerg Infect Dis 2005; 11: 821–8.

50. Sletvold H, Johnsen PJ, Simonsen GS et al. *Comparative DNA analysis of two vanA plasmids from Enterococcus faecium strains isolated from poultry and a poultry farmer in Norway*. Antimicrob Agents Chemother 2007; 51: 736–9.

51. Kopit LM, Kim EB, Siezen RJ et al. *Safety of the surrogate microorganism Enterococcus faecium NRRL B-2354 for use in thermal process validation*. Appl Environ Microbiol 2014; 80: 1899–909.

52. Qin X, Galloway-Peña JR, Sillanpaa J et al. *Complete genome sequence of Enterococcus faecium strain TX16 and comparative genomic analysis of Enterococcus faecium genomes*. BMC Microbiol 2012; 12: 135.

53. Xu X, Lin D, Yan G et al. *vanM, a new glycopeptide resistance gene cluster found in Enterococcus faecium*. Antimicrob Agents Chemother 2010; 54: 4643–7.

54. Lester CH, Frimodt-Møller N, Sørensen TL et al. *In vivo transfer of the vanA resistance gene from an Enterococcus faecium isolate of animal origin to an E. faecium isolate of human origin in the intestines of human volunteers*. Antimicrob Agents Chemother 2006; 50: 596–9.

55. Arias CA, Panesso D, Singh K V et al. *Cotransfer of antibiotic resistance genes and a hylEfm-containing virulence plasmid in Enterococcus faecium*. Antimicrob Agents Chemother 2009; 53: 4240–6.

56. Laverde Gomez JA, van Schaik W, Freitas AR et al. *A multiresistance megaplasmid pLG1 bearing a hylEfm genomic island in hospital Enterococcus faecium isolates.* Int J Med Microbiol 2011; 301: 165–75.

57. Rosvoll TCS, Lindstad BL, Lunde TM et al. *Increased high-level gentamicin resistance in invasive Enterococcus faecium is associated with aac(6')Ie-aph(2")Ia-encoding transferable megaplasmids hosted by major hospital-adapted lineages*. FEMS Immunol Med Microbiol 2012; 66: 166–76.

58. Tomita H, Tanimoto K, Hayakawa S et al. *Highly conjugative pMG1-like plasmids carrying Tn1546-like transposons that encode vancomycin resistance in Enterococcus faecium*. J Bacteriol 2003; 185: 7024–8.

59. Freitas AR, Novais C, Ruiz-Garbajosa P et al. *Clonal expansion within clonal complex 2 and spread of vancomycin-resistant plasmids among different genetic lineages of Enterococcus faecalis from Portugal*. J Antimicrob Chemother 2009; 63: 1104–11.

60. Lam MMC, Seemann T, Bulach DM et al. *Comparative analysis of the first complete Enterococcus faecium genome*. J Bacteriol 2012; 194: 2334–41.

61. Howden BP, Holt KE, Lam MMC et al. *Genomic insights to control the emergence of vancomycin-resistant enterococci*. MBio 2013; 4: e00412–3 – .

62. Domingo M-C, Huletsky A, Giroux R et al. *vanD and vanG-like gene clusters in a Ruminococcus species isolated from human bowel flora.* Antimicrob Agents Chemother 2007; 51: 4111–7.

63. Werner G, Freitas AR, Coque TM et al. *Host range of enterococcal vanA plasmids among Gram-positive intestinal bacteria.* J Antimicrob Chemother 2011; 66: 273–82.

64. Cilo BD, Ağca H, Efe K et al. *Investigation of vancomycin resistant Enterococcus faecium outbreak in neonatal intensive care unit*. Int J Clin Exp Med 2014; 7: 5342–7.

65. Marcadé G, Micol J-B, Jacquier H et al. *Outbreak in a haematology unit involving an unusual strain of glycopeptide-resistant Enterococcus faecium carrying both vanA and vanB genes*. J Antimicrob Chemother 2014; 69: 500–5.

66. Yang J, Li T, Ning Y et al. *Molecular characterization of resistance, virulence and clonality in vancomycin-resistant Enterococcus faecium and Enterococcus faecalis: A hospital-based study in Beijing, China*. Infect Genet Evol 2015; 33: 253–60.

67. Campos PA, Batistão DWF, Gontijo-Filho PP et al. *A sustained endemic outbreak of vancomycin-resistant Enterococcus faecium: A 30-month surveillance study*. Scand J Infect Dis 2014; 46: 547–54.

68. Resende M, Caierão J, Prates JG et al. *Emergence of vanA vancomycin-resistant Enterococcus faecium in a hospital in Porto Alegre, South Brazil*. J Infect Dev Ctries 2014; 8: 160–7.

69. Szakacs TA, Kalan L, McConnell MJ et al. *Outbreak of vancomycin-susceptible Enterococcus faecium containing the wild-type vanA gene*. J Clin Microbiol 2014; 52: 1682–6.

70. Lee S-C, Wu M-S, Shih H-J et al. *Identification of vancomycin-resistant enterococci clones and inter-hospital spread during an outbreak in Taiwan*. BMC Infect Dis 2013; 13: 163.

71. Thierfelder C, Keller PM, Kocher C et al. *Vancomycin-resistant Enterococcus*. Swiss Med Wkly 2012; 142: w13540.

72. Fournier S, Brossier F, Fortineau N et al. *Long-term control of vancomycin-resistant Enterococcus faecium at the scale of a large multihospital institution: a seven-year experience*. Euro Surveill 2012; 17.

73. Pusch T, Kemp D, Trevino S et al. *Controlling outbreak of vancomycin-resistant Enterococcus faecium among infants caused by an endemic strain in adult inpatients*. Am J Infect Control 2013; 41: 51–6.

74. Tuon FF, Penteado-Filho SR, Camilotti J et al. *Outbreak of vancomycin-resistant Enterococcus in a renal transplant unit*. Braz J Infect Dis 15: 403–5.

75. Cheng VCC, Chan JFW, Tai JWM et al. *Successful control of vancomycin-resistant Enterococcus faecium outbreak in a neurosurgical unit at non-endemic region.* Emerg Health Threats J 2009; 2: e9.

76. Werner G, Klare I, Fleige C et al. *Vancomycin-resistant vanB-type Enterococcus faecium isolates expressing varying levels of vancomycin resistance and being highly prevalent among neonatal patients in a single ICU*. Antimicrob Resist Infect Control 2012; 1: 21.

77. Xu H, Tian R, Chen D et al. *Nosocomial spread of hospital-adapted CC17 vancomycin-resistant Enterococcus faecium in a tertiary-care hospital of Beijing, China*. Chin Med J (Engl) 2011; 124: 498–503.

78. Henard S, Gendrin V, Simon L et al. *Control of a regional outbreak of vanA glycopeptide-resistant Enterococcus faecium, Eastern France, 2004-2009*. Int J Hyg Environ Health 2011; 214: 265–70.

79. Liu Y, Cao B, Gu L et al. *Successful control of vancomycin-resistant Enterococcus faecium nosocomial outbreak in a teaching hospital in China*. Am J Infect Control 2012; 40: 568–71.

80. Iosifidis E, Evdoridou I, Agakidou E et al. *Vancomycin-resistant Enterococcus outbreak in a neonatal intensive care unit: epidemiology, molecular analysis and risk factors*. Am J Infect Control 2013; 41: 857–61.

81. Valdezate S, Labayru C, Navarro A et al. *Large clonal outbreak of multidrug-resistant CC17 ST17 Enterococcus faecium containing Tn5382 in a Spanish hospital*. J Antimicrob Chemother 2009; 63: 17–20.

82. Panesso D, Reyes J, Rincón S et al. *Molecular epidemiology of vancomycin-resistant Enterococcus faecium: a prospective, multicenter study in South American hospitals*. J Clin Microbiol 2010; 48: 1562–9.

83. Park SY, Kang J-H, Kim J-H et al. *Vancomycin-resistant Enterococcus faecium outbreak caused by patient transfer in 2 separate intensive care units*. Am J Infect Control 2012; 40: 565–7.

84. Servais A, Mercadal L, Brossier F et al. *Rapid curbing of a vancomycin-resistant Enterococcus faecium outbreak in a nephrology department*. Clin J Am Soc Nephrol 2009; 4: 1559–64.

85. Hoshuyama T, Moriguchi H, Muratani T et al. *Vancomycin-resistant enterococci (VRE) outbreak at a university hospital in Kitakyushu, Japan: case-control studies*. J Infect Chemother 2008; 14: 354–60.

86. Dendle C, Ballard SA, Grabsch EA et al. *Outbreak of vancomycin-resistant Enterococcus faecium containing both vanA and vanB gene clusters*. J Hosp Infect 2009; 71: 379–81.

87. Al-Mohri HA, Tadros MA, Louie L et al. *Utility of direct, real-time PCR in the management of a nosocomial outbreak of vancomycin-resistant Enterococcus faecium (vanB genotype)*. Eur J Clin Microbiol Infect Dis 2008; 27: 321–2.

88. Kirdar S, Sener AG, Arslan U et al. *Molecular epidemiology of vancomycin-resistant Enterococcus faecium strains isolated from haematological malignancy patients in a research hospital in Turkey*. J Med Microbiol 2010; 59: 660–4.

89. Pereira GH, Müller PR, Zanella RC et al. *Outbreak of vancomycin-resistant enterococci in a tertiary hospital: the lack of effect of measures directed mainly by surveillance cultures and differences in response between Enterococcus faecium and Enterococcus faecalis*. Am J Infect Control 2010; 38: 406–9.

90. Lester CH, Olsen SS, Schønheyder HC et al. *Typing of vancomycin-resistant enterococci obtained from patients at Danish hospitals and detection of a genomic island specific to CC17 Enterococcus faecium.* Int J Antimicrob Agents 2010; 35: 312–4.

91. Yoon YK, Sim HS, Kim JY et al. *Epidemiology and control of an outbreak of vancomycin-resistant enterococci in the intensive care units*. Yonsei Med J 2009; 50: 637–43.

92. Zhu X, Zheng B, Wang S et al. *Molecular characterisation of outbreak-related strains of vancomycin-resistant Enterococcus faecium from an intensive care unit in Beijing, China*. J Hosp Infect 2009; 72: 147–54.

93. Khan MA, Northwood JB, Loor RGJ et al. *High prevalence of ST-78 infection-associated vancomycin-resistant Enterococcus faecium from hospitals in Asunción, Paraguay*. Clin Microbiol Infect 2010; 16: 624–7.

94. Kawalec M, Pietras Z, Daniłowicz E et al. *Clonal structure of Enterococcus faecalis isolated from Polish hospitals: characterization of epidemic clones*. J Clin Microbiol 2007; 45: 147–53.

95. Werner G, Klare I, Witte W. *The current MLVA typing scheme for Enterococcus faecium is less discriminatory than MLST and PFGE for epidemic-virulent, hospital-adapted clonal types*. BMC Microbiol 2007; 7: 28.

96. Schmidt-Hieber M, Blau IW, Schwartz S et al. *Intensified strategies to control vancomycin-resistant enterococci in immunocompromised patients*. Int J Hematol 2007; 86: 158–62.

97. Ergani-Ozcan A, Naas T, Baysan BO et al. *Nosocomial outbreak of vancomycin-resistant Enterococcus faecium in a paediatric unit at a Turkish university hospital*. J Antimicrob Chemother 2008; 61: 1033–9.

98. Comert FB, Kulah C, Aktas E et al. *First isolation of vancomycin-resistant enteroccoci and spread of a single clone in a university hospital in northwestern Turkey*. Eur J Clin Microbiol Infect Dis 2007; 26: 57–61.

99. Montesinos I, Campos S, Ramos MJ et al. [*Study of the first outbreak of vanA enterococcus faecium in the Canary Islands*]. Enferm Infecc Microbiol Clin 28: 430–4.

100. Souli M, Sakka V, Galani I et al. *Colonisation with vancomycin- and linezolid-resistant Enterococcus faecium in a university hospital: molecular epidemiology and risk factor analysis*. Int J Antimicrob Agents 2009; 33: 137–42.

101. Ergaz Z, Arad I, Bar-Oz B et al. *Elimination of vancomycin-resistant enterococci from a neonatal intensive care unit following an outbreak*. J Hosp Infect 2010; 74: 370–6.

102. Drews SJ, Richardson SE, Wray R et al. *An outbreak of vancomycin-resistant Enterococcus faecium in an acute care pediatric hospital: Lessons from environmental screening and a case-control study*. Can J Infect Dis Med Microbiol 2008; 19: 233–6.

103. Lucet J-C, Armand-Lefevre L, Laurichesse J-J et al. *Rapid control of an outbreak of vancomycin-resistant enterococci in a French university hospital*. J Hosp Infect 2007; 67: 42–8.

104. Yang K-S, Fong Y-T, Lee H-Y et al. *Predictors of vancomycin-resistant enterococcus (VRE) carriage in the first major VRE outbreak in Singapore*. Ann Acad Med Singapore 2007; 36: 379–83.

105. López M, Hormazábal JC, Maldonado A et al. *Clonal dissemination of Enterococcus faecalis ST201 and Enterococcus faecium CC17-ST64 containing Tn5382-vanB2 among 16 hospitals in Chile*. Clin Microbiol Infect 2009; 15: 586–8.

106. Worth LJ, Thursky KA, Seymour JF et al. *Vancomycin-resistant Enterococcus faecium infection in patients with hematologic malignancy: patients with acute myeloid leukemia are at high-risk*. Eur J Haematol 2007; 79: 226–33.

107. Aumeran C, Baud O, Lesens O et al. *Successful control of a hospital-wide vancomycin-resistant Enterococcus faecium outbreak in France*. Eur J Clin Microbiol Infect Dis 2008; 27: 1061–4.

108. Nebreda T, Oteo J, Aldea C et al. *Hospital dissemination of a clonal complex 17 vanB2-containing Enterococcus faecium*. J Antimicrob Chemother 2007; 59: 806–7.

109. Brossier F, Lefrançois S, Paute J et al. *Decolonisation for early control of an outbreak of vancomycin-resistant Enterococcus faecium in a geriatric rehabilitation care facility*. J Hosp Infect 2010; 76: 368–9.

110. Theilacker C, Jonas D, Huebner J et al. *Outcomes of invasive infection due to vancomycin-resistant Enterococcus faecium during a recent outbreak*. Infection 2009; 37: 540–3.

111. Borgmann S, Schulte B, Wolz C et al. *Discrimination between epidemic and non-epidemic glycopeptide-resistant E. faecium in a post-outbreak situation*. J Hosp Infect 2007; 67: 49–55.

112. Deplano A, Denis O, Nonhoff C et al. *Outbreak of hospital-adapted clonal complex-17 vancomycin-resistant Enterococcus faecium strain in a haematology unit: role of rapid typing for early control*. J Antimicrob Chemother 2007; 60: 849–54.

113. Dobbs TE, Patel M, Waites KB et al. *Nosocomial spread of Enterococcus faecium resistant to vancomycin and linezolid in a tertiary care medical center*. J Clin Microbiol 2006; 44: 3368–70.

114. Chlebicki MP, Ling ML, Koh TH et al. *First outbreak of colonization and infection with vancomycin-resistant Enterococcus faecium in a tertiary care hospital in Singapore*. Infect Control Hosp Epidemiol 2006; 27: 991–3.

115. Koh TH, Hsu L-Y, Chiu L-L et al. *Emergence of epidemic clones of vancomycin-resistant Enterococcus faecium in Singapore*. J Hosp Infect 2006; 63: 234–6.

116. Libisch B, Lepsanovic Z, Top J et al. *Molecular characterization of vancomycin-resistant Enterococcus spp. clinical isolates from Hungary and Serbia*. Scand J Infect Dis 2008; 40: 778–84.

117. Naas T, Fortineau N, Snanoudj R et al. *First nosocomial outbreak of vancomycin-resistant Enterococcus faecium expressing a VanD-like phenotype associated with a vanA genotype*. J Clin Microbiol 2005; 43: 3642–9.

118. Böröcz K, Szilágyi E, Kurcz A et al. *First vancomycin-resistant Enterococcus faecium outbreak reported in Hungary*. Euro Surveill 2005; 10: E050127.1.

119. Pendle S, Jelfs P, Olma T et al. *Difficulties in detection and identification of Enterococcus faecium with low-level inducible resistance to vancomycin, during a hospital outbreak*. Clin Microbiol Infect 2008; 14: 853–7.

120. Abbassi MS, Znazen A, Mahjoubi F et al. *Emergence of vancomycin-resistant Enterococcus faecium in Sfax: clinical features and molecular typing*. Médecine Mal Infect 2007; 37: 240–1.

121. Zárate MS, Gales A, Jordá-Vargas L et al. [*Environmental contamination during a vancomycin-resistant Enterococci outbreak at a hospital in Argentina*]. Enferm Infecc Microbiol Clin 2007; 25: 508–12.

122. Novais C, Sousa JC, Coque TM et al. *Molecular characterization of glycopeptide-resistant Enterococcus faecium isolates from Portuguese hospitals*. Antimicrob Agents Chemother 2005; 49: 3073–9.

123. Khan MA, van der Wal M, Farrell DJ et al. *Analysis of VanA vancomycin-resistant Enterococcus faecium isolates from Saudi Arabian hospitals reveals the presence of clonal cluster 17 and two new Tn1546 lineage types*. J Antimicrob Chemother 2008; 62: 279–83.

124. Stampone L, Del Grosso M, Boccia D et al. *Clonal spread of a vancomycin-resistant Enterococcus faecium strain among bloodstream-infecting isolates in Italy*. J Clin Microbiol 2005; 43: 1575–80.

125. Oh HS, Kim EC, Oh MD et al. *Outbreak of vancomycin resistant enterococcus in a hematology/oncology unit in a Korean University Hospital, and risk factors related to patients, staff, hospital care and facilities*. Scand J Infect Dis 2004; 36: 790–4.

126. Peta M, Carretto E, Barbarini D et al. *Outbreak of vancomycin-resistant Enterococcus spp. in an Italian general intensive care unit*. Clin Microbiol Infect 2006; 12: 163–9.

127. Christiansen KJ, Tibbett PA, Beresford W et al. *Eradication of a large outbreak of a single strain of vanB vancomycin-resistant Enterococcus faecium at a major Australian teaching hospital*. Infect Control Hosp Epidemiol 2004; 25: 384–90.

128. McEvoy SP, Plant AJ, Pearman JW et al. *Risk factors for the acquisition of vancomycin-resistant enterococci during a single-strain outbreak at a major Australian teaching hospital*. J Hosp Infect 2006; 62: 256–8.

129. Mascini EM, Jalink KP, Kamp-Hopmans TEM et al. *Acquisition and duration of vancomycin-resistant enterococcal carriage in relation to strain type*. J Clin Microbiol 2003; 41: 5377–83.

130. Mascini EM, Troelstra A, Beitsma M et al. *Genotyping and preemptive isolation to control an outbreak of vancomycin-resistant Enterococcus faecium*. Clin Infect Dis 2006; 42: 739–46.

131. Camargo ILBC, Gilmore MS, Darini ALC. *Multilocus sequence typing and analysis of putative virulence factors in vancomycin-resistant and vancomycin-sensitive Enterococcus faecium isolates from Brazil*. Clin Microbiol Infect 2006; 12: 1123–30.

132. Knoll M, Daeschlein G, Okpara-Hofmann J et al. *Outbreak of vancomycin-resistant enterococci (VRE) in a hematological oncology ward and hygienic preventive measures*. *A long-term study*. Onkologie 2005; 28: 187–92.

133. Routsi C, Platsouka E, Willems RJL et al. *Detection of enterococcal surface protein gene (esp) and amplified fragment length polymorphism typing of glycopeptide-resistant Enterococcus faecium during its emergence in a Greek intensive care unit.* J Clin Microbiol 2003; 41: 5742–6.

134. Corso AC, Gagetti PS, Rodríguez MM et al. *Molecular epidemiology of vancomycin-resistant Enterococcus faecium in Argentina*. Int J Infect Dis 2007; 11: 69–75.

135. Kawalec M, Gniadkowski M, Hryniewicz W. *Outbreak of vancomycin-resistant enterococci in a hospital in Gdask, Poland, due to horizontal transfer of different Tn1546-like transposon variants and clonal spread of several strains*. J Clin Microbiol 2000; 38: 3317–22.

136. Khan E, Sarwari A, Hasan R et al. *Emergence of vancomycin-resistant Enterococcus faecium at a tertiary care hospital in Karachi, Pakistan.* J Hosp Infect 2002; 52: 292–6.

137. van der Steen LF, Bonten MJ, van Kregten E et al. [*Vancomycin-resistant Enterococcus faecium outbreak in a nephrology ward*]. Ned Tijdschr Geneeskd 2000; 144: 2568–72.

138. Kawalec M, Gniadkowski M, Kedzierska J et al. *Selection of a teicoplanin-resistant Enterococcus faecium mutant during an outbreak caused by vancomycin-resistant enterococci with the vanB phenotype*. J Clin Microbiol 2001; 39: 4274–82.

139. Ozorowski T, Kawalec M, Zaleska M et al. *The effect of an antibiotic policy on the control of vancomycin-resistant enterococci outbreak and on the resistance patterns of bacteria isolated from the blood of patients in a hematology unit.* Pol Arch Med Wewnętrznej 2009; 119: 712–8.

140. Chang C-M, Wang L-R, Lee H-C et al. *Characterisation of vancomycin-resistant enterococci from hospitalised patients at a tertiary centre over a seven-year period*. J Hosp Infect 2010; 74: 377–84.

141. Granlund M, Carlsson C, Edebro H et al. *Nosocomial outbreak of vanB2 vancomycin-resistant Enterococcus faecium in Sweden*. J Hosp Infect 2006; 62: 254–6.

142. McCarthy KM, Van Nierop W, Duse A et al. *Control of an outbreak of vancomycin-resistant Enterococcus faecium in an oncology ward in South Africa: effective use of limited resources.* J Hosp Infect 2000; 44: 294–300.

143. Colak D, Naas T, Gunseren F et al. *First outbreak of vancomycin-resistant enterococci in a tertiary hospital in Turkey*. J Antimicrob Chemother 2002; 50: 397–401.

144. Panesso D, Ospina S, Robledo J et al. *First characterization of a cluster of VanA-type glycopeptide-resistant Enterococcus faecium, Colombia*. Emerg Infect Dis 2002; 8: 961–5.

145. Roger M, Faucher MC, Forest P et al. *Evaluation of a vanA-specific PCR assay for detection of vancomycin-resistant Enterococcus faecium during a hospital outbreak*. J Clin Microbiol 1999; 37: 3348–9.

146. Timmers GJ, van der Zwet WC, Simoons-Smit IM et al. *Outbreak of vancomycin-resistant Enterococcus faecium in a haematology unit: risk factor assessment and successful control of the epidemic*. Br J Haematol 2002; 116: 826–33.

147. Bartley PB, Schooneveldt JM, Looke DF et al. *The relationship of a clonal outbreak of Enterococcus faecium vanA to methicillin-resistant Staphylococcus aureus incidence in an Australian hospital*. J Hosp Infect 2001; 48: 43–54.

148. Zanella RC, Brandileone MCC, Bokermann S et al. *Phenotypic and genotypic characterization of VanA Enterococcus isolated during the first nosocomial outbreak in Brazil.* Microb Drug Resist 2003; 9: 283–91.

149. Titze-de-Almeida R, Van Belkum A, Felipe MSS et al. *Multilocus sequence typing of hospital-associated Enterococcus faecium from Brazil reveals their unique evolutionary history*. Microb Drug Resist 2006; 12: 121.

150. MacIntyre CR, Empson M, Boardman C et al. *Risk factors for colonization with vancomycin-resistant enterococci in a Melbourne hospital*. Infect Control Hosp Epidemiol 2001; 22: 624–9.

151. Lee HK, Lee WG, Cho SR. *Clinical and molecular biological analysis of a nosocomial outbreak of vancomycin-resistant enterococci in a neonatal intensive care unit*. Acta Paediatr 1999; 88: 651–4.

152. Kirkpatrick BD, Harrington SM, Smith D et al. *An outbreak of vancomycin-dependent Enterococcus faecium in a bone marrow transplant unit.* Clin Infect Dis 1999; 29: 1268–73.

153. Falk PS, Winnike J, Woodmansee C et al. *Outbreak of vancomycin-resistant enterococci in a burn unit*. Infect Control Hosp Epidemiol 2000; 21: 575–82.

154. Hanna H, Umphrey J, Tarrand J et al. *Management of an outbreak of vancomycin-resistant enterococci in the medical intensive care unit of a cancer center.* Infect Control Hosp Epidemiol 2001; 22: 217–9.

155. Schuster F, Graubner UB, Schmid I et al. *Vancomycin-resistant-enterococci--colonization of 24 patients on a pediatric oncology unit*. Klin Pädiatrie 210: 261–3.

156. Elsner HA, Sobottka I, Feucht HH et al. *Nosocomial outbreak of vancomycin-resistant Enterococcus faecium at a German university pediatric hospital*. Int J Hyg Environ Health 2000; 203: 147–52.

157. Donskey CJ, Schreiber JR, Jacobs MR et al. *A polyclonal outbreak of predominantly VanB vancomycin-resistant enterococci in northeast Ohio. Northeast Ohio Vancomycin-Resistant Enterococcus Surveillance Program.* Clin Infect Dis 1999; 29: 573–9.

158. Nourse C, Murphy H, Byrne C et al. *Control of a nosocomial outbreak of vancomycin resistant Enterococcus faecium in a paediatric oncology unit: risk factors for colonisation*. Eur J Pediatr 1998; 157: 20–7.

159. Rizkalla EA, Moore JE, Marshall SA et al. *Glycopeptide-resistant enterococci in Northern Ireland: first reported outbreak*. J Antimicrob Chemother 1997; 40: 607–8.

160. Hwang YS, Brinton BG, Leonard RB et al. *Investigation of an outbreak of vancomycin-resistant Enterococcus faecium in a low prevalence university hospital*. J Investig Med 1998; 46: 435–43.

161. Scagnelli M, Pellizer G, de Lalla F et al. *Epidemiological analysis of vancomycin-resistant enterococci in a large tertiary-care hospital in Northern Italy.* Eur J Clin Microbiol Infect Dis 2001; 20: 609–16.

162. Bonten MJ, Hayden MK, Nathan C et al. *Epidemiology of colonisation of patients and environment with vancomycin-resistant enterococci.* Lancet (London, England) 1996; 348: 1615–9.

163. Brown AR, Amyes SG, Paton R et al. *Epidemiology and control of vancomycin-resistant enterococci (VRE) in a renal unit*. J Hosp Infect 1998; 40: 115–24.

164. Torell E, Fredlund H, Törnquist E et al. *Intrahospital spread of vancomycin-resistant Enterococcus faecium in Sweden.* Scand J Infect Dis 1997; 29: 259–63.

165. Pegues DA, Pegues CF, Hibberd PL et al. *Emergence and dissemination of a highly vancomycin-resistant vanA strain of Enterococcus faecium at a large teaching hospital*. J Clin Microbiol 1997; 35: 1565–70.

166. Pearce CL, Evans MK, Peters SM et al. *Clonal diversity of vancomycin-resistant enterococci from an outbreak in a tertiary care university hospital*. Am J Infect Control 1998; 26: 563–8.

167. Chadwick PR, Oppenheim BA, Fox A et al. *Epidemiology of an outbreak due to glycopeptide-resistant Enterococcus faecium on a leukaemia unit*. J Hosp Infect 1996; 34: 171–82.

168. Lavery A, Rossney AS, Morrison D et al. *Incidence and detection of multi-drug-resistant enterococci in Dublin hospitals*. J Med Microbiol 1997; 46: 150–6.

169. Anglim AM, Klym B, Byers KE et al. *Effect of a vancomycin restriction policy on ordering practices during an outbreak of vancomycin-resistant Enterococcus faecium*. Arch Intern Med 1997; 157: 1132–6.

170. Wells CL, Juni BA, Cameron SB et al. *Stool carriage, clinical isolation, and mortality during an outbreak of vancomycin-resistant enterococci in hospitalized medical and/or surgical patients*. Clin Infect Dis 1995; 21: 45–50.

171. Quale J, Landman D, Atwood E et al. *Experience with a hospital-wide outbreak of vancomycin-resistant enterococci.* Am J Infect Control 1996; 24: 372–9.

172. Issack MI, Power EG, French GL. *Investigation of an outbreak of vancomycin-resistant Enterococcus faecium by random amplified polymorphic DNA (RAPD) assay*. J Hosp Infect 1996; 33: 191–200.

173. Dominguez EA, Davis JC, Langnas AN et al. *An outbreak of vancomycin-resistant Enterococcus faecium in liver transplant recipients.* Liver Transpl Surg 1997; 3: 586–90.

174. Edmond MB, Ober JF, Weinbaum DL et al. *Vancomycin-resistant Enterococcus faecium bacteremia: risk factors for infection.* Clin Infect Dis 1995; 20: 1126–33.

175. Jordens JZ, Bates J, Griffiths DT. *Faecal carriage and nosocomial spread of vancomycin-resistant Enterococcus faecium*. J Antimicrob Chemother 1994; 34: 515–28.

176. Montecalvo MA, Horowitz H, Gedris C et al. *Outbreak of vancomycin-, ampicillin-, and aminoglycoside-resistant Enterococcus faecium bacteremia in an adult oncology unit*. Antimicrob Agents Chemother 1994; 38: 1363–7.

177. Boyce JM, Opal SM, Chow JW et al. *Outbreak of multidrug-resistant Enterococcus faecium with transferable vanB class vancomycin resistance*. J Clin Microbiol 1994; 32: 1148–53.

178. Boyle JF, Soumakis SA, Rendo A et al. *Epidemiologic analysis and genotypic characterization of a nosocomial outbreak of vancomycin-resistant enterococci*. J Clin Microbiol 1993; 31: 1280–5.

179. Livornese LL, Dias S, Samel C et al. *Hospital-acquired infection with vancomycin-resistant Enterococcus faecium transmitted by electronic thermometers.* Ann Intern Med 1992; 117: 112–6.

180. Handwerger S, Raucher B, Altarac D et al. *Nosocomial outbreak due to Enterococcus faecium highly resistant to vancomycin, penicillin, and gentamicin.* Clin Infect Dis 1993; 16: 750–5.

SUPPLEMENTARY MATERIAL

Table S1. Clonal characteristics and epidemiological background of VREfm (n=71) and VSEfm (n=82) isolates included in this study.

VREfm/VSEfm isolates[a]	BAPS subgroup	ST	PFGE type[b]	No.	*van* gene	Country[c]	City / Region	Date	Source	Virulence								
										esp	*hyl*	*entA*	*bac32*	*bac43*	*entB*	*entP*	*entQ*	*entL*
P575	3.1	280	VREF-55	1	A	PER	NI	NI	urine	+	+	+	+					
H358	3.1	280	VREF-17	1	A	PRT	Porto, Viseu	2002-03	urine	+	+	+						
DB20546/OB19275	3.1	280	VREF-17	2	B	SIN	Singapore	2004	NI	+		+						
E0161	3.3a2	16	VREF-26	1	A	USA	Chicago	1995	faeces			+						
PAO2	3.3a2	16	VREF-41	1	A	AUS	Brisbane	1997-1999	faeces; urine	+	+							
E0481	3.3a2	16	VREF-8	1	A	NLD	Amsterdam	1999	NI	+		+						
E0805	3.3a2	16	VREF-9**	1	A	NLD	Utrecht	2000	faeces	+		+				+		
E1132	3.3a2	16	VREF-9**	1	A	USA	Oregon	2001	faeces	+	+	+						
E1651	3.3a2	16	VREF-9**	1	A	NLD	Amersfoort	2002	wound	+	+	+						
2664	3.3a2	16	VREF-38	1	A	ARG	NI	2000	stool	+		+				+		
E2480 (C68)	3.3a2	16	VREF-27	1	B	USA	Cleveland	1996	faeces	+	+	+						
Ef82	3.3a2	17	VREF-32*	1	A	USA	New York	1992	NI	+		+	+					
2391	3.3a2	17	VREF-32*	1	A	ARG	Buenos Aires	1997-98	stool	+		+						
232/09	3.3a2	17	VREF-56*	1	A	ESP	Granada	2009	blood	+	+	+						
E417	3.3a2	17	VREF-58	1	A	ECU	NI	NI	blood	+		+						
VREII	3.3a2	17	VREF-55	1	A	FIN	Helsinki	1996-98	NI			+						
265859	3.3a2	17	VREF-18	1	A	ESP	Madrid	2003	organic fluid	+								
S13	3.3a2	17	VREF-52	1	A	SAR	Riyadh	2000-03	NI		+		+			+		
A437	3.3a2	17	VREF-53	1	A	TUN	Sfax	2003	NI		+	+						
Vri16	3.3a2	17	VREF-34	1	A	BRA	NI	NI	NI	+	+	+	+					
Ef80	3.3a2	17	VREF-31	1	B	USA	New York	1991-92	NI	+	+	+						
E0532	3.3a2	17	VREF-46	1	B	AUS	NI	1998	urine	+		+						
361400	3.3a2	17	VREF-19	1	B	ESP	Madrid	2004	wound	+								
135487	3.3a2	17	VREF-19	1	B	ESP	Madrid	2005	blood	+		+						
604/06	3.3a2	17	VREF-45	1	B	ESP	Burgos	2006	abdominal fluid	+		+						
Ent35	3.3a2	17	VREF-45	1	B	ESP	Soria	2004-06	NI	+		+						
E1438	3.3a2	65	VREF-7	1	A	GRC	Athens	1999	blood	+	+	+						
2219/05	3.3a2	202	VREF-36	1	A	POL	Krakow	2005	wound	+		+	+					
UW6511	3.3a2	202	VREF-24	1	A	DEU	Berlin	2006	blood	+		+		+				
AE12	3.3a2	209	VREF-15	1	B	ITA	Northern Italy	2002	blood	+		+						
E0013	3.3a1	18	VREF-3	1	A	GBR	Oxford	1992	urine	+		+						
S30	3.3a1	18	VREF-NI	1	A	SAR	Riyadh	2000-03	NI		+	+						
E2373	3.3a1	18	VREF-22	1	A	SER	Belgrade	2005	coproculture		+	+	+					
H182	3.3a1	18	VREF-13	1	A	PRT	Porto, Coimbra	2002-03	blood, hepatic fluid			+						
48311	3.3a1	18	VREF-14	1	A	PRT	Coimbra	NI	NI			+				+		
VRE101	3.3a1	18	VREF-49	1	A	DNK	NI	2005-08	NI									
2227/05	3.3a1	18	VREF-43	1	A	POL	Krakow	2005	rectal swab		+	+	+					
C497	3.3a1	18	VREF-48	1	A	COL	NI	NI	blood		+	+						
E144	3.3a1	64	VREF-39	1	B	CHI	Santiago	2003	urine	+		+						
E76	3.3a1	64	VREF-40	1	B	CHI	Santiago	2004	urine	+		+						
H74	3.3a1	132	VREF-10	1	A	PRT	Porto, Coimbra	1999-2001	organic fluid, blood	+								
H311	3.3a1	132	VREF-54	1	A	PRT	Porto	2002	urine	+		+						
VRE100	3.3a1	173	VREF-42	1	B	AUS	Perth	2001	NI		+	+	+					
2348	2.1a	78	VREF-37	1	A	ARG	NI	1998	stool	+		+			+			
AE01	2.1a	78	VREF-11	1	A	ITA	Several cities	2001-03	blood	+		+						
E1644	2.1a	78	VREF-11	1	A	DEU	Freiburg	2002	catheter	+		+						
C853	2.1a	78	VREF-11	1	A	ESP	Barcelona	2006	NI	+		+						
P1	2.1a	78	VREF-51	1	A	PRG	Asunción	2005	NI	+		+			+			
E2365	2.1a	80	VREF-20	1	B	HUN	Budapest	2004	blood	+	+	+						
UW1806	2.1a	117	VREF-5	1	A	DEU	Berlin	1998	tracheal secretion	+				+				
VnR16	2.1a	117	VREF-28	1	A	ESP	Madrid	2008	Wound		+	+		+				
UW5905	2.1a	192	VREF-16	1	A	DEU	Freiburg	2004	blood	+	+	+						
Lin1	2.1a	203	VREF-30	1	A	USA	Alabama	2004-05	NI	+		+						
UW6337	2.1a	203	VREF-23	1	A	DEU	Tübingen	2005	tracheal secretion	+		+		+				
E422	2.1a	203	VREF-57	1	A	ECU	NI	NI	urine	+		+						
VRE2	2.1a	203	VREF-48	1	B	DNK	NI	2005-08	NI	+								
S1	2.1a	358	VREF-44	1	A	SAR	Riyadh	2000-03	NI			+						
Efm Canada	2.1a	412	VREF-50	1	A	CAN	Ontario	2005	NI	+	+	+		+				
V689	2.1a	412	VREF-50	1	A	VEN	NI	NI	wound	+		+						
P1123	2.1a	412	VREF-56	1	A	PER	NI	NI	blood	+		+						
P118	2.1a	438	VREF-51	1	A	PRG	Asunción	2006	NI	+		+			+			

VREfm/VSEfm isolates[a]	BAPS subgroup	ST	PFGE type[b]	No.	*van* gene	Country[c]	City / Region	Date	Source	Virulence								
										esp	*hyl*	*entA*	*bac32*	*bac43*	*entB*	*entP*	*entQ*	*entL*
H305	2.1b	5	VREF-12	1	A	PRT	Porto	2002	wound									
Vri22	2.1b	50	VREF-35	1	A	BRA	NI	NI	NI			+			+			
BM4147 (E0005)	2.3a	25	VREF-1	1	A	FRA	NI	1986	faeces									
BM4165	2.3a	25	VREF-2	1	A	FRA	Nancy	1987	faeces									
E1686	2.3a	114	VREF-33	1	A	BRA	S. Paulo	1998	faeces	+			+					
71689	2.3b	265	VREF-4	1	B	ESP	Madrid	1998	urine			+				+		
VREI	5	484	VREF-47	1	B	FIN	Helsinki	1996-98	NI									
E0292	7	20	VREF-25	1	A	USA	Detroit	1992-94	urine	+		+						
Texas	7	182	VREF-29	1	B	USA	Texas	1992	NI	+		+	+					
EFM4s	3.1	22	ASEF-2	1	-	ESP	Madrid	1995	blood			+			+			
EFM28s	3.1	22	ASEF-22	1	-	ESP	Madrid	1999	blood			+			+	+		
EFM22s	3.1	32	ASEF-5	1	-	ESP	Madrid	1995	blood			+			+			
EFM48	3.1	125	AREF-Q	1	-	ESP	Madrid	2000	blood			+	+		+			
EFM11s	3.1	214	ASEF-36	1	-	ESP	Madrid	2006	blood			+			+	+		
EFM12s	3.1	214	ASEF-37	1	-	ESP	Madrid	2006	blood			+			+			
EFM20s	3.1	533	ASEF-41	1	-	ESP	Madrid	2006	blood			+			+	+		
EFM5s	3.2	29	ASEF-34	1	-	ESP	Madrid	2007	blood						+			
EFM1s	3.2	97	ASEF-31	1	-	ESP	Madrid	2001	blood									
EFM39-42	3.3a2	16	AREF-T**	4	-	ESP	Madrid	2001-04	blood	+	+	+			1			
E1718	3.3a2	17	AREF-1	1	-	DNK	Aarhus	1999-2000	NI		+							
E1340	3.3a2	17	AREF-6	1	-	NOR	Bergen	1999	wound			+						
EFM34	3.3a2	17	AREF-P	1	-	ESP	Madrid	1995	blood	+		+						
EFM35	3.3a2	17	AREF-B	1	-	ESP	Madrid	1996	blood	+		+						
EFM38	3.3a2	17	AREF-8	1	-	ESP	Madrid	2008	blood	+	+	+						
EFM44	3.3a2	63	AREF-W.2	1	-	ESP	Madrid	2000	blood	+		+			+			
EFM46	3.3a2	103	AREF-W	1	-	ESP	Madrid	2001	blood	+		+				+		
EFM47	3.3a2	103	AREF-W.3	1	-	ESP	Madrid	2001	blood	+	+	+			+			
EFM1	3.3a1	18	AREF-A	1	-	ESP	Madrid	1995	blood			+			+			
EFM2	3.3a1	18	AREF-C	1	-	ESP	Madrid	1996	blood			+			+			
EFM3-29	3.3a1	18	AREF-D	13	-	ESP	Madrid	1997-2005	blood	8	1	+	1					
EFM23	3.3a1	18	AREF-D.1	1	-	ESP	Madrid	2002	blood	+	+	+						
EFM8	3.3a1	18	AREF-E	1	-	ESP	Madrid	1997	blood		+	+						
EFM13	3.3a1	18	AREF-G	1	-	ESP	Madrid	1998	blood			+			+			
EFM14	3.3a1	18	AREF-Z	1	-	ESP	Madrid	1998	blood			+			+			
EFM15	3.3a1	18	AREF-J	1	-	ESP	Madrid	1999	blood			+						
EFM17,19	3.3a1	18	AREF-N	2	-	ESP	Madrid	2000	blood	1		+						
EFM18	3.3a1	18	AREF-M	1	-	ESP	Madrid	2000	blood			+			+			
EFM20	3.3a1	18	AREF-Y	1	-	ESP	Madrid	2001	blood		+	+				+		+
EFM24	3.3a1	18	AREF-U	1	-	ESP	Madrid	2002	blood			+						
EFM25	3.3a1	18	AREF-1	1	-	ESP	Madrid	2003	blood			+						
EFM26,28	3.3a1	18	AREF-2	2	-	ESP	Madrid	2004	blood	1	1	+						
EFM27	3.3a1	18	AREF-3	1	-	ESP	Madrid	2004	blood		+	+						
EFM30	3.3a1	18	AREF-4	1	-	ESP	Madrid	2006	blood	+	+	+						
EFM31	3.3a1	18	AREF-5	1	-	ESP	Madrid	2006	blood	+	+	+						
EFM32	3.3a1	18	AREF-6	1	-	ESP	Madrid	2007	blood		+							
EFM33	3.3a1	18	AREF-7	1	-	ESP	Madrid	2008	blood			+						
EFM2s	3.3a1	18	ASEF-9	1	-	ESP	Madrid	1996	blood			+						
EFM25s	3.3a1	18	ASEF-12	1	-	ESP	Madrid	1997	blood			+			+			
EFM3s	3.3a1	18	ASEF-24	1	-	ESP	Madrid	2000	blood									
EFM32s	3.3b	102	ASEF-29	1	-	ESP	Madrid	2001	blood			+			+	+		
EFM45	3.3b	102	AREF-S	1	-	ESP	Madrid	2001	blood			+			+	+		
EFM27s	3.3b	888	ASEF-20	1	-	ESP	Madrid	1999	blood			+	+		+	+		+
EFM49	2.1a	203	AREF-9	1	-	ESP	Madrid	2006	blood	+	+	+						
EFM50	2.1a	203	AREF-9.3	1	-	ESP	Madrid	2006	blood			+						
EFM51	2.1a	203	AREF-9.1	1	-	ESP	Madrid	2007	blood	+		+						
EFM52	2.1a	266	AREF-10	1	-	ESP	Madrid	2004	blood			+			+			
EFM17s	2.1a	442	ASEF-39	1	-	ESP	Madrid	2005	blood			+			+			
EFM33s	2.1b	46	ASEF-30	1	-	ESP	Madrid	2001	blood						+	+		
EFM7s	2.1b	69	ASEF-35	1	-	ESP	Madrid	2003	blood			+			+			
EFM8s	2.1b	69	ASEF-44	1	-	ESP	Madrid	2005	blood			+			+	+		
EFM26s	2.3a	21	ASEF-13	1	-	ESP	Madrid	1997	blood			+			+			
EFM30s	2.3a	25	ASEF-26	1	-	ESP	Madrid	2000	blood						+			
EFM21s	2.3a	71	ASEF-3	1	-	ESP	Madrid	1995	blood			+			+			
EFM13s	2.3a	420	ASEF-38	1	-	ESP	Madrid	2007	blood			+			+			

VREfm/VSEfm isolates[a]	BAPS subgroup	ST	PFGE type[b]	No.	*van* gene	Country[c]	City / Region	Date	Source	Virulence								
										esp	*hyl*	*entA*	*bac32*	*bac43*	*entB*	*entP*	*entQ*	*entL*
EFM29s	2.3b	247	ASEF-25	1	-	ESP	Madrid	2000	blood			+			+			
EFM15s	1.1	474	ASEF-42	1	-	ESP	Madrid	2004	blood			+			+	+		
EFM31s	1.2	74	ASEF-28	1	-	ESP	Madrid	2001	blood						+	+		
EFM9s	1.2	85	ASEF-17	1	-	ESP	Madrid	1997	blood						+	+		
EFM6s	1.2	96	ASEF-32	1	-	ESP	Madrid	2002	blood						+	+		+
EFM10s	1.2	178	ASEF-18	1	-	ESP	Madrid	1997	blood			+	+		+	+	+	
EFM14s	1.2	178	ASEF-19	1	-	ESP	Madrid	1999	blood						+	+	+	
EFM24s	1.2	674	ASEF-15	1	-	ESP	Madrid	1997	blood						+			
EFM54	6	419	AREF-11	1	-	ESP	Madrid	2005	blood	+	+	+						
EFM34s	7	675	ASEF-40	1	-	ESP	Madrid	2007	blood						+	+		

Abbreviations: BAPS, Bayesian analysis of Population Structure; CC, clonal complex; ST, sequence type; VSEF, vancomycin-susceptible *Enterococcus faecium*; AREF, ampicillin-resistant *Enterococcus faecium*; ASEF, ampicillin-susceptible *Enterococcus faecium*; NI, not identified. [a]The original references describing the isolates included in this study can be found in Table 1. [b]PFGE types were arbitrarily designated by main phenotype (VREF, ASEF or AREF) followed by a number or capital letter. *The PFGE patterns VREF-32 and VREF-56 share common bands (≤ 8 bands difference). **VREF-9 and AREF-T are the same PFGE pattern (≤ 6 bands difference). [c]ARG, Argentina; AUS, Australia; BRA, Brazil; CAN, Canada; CHI, Chile; COL, Colombia; DEU, Germany; DNK, Denmark; ECU, Ecuador; ESP, Spain; FIN, Finland; FRA, France; GBR, Great Britain; GRC, Greece; HUN, Hungary; ITA, Italy; NLD, The Netherlands; NOR, Norway ; PER, Peru; POL, Poland; PRG, Paraguay; PRT, Portugal; SAR, Saudi Arabia; SER, Serbia; SIN, Singapore; TUN, Tunisia; USA, United States of America; VEN, Venezuela. [d]Positive virulence (*esp, hyl*) and bacteriocin (*entA, entB, entP, entQ, entL, bac32, bac43*) genes appear with a (+).

Table S2. Oligonucleotides and PCR conditions for the search of virulence, bacteriocin, replicase and toxin-antitoxin genes.

No	Primer	Oligonucleotide sequence (5'to 3')	GenBank accession number (gene)	Description (examples)	Species	Position of amplicon (FW/RV)	Amplicon size	PCR conditions	Reference
			AB374546	Aggregation substance (*prgB*) in pMG2200	*Enterococcus faecalis*	96511-96531 / 96888-96868		MultiplexPCR (*asa, esp, hyl*):	
1	asa-1	GCA CGC TAT TAC GAA CTA TGA	X62656	Aggregation substance (*asp1*) in pPD1	*E. faecalis*	2636-2656 / 3013-2993	378 bp		127
2	asa-2	TAA GAA AGA ACA TCA CCA CGA	X17214	*asa1* gene product in pAD1	*E. faecalis DS16*	3205-3225 / 3582-3562		1cycle of 10 min at 94ºC; 30 cycles of 1min at 94ºC,	
			AE016833	Aggregation substance (*asa1*) in pTEF1	*E. faecalis V583*	36201-36221/ 36578-36558		1min at 56ºC, 1min at 72ºC; 1cycle of 10 min at 72ºC	
3	hyl-1	ACA GAA GAG CTG CAG GAA ATG	AF544400	Putative hyaluronidase (hyl) in *E. faecium* CV133	*Enterococcus faecium*	1026-1046 / 1301-1281	276 bp		127
4	hyl-2	GAC TGA CGT CCA AGT TTC CAA							
5	esp-1	AGA TTT CAT CTT TGA TTC TTG G	AF454824	Enterococcal surface protein (*esp*) in *E. faecalis* PAI	*E. faecalis*	56102-56123 / 56612-56592	511bp		127
6	esp-2	AAT TGA TTC TTT AGC ATC TGG	AF443999	Surface protein Esp variant (*esp*) in *E. faecium* E0300	*E. faecium*	92-113 / 602-582			
7	acm-F2	CAG GCA GAG ATA TCA GCA G	AY135217	Collagen adhesin precursor in *E. faecium* TX2555	*E. faecium*	489-507	1496 bp	1cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC,	128
8	acm-R1	ATT CTC ATT TGT AAC GAC TAG C				1984-1963		30 s at 56ºC, 1min at 72ºC; 1cycle of 10 min at 72ºC	
9	EntA-F	GTA CCA CTC ATA GTG GA	X94181	Enterocin A in *E. faecium* CTC492	*E. faecium*	160-176	451 bp	1cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC,	129
10	EntA-R	GAG ATT TAT CTC CAT AAT CT		Enterocin A immunity in *E. faecium* CTC492		610-591		30 s at 50ºC, 30 s at 72ºC; 1cycle of 10 min at 72ºC	
11	EntB-F	GAA AAT GAT CAC AGA ATG CCT A	EFU87997	Enterocin B in *E. faecium* T136	*E. faecium*	146-167	159 bp	1cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC,	129
12	EntB-R	GTT GCA TTT AGA GTA TAC ATT TG				304-282		30 s at 56ºC, 30 s at 72ºC; 1cycle of 10 min at 72ºC	
13	EntP-F	ATG AGA AAA AAA TTA TTT AGT TT	AF005726	Enterocin P precursor in *E. faecium* P13	*E. faecium*	173-195	216 bp	1cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC,	129
14	EntP-R	TTA ATG TCC CAT ACC TGC CAA ACC				388-365		30 s at 50ºC, 30 s at 72ºC; 1cycle of 10 min at 72ºC	
15	EntQ-F	AGG GCT ACT TGG ATA GTA CAC	NC_008259	Enterocin Q in pCIZ2	*E. faecium*	45-66	678 bp	1cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC,	130
16	EntQ-R	AAC AAG AAA ATT GCG GCT G		Enterocin EntQ ABC-transporter in pCIZ2		722-704		30 s at 52ºC, 30 s at 72ºC; 1cycle of 10 min at 72ºC	
17	EntL50A/B-F	TCC CTA CAG TCT CCC TTCC	AJ223633	Enterocin L50A and L50B in pCIZ1	*E. faecium*	877-895 / 1753-1735	877 bp	1cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC,	130
18	EntL50A/B-R	TCT AGC GTT AAG CCG AAT G	QI98088	Enterocin I(*entI*) and Enterocin J (*entJ*) in pEF1	*E. faecium 6T1a*	1331-1349 / 2206-2188		30 s at 52ºC, 30 s at 72ºC; 1cycle of 10 min at 72ºC	
19	Bac32-F	AAT GGT GGA GTG GTT GAA GC	AB205024	Bacteriocin and immunity proteins in p200B	*E. faecium*	162-181	462 bp	1cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC,	This study
20	Bac32-R	GAG TGA TTA TTT TCG CCC GT				623-604		30 s at 56ºC, 30 s at 72ºC; 1cycle of 10 min at 72ºC	
21	Bac43-F	CGA AAA GG AAA AAC AAT CAT G	AB178871	*bacA* and *bacB* in pB82	*E. faecium*	104-124	576 bp	1cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC,	131
22	Bac43-R	TCC CAT TTT CAT TTT ATT CC				679-660		30 s at 50ºC, 30 s at 72ºC; 1cycle of 10 min at 72ºC	
			X17655	Unnamed protein product; repR gene product (AA 1-496) in pIP501	*Streptococcus agalactiae*	695-714 / 1318-1299			
			U83488	RepR in pGB354	*S.agalactiae*	79-98 / 702-698			
			AF007787	RepE (replication protein) in pAMβ1	*E. faecalis*	3188-3207 / 3811-3792			
			X92945	Putative RepS protein in pRE25 (orf6)	*E. faecalis RE25*	4740-4759 / 5363-5344			
23	rep1-F	TCG CTC AAT CAC TAC CAA GC	AE016833	RepE protein in pTEF1	*E. faecalis V583*	45996-46015 / 46619-46600		1cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC,	132
24	rep1-R	CTT GAA CGA GTA AAG CC CTT	AM932524	Putative replication protein (repE) in pIP816; putative, identical to pAMβ1oriR	*E. faecium BM4147*	17694-17713 / 18317-18298	624 bp	30 s at 52ºC, 30 s at 72ºC; 1cycle of 10 min at 72ºC	
			AY357120	RepS (initiation of plasmid replication) in pSM19035	*S. pyogenes*	712-731/ 1335-1316			
			AY357120	RepS, "copy 2", (initiation of plasmid replication) in pSM19035	*S. pyogenes*	16116-16135 / 16739-16720			
			X66468	RepS in pMD101	*S. pyogenes*	712-731/ 1335-1316			
			X64695	repS in pBT233	*S. pyogenes*	577-596 / 1200-1181			
			EF450709	RepS in S.aureus	*Staphylococcus aureus CM05*	312-331/ 935-916			
			AB290882	Putative repE protein in pKL0018	*Lactococcus garvieae*	75-94 / 698-679			
			DQ198088	RepR in pEF1;putative Rep protein of pRE25	*E. faecium 6T1a*	16626-16645 / 17255-17230			
			X92945	Putative Rep protein in pRE25 (orf1)	*E. faecalis RE25*	363-382 / 992-967			
25	rep2-F	GAG AAC CAT CAA GGC GAA AT	AM932524	Putative replication protein (repE) in pIP816	*E. faecium BM4147*	29062-29081 / 29691-29666	630 bp	1cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC,	132
26	rep2-R	ACC AGA ATA AGC ACT ACG TAC AAT CT	AM296544	Replication protein (repR) in pVEF1	*E. faecium 399/F99/H8*	23818-23837 / 24447-24422		30 s at 52ºC, 30 s at 72ºC; 1cycle of 10 min at 72ºC	
			AM410096	Putative replication protein (repR) in pVEF2	*E. faecium 399/F99/A9*	23906-23925 / 24535-24510			
			AM931300	Putative replication protein (repR) in pVEF3	*E. faecium 399/S99/A7*	30095-30114 / 30724-30699			
			NC_010599	Replication protein Rep63A in pAW63; similar to *B. anthracis* plasmid pXO2 RepS	*Bacillus thuringiensis*	42922-42941/ 43325-43298			
27	rep3-F	ACA TTT TCC TCA AAG AAC AT	DQ242517	Replication protein Rep165 in pBMB165	*B. thuringiensis*	15751-15770 / 16154-16128	404 bp	1cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC,	132
28	rep3-R	CCT AAT GTA TAT AAT TTT GGT ACA TAT	CP000047	Replication protein in pBT9727	*B. thuringiensis*	44281-44300 / 44684-44658		30 s at 49ºC, 30 s at 72ºC; 1cycle of 10 min at 72ºC	
			NC_002146	Hypothetical protein in pX02, similar to *Bacillus thuringiensis* RepS protein	*Bacillus anthracis*	33445-33464 / 33848-33822			
29	rep4-F	ACT ATG TCG TTG AGT CTA ATG ACT	U26268	Putative replication protein RepB in pMBB1	*E. faecium*	1121-1144 / 1544-1516	424 bp	1cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC,	132
30	rep4-R	AGC AAG ATA GAA TAT TTA CTT TTA AGT TT						30 s at 56ºC, 30 s at 72ºC; 1cycle of 10 min at 72ºC	
			BX571858	Replication protein in pSAS	*S. aureus*	17667-17689 / 18303-18281			
31	rep5-F	ATG TGT AAT AAA TTA AAA GAG CA	AP004832	Replication protein (Rep) in pMW2	*S. aureus*	2218-2240 / 2854-2832	637 bp	1cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC,	132
32	rep5-R	ATT GTC TTG ATT TAT CTA TCT TG	AP003139	Replication protein (Rep) in pN315	*S. aureus*	21660-21682 / 22296-22274		30 s at 52ºC, 30 s at 72ºC; 1cycle of 10 min at 72ºC	
			AB304512	Replication protein (Rep) in pTZ2162	*S. aureus*	2617-2639 / 3253-3231			
			AJ223161	Rep protein in pS86	*E. faecalis S-86*	607-629 / 1157-1134			
33	rep6-F	ACG AAT GAA AGA TAA AGG AGT AG	AF503772	RepE replication protein in pAM α1	*E. faecalis*	4533-4556 / 5083-5061	551 bp	1cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC,	132
34	rep6-R	TAA ATT CTA GTT TGG CAA TCT TAT	AF109375	RepA in p703/5	*E. faecalis KBL703*	812-834 / 1362-1339		30 s at 52ºC, 30 s at 72ºC; 1cycle of 10 min at 72ºC	
			AY842500	Replication protein (repE) in pEF47	*E. faecalis 47/3*	27-49 / 577-554			

Table S2. Oligonucleotides and PCR conditions for the search of virulence, bacteriocin, replicase and toxin-antitoxin genes (Cont).

No	Primer	Oligonucleotide sequence (5' to 3')	GenBank accession number (gene)	Description (examples)	Species	Position of amplicon (FW/RV)	Amplicon size	PCR conditions	Reference
			AY842501	Replication protein (repE) in pLRC255	*Streptococcus gallolyticus LRC0255*	27-49 / 577-554			
			NC_003393	Hypothetical protein, repC protein in pT181	*S. aureus*	162-181 / 388-369			
			NC_007791	Replication initiation protein in pUSA02	*S. aureus*	3681-3700 / 3907-3888			
			CP000257	Replication initiation protein in pUSA03	*S. aureus*	3681-3700 / 3907-3888			
			X06627	ORF (repE) in pSI94	*S. aureus*	3683-3702 / 3909-3890			
			AB369999	Replication protein in pTZ4	*S. aureus*	1505-1524 / 1731-1712			
			AJ888003	Plasmid replication protein (repC) in pSTE2	*S. aureus*	2061-2080 / 2287-2268			
			NC_002129	Pot. replication protein (CDS2) in pC221	*S. aureus*	1505-1524 / 1731-1712			
			J03323	REP N protein (rep N) in pCW7	*S. aureus*	511-530 / 737-718			
			EU36562#22	RepC in pKH16 and pKH17	*S. aureus*	3968(9)-3987(8) / 4194(5)-4175(6)			
			U36910	Replication protein in pJ3358	*S. aureus*	1138-1157 / 1364-1345			
35	rep(7)-F	AGA CGT AAT ATG CGT RTT GA	U38656	pKH1	*S. aureus*	4081-4100 / 4307-4288	227 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC, 30 s at 49ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	[132]
36	rep(7)-R	CCA AAA TAY TTY GTT TCT GG	U38428	Replication protein (repC) in pKH6	*S. aureus*	3966-3985 / 4192-4173			
			U38429	Replication protein (rep) in pKH7	*S. aureus*	387-406 / 613-594			
			M21929	Replication protein in pUB112	*S. aureus*	501-520 / 727-708			
			M90090	Replication initiator protein (rep) in pSBK203	*S. aureus*	313-332 / 539-520			
			M16217	RepC in pNS1	*S. aureus*	3390-3409 / 3616-3597			
			AE015930	RepC protein polypeptide A in pSE12228-01	*Staphylococcus epidermidis*	3681-3700 / 3907-3888			
			M16902	Rep protein in pTZ12	*Bacillus subtilis*	431-450 / 657-638			
			X92946	repD in pK214	*Lactococcus lactis*	19322-19341 / 19548-19529			
			X68412	Replication initiator protein (rep) in pWDB100	*Listeria monocytogenes*	561-580 / 787-768			
			AY939911	Replication initiator protein (repD) in plasmid	*Enterococcus casseliflavus HZ95*	7350-7369 / 7576-7557			
			X92945	Putative Rep protein in pRE25 (orf11)	*E. faecalis*	10118-10137 / 10344-10325			
			U83488	Putative replication initiation protein (repB) in pGB354	*S. agalactiae*	5460-5479 / 5686-5667			
37	rep(8)-F	TAG ATA CGA CAA AAG AAG AAT TAC A	AE002565	Replication-associated protein (EP0002) in pAM373	*E. faecalis*	1743-1764 / 2137-2113	395 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC, 30 s at 52ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	[132]
38	rep(8)-R	CCA ATC ATG TAA TGT TAC AAC C	AJ490170	Replication-associated protein A (repA) in pEJ97-1	*E. faecalis*	7448-7472 / 7842-7821			
			D78016	REP A in pPD1; ORF2; pheromone related transfer gene	*E. faecalis*	1618-1637 / 1818-1797			
			AY855841	PrgW ("replication") in pCF10	*E. faecalis*	3664-3683 / 3864-3843			
39	rep(9)-F	GCT CGA TCA RTT TTC AGA AG	L01794	Replication-associated protein (repA) in pAD1	*E. faecalis*	333-354 / 533-514	201bp	1 cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC, 30 s at 52ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	[132]
40	rep(9)-R	CGC AAA CAT TTG TCW ATT TCT T	AE016833	Replication-associated protein RepA (repA-1) in pTEF1	*E. faecalis V583*	733-752 / 933-912			
			AE016831	Replication-associated protein RepA (repA-2) in pTEF2	*E. faecalis V583*	735-754 / 935-914			
			AB374546	Plasmid replication protein (repA) in pMG2200	*E. faecalis*	83638-83657 / 83838-83817			
			M13761	Replication protein in pIM13	*Bacillus subtilis*	413-432 / 795-776			
			M17990	RepL in pE5	*S. aureus*	413-432 / 795-776			
41	rep(10)-F	TAT AAA GGC TCT CAG AGG CT	CP000258	Replication and maintenance protein in pUSA03	*S. aureus*	6705-6724 / 7087-7068	383 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC, 30 s at 54ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	[132]
42	rep(10)-R	CCA AAT TCG AGT AAG AGG TA	DQ088624	Replication protein (RepL) in pWBG738	*S. aureus*	7190 / 453-434			
			EU350089/90	Rep protein in pKH19/20	*S. aureus*	413-432 / 795-776			
			AP006718	Replication and maintenance protein in pSHaeB	*Staphylococcus haemolyticus*	1525-1544 / 1907-1888			
			U82607	Plasmid replication protein in pPV141	*Staphylococcus chromogenes*	1607-1626 / 1989-1970			
43	rep(10b)-F	TAA ATA AAG ACT CAG GAG AAG TA	NC_001994	Replication protein in pSK3	*S. aureus*	922-944 / 1122-1103	201bp	1 cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC, 30 s at 54ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	[132]
44	rep(10b)-R	TAG CAA GTT CTC GAA CTG TT	U96610	Replication protein in pSK6	*S. aureus*	816-838 / 1016-997			
			X55798	ORF 154 in pOX2000	*S. aureus*	633-655 / 833-814			
45	rep(12)-F	GAG CCT ATA ACA GAG TAC ACA	DQ363750	Replication protein ori43 in pBMB67	*B. thuringiensis*	21249-21269 / 21819-21799	571bp	1 cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC, 30 s at 52ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	[132]
46	rep(12)-R	CAA ATA TAG GCT TTG TAG TTC	AY278324	Replication protein in *B. thurigiensis*	*B. thuringiensis*	1506-1076 / 1626-1606			
			M60513	Replication protein in *B. thurigiensis* HD263	*B. thuringiensis*	853-873 / 1423-1403			
			NC_002013	Hypothetical protein in pC194	*S. aureus*	155-176 / 557-538	403 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC, 30 s at 46ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	[132]
47	rep(13)-1	ATG ATG CAA TAT ATT AAG CA	M37889	Replication protein in pSK89	*S. aureus*	513-534 / 915-896			
48	rep(13)-2	TAC CAG AAT AYT TAG CCA TTT C	AP008935	Putative replication initiation protein in pSSP1	*Staphylococcus saprophyticus*	34138-34159 / 34540-34521			
			EF537646	Replication protein in pWBG1773	*S. aureus*	970-989 / 1372-1351			
49	rep(14a)-F	GAT ATT GCT TGC GAT ATT	AB038522	Replication protein in pEFNP1	*E. faecium*	661-678 / 1128-1110	468 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC, 30 s at 50ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	This study
50	rep(14a)-R	CTC TCG RAA ATG ATT CGT C	EU370688	Replication initiation protein (*repA*) in pJS42	*E. faecium*	25-42 / 492-474			
			EU327398	Replication initiation protein (*rep*) in pRI1	*E. faecium*	3248-3266 / 3715-3698			
51	rep(15)-1	CAG TAG AAG AAA ATT ATA AAG AAC	AE017171	Replication initiation protein in pLW043	*S. aureus*	120-143 / 447-427	328 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30s at 94ºC, 30 s at 52ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	[132]
52	rep(15)-2	GTT ATG GCT GGT TTT AAT AAA	AF051917	Putative replication initiation protein Rep in pSK41	*S. aureus*	13224-13247 / 13551-13531			
			NC_007792	Replication initiation protein in pUSA03	*S. aureus*	120-143 / 447-427			

Table S2. Oligonucleotides and PCR conditions for the search of virulence, bacteriocin, replicase and toxin-antitoxin genes (Cont).

No	Primer	Oligonucleotide sequence (5'to 3')	GenBank accession number (gene)	Description (examples)	Species	Position of amplicon (FW/RV)	Amplicon size	PCR conditions	Reference
			BX571858	Replication-associated protein in pSAS, similar to *S. aureus* plasmid pSK1	*S. aureus*	4737-4761/ 5328-5309			
53	rep(16)-1	CAG GAA AAC ACT TCG TTT AT	AB304512	Replication associated protein in pTZ2162	*S. aureus*	148-167 / 739-715	592 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30 s at 94ºC,	[132]
54	rep(16)-2	CTT CTA TAT CAC TAT CAT TGT CAT T	CP000737	Protein of unknown function DUF536 in pSJH101	*S. aureus JH1*	6071-6090 / 6662-6630		30 s at 52ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	
			CP000704	Protein of unknown function DUF536 in pSJH901	*S. aureus JH9*	20880-20899 / 21471-21447			
55	rep(17)-F	TAC TAA CTG TTG GTA ATT CGT TAA AT	AF507977	Putative RepA in pRUM; similar to regulatory protein PrgW from pCF10	*E. faecium U37*	20562-20587 / 21063-21043	502 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30 s at 94ºC,	[132]
56	rep(17)-R	ATC AAG GAC TCA ACC GTA ATT	GQ900487	Plasmid SAP_083B	*E. faecium*	42250-42275 / 42751-42731		30 s at 52ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	
			EU376117	Putative RepA in plasmid = "pRUM"	*E. faecium VRE3*	1470-1495 / 1971-1951			
57	rep(18a)-F	ACA CCA GTC GAA ATG AAT TT	AF408195	Putative replication protein (repA) in pEF418	*E. faecalis 418*	258-277	462 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30 s at 94ºC,	[132]
58	rep(18a)-R	AGG AAT ATC AAG TAA TTC ATG AAA GT				719-694		30 s at 52ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	
			AF164559	Replication protein (*repA*) in pEF1071	*E. faecalis BFE 1071*	7933-7952 / 8353-8332			
59	rep(18b)-F	GTG TCT AGA ATG CGT GAA AAA GG	AB178871	Replication protein (*repA*) in pB82	*E. faecium*	4250-4272 / 4670-4649	421bp	1 cycle of 10 min at 94ºC; 35 cycles of 30 s at 94ºC,	This study
60	rep(18b)-R	CTA ATA ATT CAA GAA AGT CTT C	EU370687	Replication initiation protein (*repA*) in pJS33	*E. faecium JH95*	1-19 / 421-400		30 s at 54ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	
			AY063485	*repA* protein in plasmid encoded-bacteriocin locus	*E. faecalis FAIR-E 309*	2786-2805 / 3206-3185			
61	rep(18c)-F	TGT TCT AAA ATG AAA AGA AAA GG	DQ832184	Putative plasmid replication protein in pCIZ2	*E. faecium L50*	5783-5805 / 6209-6188	427 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30 s at 94ºC,	This study
62	rep(18c)-R	CTA ATA GAG CTC TAA AAT CTT C						30 s at 56ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	
			NC_009130	Hypothetical protein in pLEW6932, similar to *S. saprophyticus* pSSP2 rep protein	*Staphylococcus spp.*	40295-40316 / 40847-40829	552 bp		
63	rep(19)-1	GWG ATC GCT TAR AYT TAT CTA T	NC_007931	Putative replication initiation protein Rep in pSAB79	*S. aureus*	131-152 / 671-653	540 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30 s at 94ºC,	[132]
64	rep(19)-2	YMT TGT TST GGM AAT TCT T	NC_005127	Hypothetical protein (repA) in pUB101 replication initiation	*S. aureus*	3639-3657 / 4194-4173	555 bp	30 s at 46ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	
			CP000737	Replication initiator A domain protein in pSJH101	*S. aureus JH1*	4881-4900 / 5422-5401	541bp		
			CP000704	Replication initiator A domain protein in pSJH901	*S. aureus JH9*	19690-19709 / 20231-20210	541bp		
65	rep(20)-F	AGTCGAAAATATCCAGAACCTG	HM565183	Replication-associated protein RepA in pLG1 from 64/3xUW2774	*E. faecium*	40776-40797	135 bp	1 cycle of 10 min at 94ºC; 25 cycles of 30 s at 94ºC,	Werner G,
66	rep(20)-R	AACCAGTCTTTCGTAATAGTGC				40910-40889		30 s at 60ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	personal communication
67	rep(21)-F	TCG TTT CAC TCA TTG GAC ACC	AM296544	Plasmid replication protein in pVEF1	*E. faecium*	207-227 / 406-388	200 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30 s at 94ºC,	This study
68	rep(21)-R	TCA TTG TCG GCA TAA AGC C	AM410096	Plasmid replication protein in pVEF2	*E. faecium*	207-227 / 406-388		30 s at 52ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	
69	rep(22)-F	GTA TTA ACA CAC TGG ACT C	AB183714	Replication protein in pHTβ	*E. faecium*	49977-49995 / 50175-50157	199 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30 s at 94ºC,	This study
70	rep(22)-R	TCA GTG TAG GCA ATA ACC C	AB206333	Rep protein in pMG1	*E. faecium*	77133-77311/ 77911-77893		30 s at 52ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	
71	Fst-F	AGC AGC TTA CTT TCG GCT ATC GTC T	L01794	partoxin and antidote in pAD1	*E. faecalis*	4174-4198	146 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30 s at 94ºC,	Weaver K,
72	Fst-R	TAA TGC GGC AGC TCG CCT CGA TT				4319-4297		30 s at 70ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	personal communication
73	ε-F	TTA ACG AAT TAT CGG CAA GC	AY357120	Antidote of epsilon-zeta PSK system in pSM19035	*S. pyogenes*	6784-6803	1063 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30 s at 94ºC,	[133]
74	ζ-R	GTG GTT TAG GTG GCT GCA AG		Toxin of epsilon-zeta PSK system in pSM19035		7846-7827		30 s at 52ºC, 1 min at 72ºC; 1 cycle of 10 min at 72ºC	
75	ω-F	GGG AAA TTT AGG CGC ACA	AY357120	Transcriptional repressor (omega) in pSM19035	*S. pyogenes*	6520-6537	150 bp	1 cycle of 10 min at 94ºC; 25 cycles of 30 s at 94ºC,	This study
76	ω-R	AAT GGC GGA AAC GTA AAA GA				6669-6650		30 s at 52ºC, 1 min at 72ºC; 1 cycle of 10 min at 72ºC	
77	Axe-Txe-F	CTG ACC CTT TCC TTA CTT CCG	NC_005000	Toxin and antitoxin of Axe-Txe in pRUM	*E. faecium*	4937-4957	557 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30 s at 94ºC,	[134]
78	Axe-Txe-R	GGG TGA AAG GAA TGG AAG CAG				5493-5473		30 s at 52ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	
79	mazF-F	CAA CTG TTC CTT TCT TCG TTG CTCC	NC_000913	Toxin of the ChpA-ChpR TA system in *E. coli* K-12	*Escherichia coli*	2908831-2908855	444 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30 s at 94ºC,	[134]
80	mazE-R	GAT GAT GAA GTG AAG ATT GAC CTGG		Antitoxin of the ChpA-ChpR TA system in *E. coli* K-12		2909274-2909250		30 s at 54ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	
81	relE-F	CAG AGA ATG CGT TTG ACC G	NC_000913	Toxin of the RelE-RelB TA system in *E. coli* K-12	*E. coli*	1643371-1643389	457 bp	1 cycle of 10 min at 94ºC; 35 cycles of 30 s at 94ºC,	[134]
82	relB-R	GGT GTA ACT CCT TCT GAA GCG		Antitoxin of the RelE-RelB TA system in *E. coli* K-12		1643827-1643807		30 s at 54ºC, 30 s at 72ºC; 1 cycle of 10 min at 72ºC	

Table S3. Controls (strains) of *E. faecium* used in this study.

Strain	Main features	Applicability	Origin/Reference
E. faecium GE-1	*rif fus* (plasmid free); ST515	Conjugation recipient strain	[135]
E. faecium BM4105RF	*rif fus* (plasmid free); ST172	Conjugation recipient strain - derivative of *E. faecium* BM4105	[136]
E.faecium UA2-1	Van^r (*van* A)	Control of *vanA* amplification and hybridization	from B.E. Murray
E. faecalis V583	Van^r (*vanB*) - First VRE clinical isolate recovered in the USA	Control of pTEF1, pTEF2, pTEF3 plasmids	[137]
E. faecium C68	Van^r, Amp^r, Erm^r, Gm^r, Sm^r, Tc^r	Control of hyl_{Efm}	[138]
E. faecium L50	pCIZ2	Control of EntL50A, EntL50B, EntP and EntQ bacteriocins	[139]

Table S4.Controls (plasmids) of *E. faecium* used in this study.

Plasmid	Size (kb)	Main Features	Original Host / Source	Date of isolation	Reference
pIP501	30,6	Cm^r, Em^r, Lin^r, Pr^r; ω-ε-ζ system	*Streptococcus agalactiae* (clinical strain)	1975	[140]
pAMβ1	26,5	MLS^r; ω-ε-ζ system	*Enterococcus faecalis* DS5 (clinical strain)	1974	[141,142]
pRE25	50,2	Km^r, Sm^r, Cm^r, Em^r, Lm^r, Tet^r	*Enterococcus faecalis* RE25 (dry sausage)	1995	[143]
pEF1	21,3	Bacteriocin cluster	*Enterococcus faecium* 6T1a (Spanish-style green olive fermentation)	≤1998	[144,145]
pCIZ2	7,4	$EntQ^+$, Imm^+	*Enterococcus faecium* L50 (dry fermented sausage)	≤1995	[130]
pVEF1/2	40	Van^r; ω-ε-ζ system	*Enterococcus faecium* 399.F99.H8/A9 (farmer and poultry)	1999	[146]
pRUM	24,8	Cm^r, Em^r, Sm^r, St^r; Axe-Txe system	*Enterococcus faecium* U37 (clinical strain from USA)	≤1998	[147]
pMG1	65,1	HLRGm	*Enterococcus faecium* (clinical strain from Japan)	≤1998	[148]
pAD1	59,6	*cyl*, *uvr*, *par*	*Enterococcus faecalis* DS16 (clinical strain from USA)	≤1979	[149,15]
pAM373	36	Tet^r	*Enterococcus faecalis* RC73 (clinical strain from USA)	≤1985	[151,152]
pCF10	65	Tet^r	*Enterococcus faecalis* (clinical strain from USA)	≤1981	[153]
pTEF1	66,3	Aminoglycoside^r, Em^r (Tn*4001*-like)	*Enterococcus faecalis* V583 (clinical strain from USA)	1987	[137]
pTEF2	57,7	*prgA-prgB-prgC* (pCF10 similarity)	*Enterococcus faecalis* V583 (clinical strain from USA)	1987	[137]
pTEF3	17,9	IS*256*, IS*1216*	*Enterococcus faecalis* V583 (clinical strain from USA)	1987	[137]
pS86	5,2	cryptic	*Enterococcus faecalis* S-86 (clinical strain from Spain)	≤2000	[154]
pEF418	ND	multiple IS*1216V*; Lm^r	*Enterococcus faecalis* 418	≤2007	Gentry-Weeks *et al*. unpublished
pMV158	5,5	Tet^r	*Streptococcus agalactiae* (clinical strain from USA)	1975	[155]
pAW63	71,8	IS*231L*	*Bacillus thuringiensis subsp. kurstaki* HD73	≤1981	[156]
pMBB1	2,8	cryptic	*Enterococcus faecium* 226 from USA (unknown origin)	≤1996	[157]
pSAS	20,6	*blaZ, cadD*	*Staphylococcus aureus* MSSA476 (community-acquired invasive strain from UK)	1998	[158]
pUSA02	4,4	Tet^r	*Staphylococcus aureus* FPR3757multidrug resistant USA300 strain	≤2006	[159]
pUSA03	37,1	MLS^r	*Staphylococcus aureus* FPR3757multidrug resistant USA300 strain	≤2006	[159]
pIM13	2,2	MLS^r	*Bacillus subtillis* BD1109	1980	[160]
pC194	2,9	Cm^r	*Staphylococcus aureus*	≤1978	[161]
pRI1	6	IS*Efa4*	*Enterococcus faecium* 9631160-1 of poultry origin	≤1998	[162]
pUB101	21,8	beta-lactamase-fusidic acid resistance; *cadDX*	*Staphylococcus aureus* WBG1576 from Australia	≤1998	[163]

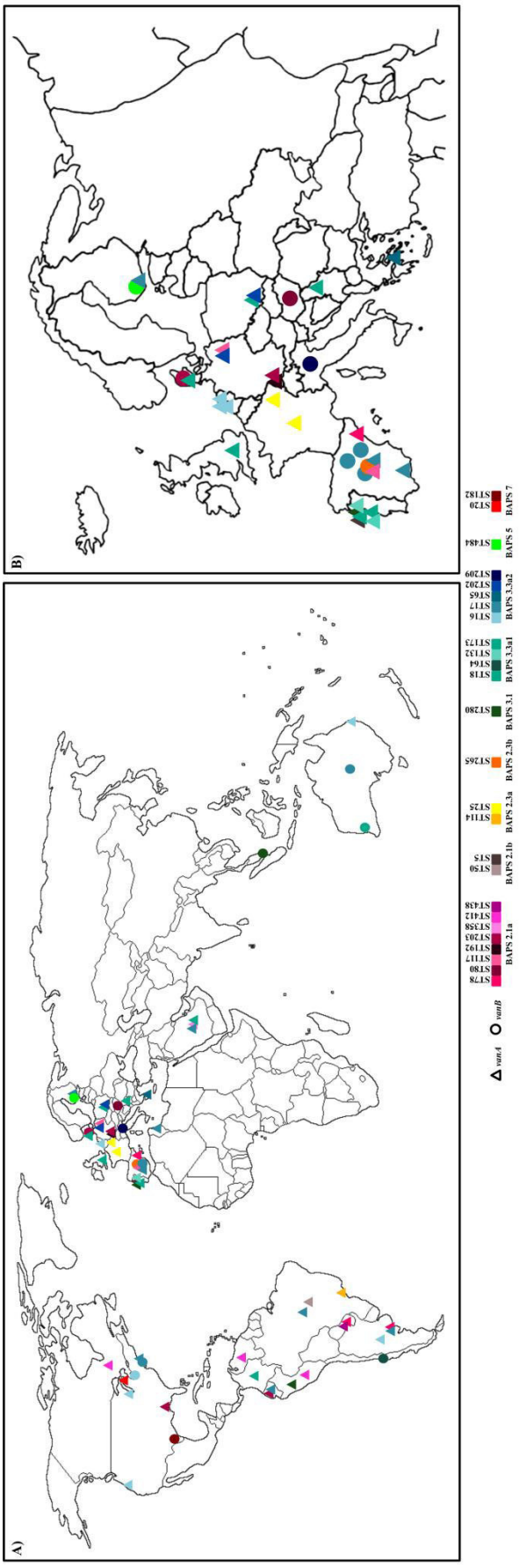
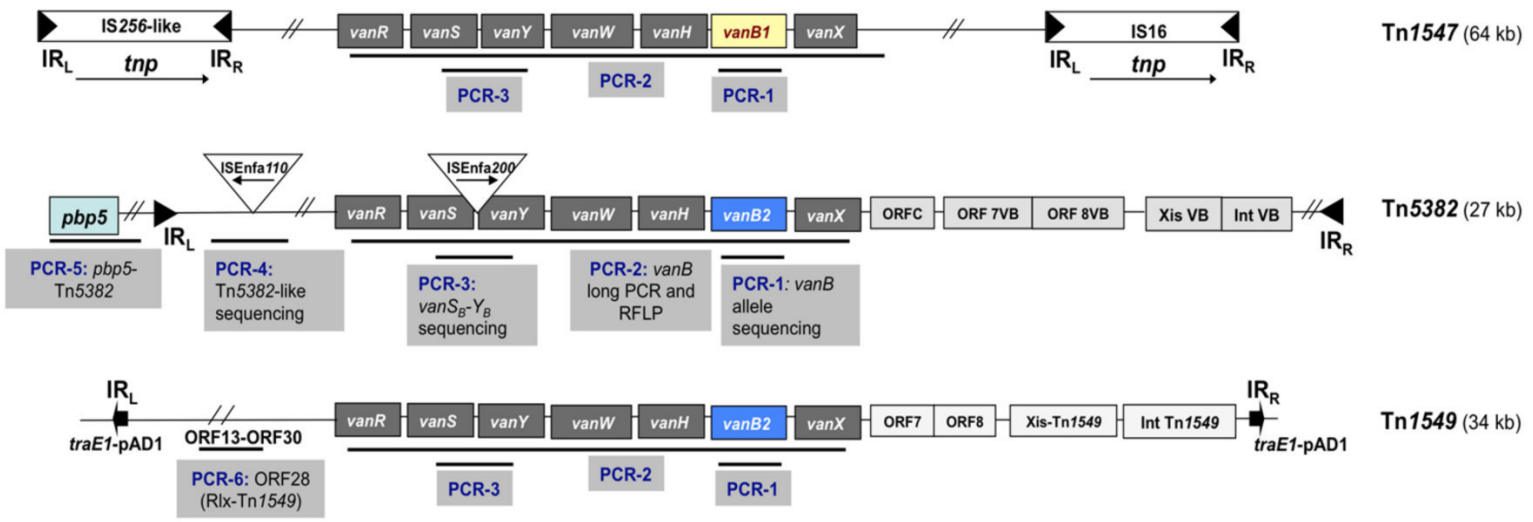


Figure S1. Geographic distribution of vancomycin-resistant *Enterococcus faecium* isolates (53 *vanA* and 18 *vanB*) in the five continents (A) and in Europe (B).



Transposon	*vanB* (PCR-1)	Transposon PCR mapping[a] *van* RSYWHBX (PCR-2)-- RFLP	*vanSB-vanYB* (PCR-3)	Tn*5382*-like (PCR-4)	*pbp5*-Tn*5382* (PCR-5)	ORF28-Tn*1549* (PCR-6)	Type designation	N.º of isolates	MLST (BAPS subgroups)	Country	Date	Location
Tn*1547*	*vanB1*	RFLP-1	*vanB1*	-	-	-	A	2	ST17 (3.3a2), ST182 (7)	USA	1992	Chr
Tn*1549*/Tn*5382*	*vanB2*	RFLP-2	*vanB2*	-	-	+	B	1	ST265 (2.3b)	ESP	1998	Pl
	vanB2	RFLP-2	*vanB2*	+	-	+	C	3	ST17 (3.3a2), ST280 (3.1)	ESP, SIN	2004-05	Pl
	vanB2	RFLP-2	*vanB2*	+	-	+	C	6	ST17 (3.3a2), ST80/ST203 (2.1a), ST280 (3.1)	ESP, HUN, DEN, AUS, SIN	1998-2008	Chr
	vanB2	RFLP-2	*vanB2*	++[b]	-	+	D	2	ST64 (3.3a1)	CHI	2003-04	Chr
	vanB2	RFLP-2*	*vanB2**[c]	+	+	+	E	3	ST16 (3.3a2), ST173 (3.3a1), ST484 (5)	FIN, USA, AUS	1996-2001	Chr
Tn*1549*/Tn*5382*	*vanB1*	RFLP-3	*vanB1*[d]	+	-	+	F	1	ST209 (3.3a2)	ITA	2002	Chr

Figure S2. Diversity of *vanB* transposons.

Abbreviations: CC, clonal complex; ST, sequence type; AUS, Australia; CHI, Chile; DNK, Denmark; ESP, Spain; FIN, Finland; HUN, Hungary; ITA, Italy; SIN, Singapore; USA, The United States; Pl, plasmid; Chr, chromosome. [a]+, amplification; -, no amplification; ++, amplification of sequences larger than those of the expected size. [b]Sequencing of the fragment revealed the presence of IS*Enfa110*. [c]Sequencing of the fragment revealed the presence of IS*Enfa200*. [d]Sequencing of *vanS*B-*vanY*B revealed a new *vanB1* type containing a C—T at 2,293 position within *vanS*B-*vanY*B (GenBank accession number KT201628). This variant resulted from recombination between Tn*1549* (*vanRS*) and Tn*1547* (*vanS*), with ends of flanking genes *vanR* and *vanX* being identical to those of pMG2200::*vanB2*-Tn*1549* from a Japanese *E. faecalis* isolate (GenBank accession number AB374546).

	Tn*1546* type	Tn*1546* PCR mapping* p1p2 (22-1330)	p3p4 (1222-2353)	p5p6 (2227 3525)	p7p8 (2769-4042)	p9p10 (3569-4793)	p11p12 (4675-6353)	p13p14 (6229-8021)	p15p16 (6979 8920)	p17p18 (8889-10473)	p19p1 (10403-10830)	no. of isolates	Sequence types (BAPS subgroups)	Country (ies)	Date	Location	*van4* plasmid size in kb
Tn*1546* variants with IS*1251*	A[a]	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	6	ST78 (2.1a), ST50 (2.1b), ST25 (2.3a)	FRA, DEU, ESP, IT, BRA	1986-2006	P	25 (n=2); >200 (n=4)
	D[b]	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	5	ST192 (2.1a), ST203 (2.1a), ST5 (2.1b), ST16 (3.3a2)	DEU, NLD, PT, AUS	1999-2005	P	30-60 (n=4), >150 (n=2)
	M[a]	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	1	ST17 (3.3a2)	FIN	1996-1998	P	300
	F[b]	(-)	(+)	(+)	(+)	(+)	(++)[g]	(+)	(+)	(+)	(+)	10	ST16/ST17/ST65 (3.3a2), ST114 (2.3a), ST20 (7)	ARG, GRC, BRA, ECU, USA	1992-2006	P	35-55 (n=9); 170-200 (n=3)
	F1	(-)	(-)	(+)	(+)	(+)	(++)[g]	(+)	(+)	(+)	(+)	2	ST18 (3.3a1), ST202 (3.3a2)	POL	2005	P	60
	F2	(-)	(+)	(-)	(-)	(+)	(++)[g]	(+)	(+)	(+)	(+)	1	ST78 (2.1a)	ARG	1998	P	50
	F3	(-)	(-)	(-)	(-)	(+)	(++)[g]	(+)	(+)	(+)	(+)	1	ST18 (3.3a1)	SER	2005	P	40
	F4	(-)	(+)	(+)	(+)	(+)	(-)[g]	(+)	(+)	(+)	(+)	4	ST17 (3.3a2), ST78/ST412/ST438 (2.1a)	CAN, BRA, PAR	2005-2006	P	50-55 (n=3); >200 (n=1)
	F5	(-)	(-)	(-)	(-)	(-)	(-)[g]	(+)	(+)	(+)	(+)	1	ST18 (3.3a1)	DNK	2006-2008	P	200
	F6	(+)	(+)	(-)	(+)	(+)	(++)[g]	(+)	(+)	(+)	(+)	1	ST280 (3.1)	PER	2006-2008	P	30
	B.1[c]	(+)	(+)	(+)	(+)	(+)	(++)[g]	(+)	(++)[h]	(+)	(+)	1	ST412 (2.1a)	VEN	2006-2008	P	30
	E.1[c]	(-)	(+)	(+)	(+)	(+)	(++)[g]	(+)	(++)[h]	(+)	(+)	1	ST412 (2.1a)	PER	2006-2008	P	30
Tn*1546* variants with IS*1216*	PP-13[d]	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(-)[h]	(-)	(+)	1	ST132 (3.3a1)	PRT	2001	P	50
	PP-23[d]	(-)	(-)	(+)	(-)	(-)	(+)	(+)	(-)[h]	(-)	(+)	1	ST132 (3.3a1)	PRT	2002	P	50
	G[a]	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(-)[h]	(+)	(+)	1	ST18 (3.3a1)	GRB	1992	P	50
	X[a]	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(-)[h]	(-)	(+)	2	ST16 (3.3a2)	NLD	2000-2002	P	30 (n=1); 85 (n=1)
	PP-16[d]	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)[h]	(+)	(+)	3	ST17/ST202 (3.3a2), ST203 (2.1a)	DEU, ECU, ESP	2006-2009	P	30 (n=2); 40 (n=1)
	Y[e]	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(++)[h]	(+)	(+)	1	ST17 (3.3a2)	ESP	2003	P	40
	B[e]	(+)	(+)	(+)	(+)	(+)	(-)[h]	(+)	(-)[h]	(+)	(+)	1	ST17 (3.3a2)	SAR	2000-03	P	55
	T[e]	(-)	(-)	(-)	(-)	(++)[h]	(+)	(+)	(++)[h]	(+)	(+)	1	ST17 (3.3a2)	TUN	2003	P	145
	J[e]	(-)	(+)	(+)	(+)	(+)	(-)[h]	(+)	(+)	(+)	(+)	2	ST18 (3.3a1)	COL, SAR	2000-2006	P	60
Tn*1546* with with IS*Efl1*	PP-4[d]	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(++)[g]	(+)	2	ST18 (3.3a1), ST117 (2.1a)	ESP, PRT	2002-2008	P	90
	PP-5[d]	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(++)[i]	(-)	2	ST18 (3.3a1), ST280 (3.1)	PRT	2003	P	85 (n=2)
	C[f]	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(++)[i]	(+)	1	ST117 (2.1a)	DEU	1998	C	Not applicable
IS*1485*	H[e]	(++)[j]	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	1	ST358 (2.1a)	SAR	2000-2003	P	50

Figure S3. Tn*1546* diversity of *vanA E. faecium* isolates.

Abbreviations: IS, insertion sequence; MLST, multilocus sequence typing; CC, clonal complex; ST, sequence type; ARG, Argentina; AUS, Australia; BRA, Brazil; COL, Colombia; DEU, Germany; DNK, Denmark; ECU, Ecuador; ESP, Spain; FIN, Finland; FRA, France; GBR, Great Britain; GRC, Greece; ITA, Italy; NLD, The Netherlands; PER, Peru; POL, Poland; PRT, Portugal; SER, Serbia; TUN, Tunisia; USA, United States of America; VEN, Venezuela; P, plasmid; C, chromosome. [*](+) amplification; (-) no amplification; (++) amplification of sequences larger than those of the expected sizes. [a]Tn*1546* types A, D, G, M, T and X were designated as previously described by Woodford *et al.* [164]. [b]Tn*1546* variants similar to "type F" were previously described as F1/F2 types [165] or the Greek type II [166]. In this study we assigned six subvariants of "type F" designated as F1, F2, F3, F4, F5 and F6. [c]For those variants that did not have a specific previously described type, we used designations similar to previous ones that have similar profiles: types “B.1” and “E.1” from South America are similar to types “B” and “E”, respectively, described by Woodford *et al;* or used new designations as type “Y” following the letter numbering criteria of Woodford *et al.* [164]. [d]Tn*1546* types "PP-4", "PP-5", "PP-13", "PP-16" and “PP-23” were classified as described [167], being types "PP-4" and "PP-5" widely disseminated in Portuguese hospitals. PP-16 has been also designated as "type A3" [168]. [e]Tn*1546* types B, H* and J* were recently described by Khan MA *et al*. [65]. [f]Tn*1546* type C was previously described by Werner *et al.* [168]. [g]IS*1251* was identified after sequencing the *vanS-vanH* amplicon or the fragment resulting of the amplification using IS*1251*-F and p11 [165,166]. [h]The presence of IS*1216* within *vanX-vanY* region was determined after sequencing the *vanX-vanY* amplicon or IS*1216*-R and p15 when first amplification was negative. [i]Positive amplification using IS*Ef1*-F and p18 as described [167]. [i*]The Spanish isolate VnR16 had single copies of IS*Ef1*,and IS*1216* within *vanY-vanZ* region. [j]IS*1485* was identified after sequencing p1-p2 amplicon fragment [65].

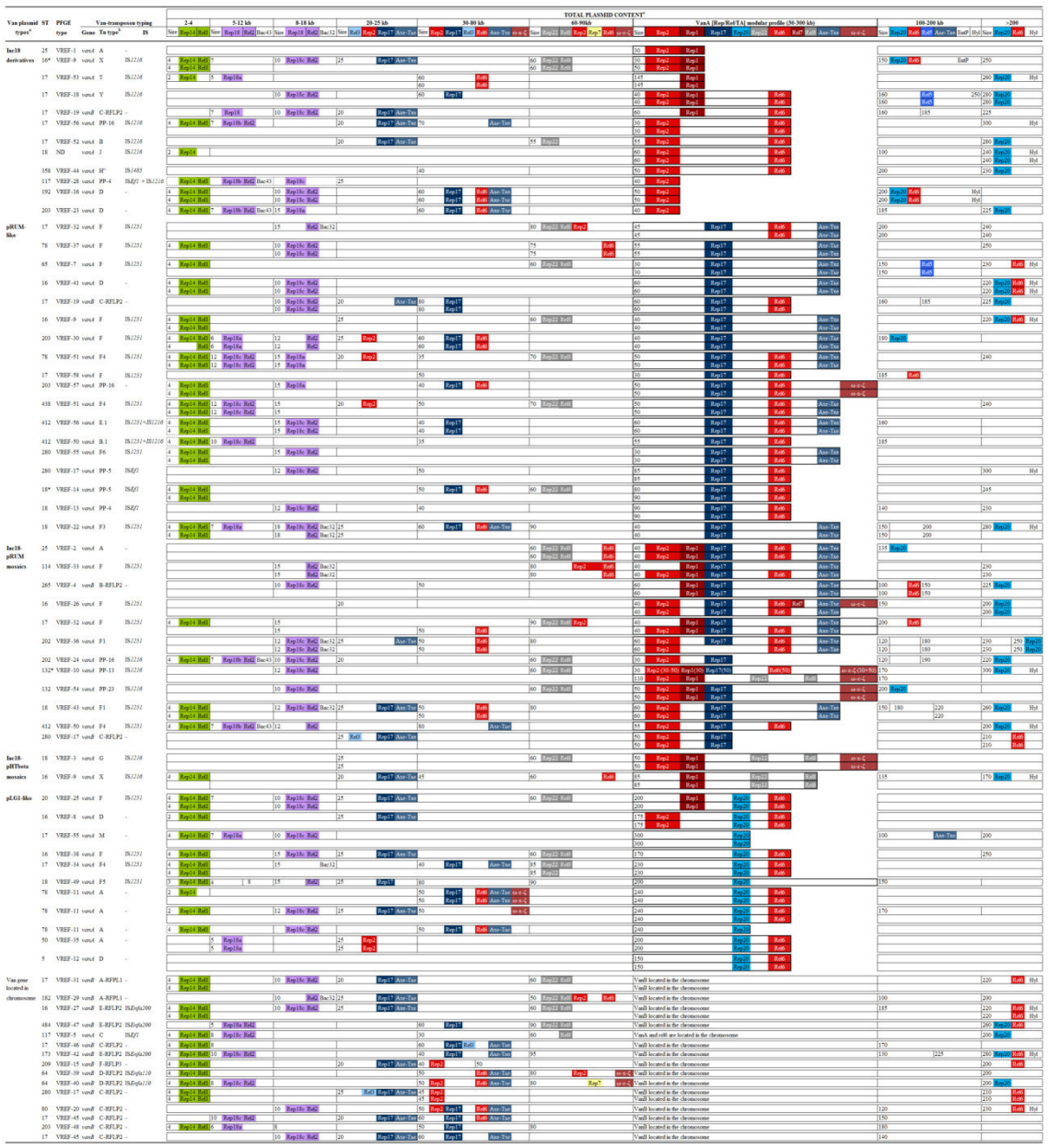
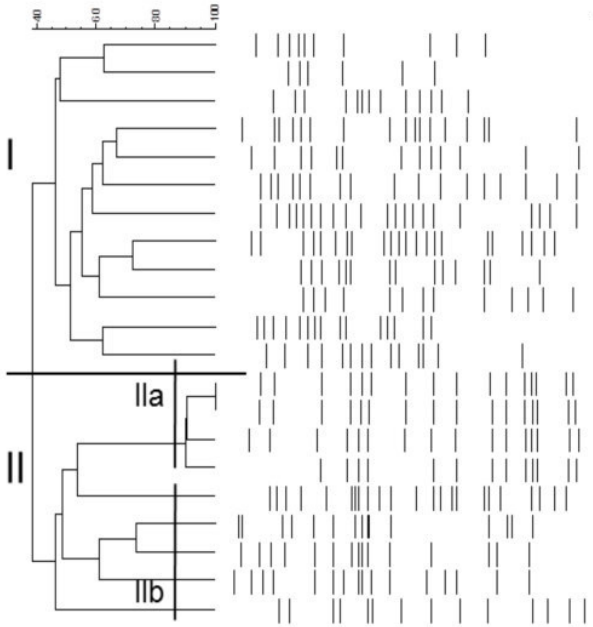


Figure S4. Plasmid diversity among VanA and VanB *E. faecium* isolates from different countries (1986-2012) (Due to the size of the table it will be provided only in digital format)

Abbreviations: ST, sequence type; Tn, transposon; IS, insertion sequence; Rep (replicases); Rel (relaxases). [a]The isolates were organized in the table according the family of the plasmid that carry the *van* genes. Isolates marked with (*) carry plasmids which size is increased after conjugation. [b]Variants of Tn*1546* and Tn*1547*/Tn*1549*/Tn*5382* were designated by capital letters as described in Fig. S2 and S3. The backbone of VanB transposons was also designated according the RFLP profile of the van*RSYWHBX* cluster by numbers (e.g. RFLP1-RFLP3)[6]. [c]The diversity of the plasmids present in each isolate is organized according their size and the modules Rep, Rel and TA: Rep (plain cells), rel (cells filled with dots) and TA (cells with diagonal stripes). Genes belonging to the same plasmid are represented with the same colour and those belonging to the same plasmid family with the same range of colours. The Rep/Rel/TA genes nomenclature has been previously described.[11,25,26] Plasmid families are represented in different colours. They include green (Rolling-Circle; rep14/pRI1-like, rel1/pRI1), violet (small-theta replicating plasmids; rep18a/pEF418, rep18b/pB82, rep18c/pCIZ2, rel2/pCIZ2), red (Inc18; rep1/pIP501, rep2/pRE25/pEF1, rel6/pEF1, rel7/pIP501, TA-Inc18-ω-ε-ζ); different blue tones for RepA_N subfamilies, dark blue (pRUM; rep17/pRUM, rel3/pRUM, TA-pRUM-Axe-Txe), turquoise (pLG1, rep20/pLG1), and light blue (pheromone-responsive, rel5/pAD1); grey (pHTß/pMG1, rep22/pHTß, rel8/pHTß) and yellow (pK214 from the Rep_3 family of *Lactococcus lactis* rep7/pK214). Toxin-antitoxin systems and virulence/bacteriocins genes located on plasmids included Axe-Txe from pRUM and ε-ζ from Inc18 plasmids, and *hyl/bac32/bac43/entP*, respectively. Genes hybridizing in the same band as *vanA/B* plasmids appear in bold cells. In a few cases, hybridization of specific probes was not achieved and amplified genes by PCR appear in dot cells.



Plasmid size (kb)	Tn*1546* type	IS	Rep content	Rel content	TA content	Country	Date	ST	no. of isolates
			pRUM pIP501 pRE25 pHTβ	pEF1 pIP501 pHTβ	Axe-Txe ω-ε-ζ				
45	F	IS*1251*				USA	1992	17	1
30	F	IS*1251*				GRC	1999	65	1
60	D	-				AUS	1996-99	16	2
40	F3	IS*1251*				SER	2005	18	1
40	F	IS*1251*				USA	2005	203	1
50	D	-				DEU	2004	192	1
50	F2	IS*1251*				ARG	1998	78	1
60	F1	IS*1251*				POL	2005	18	1
40	F	IS*1251*				USA	2001	16	1
40	F	IS*1251*				USA	1995	16	1
60	F1	IS*1251*				POL	2005	202	1
90	PP-5	IS*Efl*				PRT	NI	18	1
40	F	IS*1251*				BRA	1998	114	1
40	F	IS*1251*				ARG	1998	17	2
40	A	-				FRA	1988	25	1
30	A	-				FRA	1986	25	1
50	G	IS*1216*				GRB	1992	18	1
65						JAP	Control plasmid - pMG1		
50	PP-13	IS*1216*				PRT	2001	132	1
50	X	IS*1216*				NLD	2002	16	1
40	Y	IS*1216*				ESP	2003	17	1

Figure S5. ***Cla*I-digested plasmid DNA of representative vancomycin-resistant VanA-type *E. faecium*.**

Abbreviations: IS, insertion sequence; Rep, replicase; Rel, relaxase; TA, toxin-antitoxin; ST, sequence type; NI, not identified: ARG, Argentina; AUS, Australia; BRA, Brazil; DEU, Germany; ESP, Spain; FRA, France; GBR, Great Britain; GRC, Greece; JAP, Japan; NLD, The Netherlands; POL, Poland; PRT, Portugal; SER, Serbia; USA, United States of America. Plasmid genes (*rep, rel, TA*) previously associated with a given plasmid type (prototype) are represented with different colours based on the same criteria followed in Figure 1.

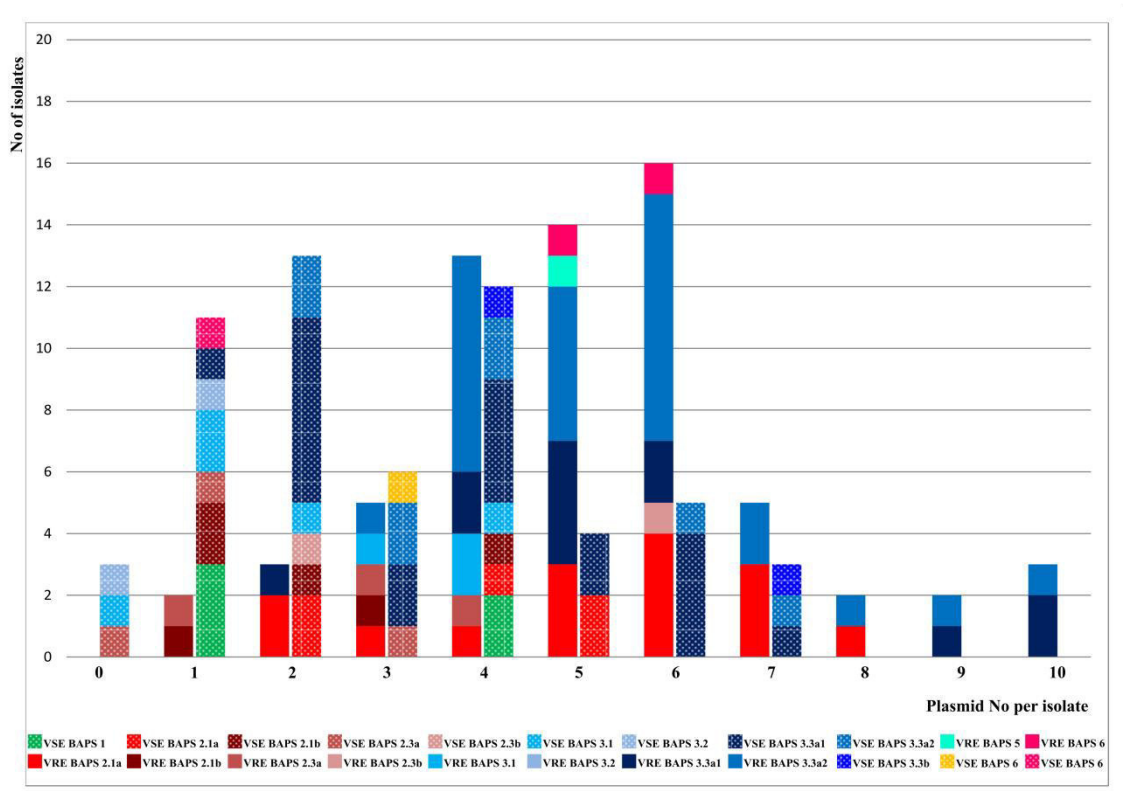


Figure S6. Plasmid number of the VREfm and VSEfm included in this study.

The number of plasmids was inferred by visualization of PFGE pattern of S1-digested genomic DNA. The VREfm (coloured cells, n=71) and VSEfm (dot coloured cells, n=82) isolates analysed were organized according phylogenomic groups inferred by BAPS.

Figure S7. Diversity of plasmids among VSEfm isolates.

Abbreviations: BAPS, Bayesian Analysis of Population Structure; ST, sequence type; ARE, ampicillin-resistant *E. faecium*; ASE, ampicillin-susceptible *E. faecium*.[a]The diversity of the plasmids present in each isolate is organized according their size and the modules Rep, Rel and TA: Rep (plain cells), rel (cells filled with dots) and TA (cells with diagonal stripes). Genes belonging to the same plasmid are represented with the same color and those belonging to the same plasmid family with the same range of colors. For Rep/Rel/TA genes nomenclature consult the references [132,169,170]. Plasmid families are represented in different colours. They include green (Rolling-Circle; rep14/pRI1-like, rel1/pRI1), violet (small-theta replicating plasmids; rep18a/pEF418, rep18b/pB82, rep18c/pCIZ2, rel2/pCIZ2), red (Inc18; rep1/pIP501, rep2/pRE25/pEF1, rel6/pEF1, rel7/pIP501, TA-Inc18-ω-ε-ζ); different blue tones for RepA_N subfamilies, dark blue (pRUM; rep17/pRUM, rel3/pRUM, TA-pRUM-Axe-Txe), turquoise (pLG1, rep20/pLG1), and light blue (pheromone-responsive, rel5/pAD1); and grey (pHTß/pMG1, rep22/pHTß, rel8/pHTß). Toxin-antitoxin systems and virulence genes located on plasmids included Axe-Txe from pRUM and ε-ζ from Inc18 plasmids, and *hyl*, respectively.

References

1. Vankerckhoven V, Van Autgaerden T, Vael C, et al. Development of a multiplex PCR for the detection of asa1, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among European hospital isolates of Enterococcus faecium. J Clin Microbiol 2004; 42: 4473–9.

2. Nallapareddy SR, Weinstock GM, Murray BE. Clinical isolates of Enterococcus faecium exhibit strain-specific collagen binding mediated by Acm, a new member of the MSCRAMM family. Mol Microbiol 2003; 47: 1733–47.

3. Strompfová V, Lauková A, Simonová M, Marcináková M. Occurrence of the structural enterocin A, P, B, L50B genes in enterococci of different origin. Vet Microbiol 2008; 132: 293–301.

4. Criado R, Diep DB, Aakra A, et al. Complete sequence of the enterocin Q-encoding plasmid pCIZ2 from the multiple bacteriocin producer Enterococcus faecium L50 and genetic characterization of enterocin Q production and immunity. Appl Environ Microbiol 2006; 72: 6653–66.

5. Todokoro D, Tomita H, Inoue T, Ike Y. Genetic analysis of bacteriocin 43 of vancomycin-resistant Enterococcus faecium. Appl Environ Microbiol 2006; 72: 6955–64.

6. Jensen LB, Garcia-Migura L, Valenzuela AJS, Løhr M, Hasman H, Aarestrup FM. A classification system for plasmids from enterococci and other Gram-positive bacteria. J Microbiol Methods 2010; 80: 25–43.

7. Johnsen PJ, Østerhus JI, Sletvold H, et al. Persistence of animal and human glycopeptide-resistant enterococci on two Norwegian poultry farms formerly exposed to avoparcin is associated with a widespread plasmid-mediated vanA element within a polyclonal enterococcus faecium population. Appl Environ Microbiol 2005; 71: 159–68.

8. Moritz EM, Hergenrother PJ. Toxin-antitoxin systems are ubiquitous and plasmid-encoded in vancomycin-resistant enterococci. Proc Natl Acad Sci U S A 2007; 104: 311–6.

9. Miranda AG, Singh K V, Murray BE. Determination of the chromosomal size of three different strains of Enterococcus faecalis and one strain of Enterococcus faecium. DNA Cell Biol 1992; 11: 331–5.

10. Poyart C, Trieu-Cuot P. Heterogeneric conjugal transfer of the pheromone-responsive plasmid pIP964 (IncHlyI) of Enterococcus faecalis in the apparent absence of pheromone induction. FEMS Microbiol Lett 1994; 122: 173–9.

11. Paulsen IT. Role of Mobile DNA in the Evolution of Vancomycin-Resistant Enterococcus faecalis. Science (80-) 2003; 299: 2071–4.

12. Carias LL, Rudin SD, Donskey CJ, Rice LB. Genetic linkage and cotransfer of a novel, vanB-containing transposon (Tn5382) and a low-affinity penicillin-binding protein 5 gene in a clinical vancomycin-resistant Enterococcus faecium isolate. J Bacteriol 1998; 180: 4426–34.

13. Cintas LM, Casaus P, Holo H, Hernandez PE, Nes IF, Håvarstein LS. Enterocins L50A and L50B, two novel bacteriocins from Enterococcus faecium L50, are related to staphylococcal hemolysins. J Bacteriol 1998; 180: 1988–94.

14. Horodniceanu T, Bouanchaud DH, Bieth G, Chabbert YA. R plasmids in Streptococcus agalactiae (group B). Antimicrob Agents Chemother 1976; 10: 795–801.

15. Clewell DB, Yagi Y, Dunny GM, Schultz SK. Characterization of three plasmid deoxyribonucleic acid molecules in a strain of Streptococcus faecalis: identification of a plasmid determining erythromycin resistance. J Bacteriol 1974; 117: 283–9.

16. Swinfield TJ, Oultram JD, Thompson DE, Brehm JK, Minton NP. Physical characterisation of the replication region of the Streptococcus faecalis plasmid pAM beta 1. Gene 1990; 87: 79–90.

17. Schwarz F V, Perreten V, Teuber M. Sequence of the 50-kb conjugative multiresistance plasmid pRE25 from Enterococcus faecalis RE25. Plasmid 2001; 46: 170–87.

18. Floriano B, Ruiz-Barba JL, Jiménez-Díaz R. Purification and genetic characterization of enterocin I from Enterococcus faecium 6T1a, a novel antilisterial plasmid-encoded bacteriocin which does not belong to the pediocin family of bacteriocins. Appl Environ Microbiol 1998; 64: 4883–90.

19. Ruiz-Barba JL, Floriano B, Maldonado-Barragán A, Jiménez-Díaz R. Molecular analysis of the 21-kb bacteriocin-encoding plasmid pEF1 from Enterococcus faecium 6T1a. Plasmid 2007; 57: 175–81.

20. Sletvold H, Johnsen PJ, Simonsen GS, Aasnaes B, Sundsfjord A, Nielsen KM. Comparative DNA analysis of two vanA plasmids from Enterococcus faecium strains isolated from poultry and a poultry farmer in Norway. Antimicrob Agents Chemother 2007; 51: 736–9.

21. Grady R, Hayes F. Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multidrug-resistant, clinical isolate of Enterococcus faecium. Mol Microbiol 2003; 47: 1419–32.

22. Ike Y, Tanimoto K, Tomita H, Takeuchi K, Fujimoto S. Efficient transfer of the pheromone-independent Enterococcus faecium plasmid pMG1 (Gmr) (65.1 kilobases) to Enterococcus strains during broth mating. J Bacteriol 1998; 180: 4886–92.

23. Tomich PK, An FY, Damle SP, Clewell DB. Plasmid-related transmissibility and multiple drug resistance in Streptococcus faecalis subsp. zymogenes strain DS16. Antimicrob Agents Chemother 1979; 15: 828–30.

24. Clewell DB, Tomich PK, Gawron-Burke MC, Franke AE, Yagi Y, An FY. Mapping of Streptococcus faecalis plasmids pAD1 and pAD2 and studies relating to transposition of Tn917. J Bacteriol 1982; 152: 1220–30.

25. Clewell DB, An FY, White BA, Gawron-Burke C. Streptococcus faecalis sex pheromone (cAM373) also produced by Staphylococcus aureus and identification of a conjugative transposon (Tn918). J Bacteriol 1985; 162: 1212–20.

26. De Boever EH, Clewell DB, Fraser CM. Enterococcus faecalis conjugative plasmid pAM373: complete nucleotide sequence and genetic analyses of sex pheromone response. Mol Microbiol 2000; 37: 1327–41.

27. Christie PJ, Korman RZ, Zahler SA, Adsit JC, Dunny GM. Two conjugation systems associated with Streptococcus faecalis plasmid pCF10: identification of a conjugative transposon that transfers between S. faecalis and Bacillus subtilis. J Bacteriol 1987; 169: 2529–36.

28. Martínez-Bueno M, Valdivia E, Gálvez A, Maqueda M. pS86, a new theta-replicating plasmid from Enterococcus faecalis. Curr Microbiol 2000; 41: 257–61.

29. del Solar G, Diaz R, Espinosa M. Replication of the streptococcal plasmid pMV158 and derivatives in cell-free extracts of Escherichia coli. Mol Gen Genet 1987; 206: 428–35.

30. Wilcks A, Jayaswal N, Lereclus D, Andrup L. Characterization of plasmid pAW63, a second self-transmissible plasmid in Bacillus thuringiensis subsp. kurstaki HD73. Microbiology 1998; 144 (Pt 5: 1263–70.

31. Wyckoff HA, Barnes M, Gillies KO, Sandine WE. Characterization and sequence analysis of a stable cryptic plasmid from Enterococcus faecium 226 and development of a stable cloning vector. Appl Environ Microbiol 1996; 62: 1481–6.

32. Holden MTG, Feil EJ, Lindsay JA, et al. Complete genomes of two clinical Staphylococcus aureus strains: evidence for the rapid evolution of virulence and drug resistance. Proc Natl Acad Sci U S A 2004; 101: 9786–91.

33. Diep BA, Gill SR, Chang RF, et al. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus. Lancet 2006; 367: 731–9.

34. Monod M, Denoya C, Dubnau D. Sequence and properties of pIM13, a macrolide-lincosamide-streptogramin B resistance plasmid from Bacillus subtilis. J Bacteriol 1986; 167: 138–47.

35. Horinouchi S, Weisblum B. Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. J Bacteriol 1982; 150: 815–25.

36. Garcia-Migura L, Hasman H, Jensen LB. Presence of pRI1: a small cryptic mobilizable plasmid isolated from Enterococcus faecium of human and animal origin. Curr Microbiol 2009; 58: 95–100.

37. O'Brien FG, Price C, Grubb WB, Gustafson JE. Genetic characterization of the fusidic acid and cadmium resistance determinants of Staphylococcus aureus plasmid pUB101. J Antimicrob Chemother 2002; 50: 313–21.

38. Woodford N, Adebiyi AM, Palepou MF, Cookson BD. Diversity of VanA glycopeptide resistance elements in enterococci from humans and nonhuman sources. Antimicrob Agents Chemother 1998; 42: 502–8.

39. Willems RJ, Top J, van den Braak N, et al. Molecular diversity and evolutionary relationships of Tn1546-like elements in enterococci from humans and animals. Antimicrob Agents Chemother 1999; 43: 483–91.

40. Demertzi E, Palepou MF, Kaufmann ME, Avlamis A, Woodford N. Characterisation of VanA and VanB elements from glycopeptide-resistant Enterococcus faecium from Greece. J Med Microbiol 2001; 50: 682–7.

41. Novais C, Freitas AR, Sousa JC, Baquero F, Coque TM, Peixe L V. Diversity of Tn1546 and its role in the dissemination of vancomycin-resistant enterococci in Portugal. Antimicrob Agents Chemother 2008; 52: 1001–8.

42. Werner G, Klare I, Fleige C, Witte W. Increasing rates of vancomycin resistance among Enterococcus faecium isolated from German hospitals between 2004 and 2006 are due to wide clonal dissemination of vancomycin-resistant enterococci and horizontal spread of vanA clusters. Int J Med Microbiol 2008; 298: 515–27.

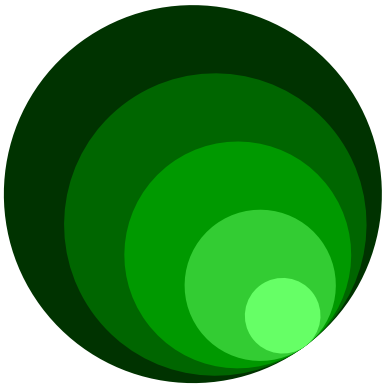
43. Khan MA, van der Wal M, Farrell DJ, et al. Analysis of VanA vancomycin-resistant Enterococcus faecium isolates from Saudi Arabian hospitals reveals the presence of clonal cluster 17 and two new Tn1546 lineage types. J Antimicrob Chemother 2008; 62: 279–83.

44. Freitas AR, Novais C, Tedim AP, et al. Microevolutionary Events Involving Narrow Host Plasmid Influences Local Fixation of Vancomycin-Resistance in Enterococcus. PLoS One 2013: e60589.

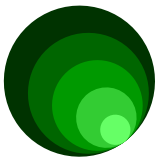
45. Goicoechea P, Romo M, Coque TM, et al. Identification of enterococcal plasmids by multiplex-PCR-based relaxase typing (Abstract number: P1669). In: 18th European Congress of Clinical Microbiology and Infectious Diseases. Barcelona, Spain, 2008.

It is beyond a doubt that all our knowledge begins with experience.

Immanuel Kant



Chapter 5



Microevolutionary Events Involving Narrow Host Plasmids Influences Local Fixation of Vancomycin-Resistance in *Enterococcus* Populations

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Performed most experimental work: A.R.F. Partially contributed to clonal and transposon characterization: C.N., A.P.T. Conceived and designed the experiments: A.R.F., L. P., T.M.C. Analyzed the data: A.R.F., C.N., A.P.T., M.V.F., F.B., L.P., T.M.C. Contributed reagents/materials/analysis tools: A.R.F., M.V.F., L.P., T.M.C. Wrote the paper: A.R.F., F.B., T.M.C.

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ABSTRACT

Vancomycin-resistance in enterococci (VRE) is associated with isolates within ST18, ST17, ST78 *Enterococcus faecium* (Efm) and ST6 *Enterococcus faecalis* (Efs) human adapted lineages. Despite of its global spread, vancomycin resistance rates in enterococcal populations greatly vary temporally and geographically. Portugal is one of the European countries where Tn*1546* (*vanA*) is consistently found in a variety of environments. A comprehensive multi-hierarchical analysis of VRE isolates (75 Efm and 29 Efs) from Portuguese hospitals and aquatic surroundings (1996–2008) was performed to clarify the local dynamics of VRE. Clonal relatedness was established by PFGE and MLST while plasmid characterization comprised the analysis of known relaxases, rep initiator proteins and toxin-antitoxin systems (TA) by PCR-based typing schemes, RFLP comparison, hybridization and sequencing. Tn*1546* variants were characterized by PCR overlapping/sequencing. Intra- and inter-hospital dissemination of Efm ST18, ST132 and ST280 and Efs ST6 clones, carrying rolling-circle (pEFNP1/pRI1) and theta-replicating (pCIZ2-like, Inc18, pHTβ-like, two pRUM-variants, pLG1-like, and pheromone-responsive) plasmids was documented. Tn*1546* variants, mostly containing IS*Ef1* or IS*1216*, were located on plasmids (30–150 kb) with a high degree of mosaicism and heterogeneous RFLP patterns that seem to have resulted from the interplay between broad host Inc18 plasmids (pIP501, pRE25, pEF1), and narrow host RepA_N plasmids (pRUM, pAD1-like). TAs of Inc18 (ω-ε-ζ) and pRUM (Axe-Txe) plasmids were infrequently detected. Some plasmid chimeras were persistently recovered over years from different clonal lineages. This work represents the first multi-hierarchical analysis of VRE, revealing a frequent recombinatorial diversification of a limited number of interacting clonal backgrounds, plasmids and transposons at local scale. These interactions provide a continuous process of parapatric clonalization driving a full exploration of the local adaptive landscape, which might assure long-term maintenance of resistant clones and eventually fixation of Tn*1546* in particular geographic areas.

INTRODUCTION

Since its first description in the late 80's, vancomycin-resistant enterococci (VRE) have been increasingly reported worldwide, but presenting remarkable geographical and temporal differences in local rates (http://www.cddep.org/ResistanceMap/bug-drug/EFa-VC) [1–3]. Vancomycin-resistant *Enterococcus faecium* (VREfm) became endemic in most North American hospitals since the mid 90's [1,2,4–6] while their overall occurrence in Europe remained low until recently, when VRE nosocomial outbreaks started to be increasingly reported in some European countries (Annual Report of the European Antimicrobial Resistance Surveillance Network, EARS-Net, 2009)[1,3,7,8]. Despite *E. faecium* (Efm) being less frequently found than *Enterococcus faecalis* (Efs) in clinical isolates, it is far more frequently resistant to vancomycin, one of the last-line intravenous antibiotic resources for therapy. However, although the rate of vancomycin-resistant *E. faecalis* (VREfs) has remained low, they are steadily increasing in both the US and in EU countries (http://www.cddep.org/ResistanceMap/bug-drug/EFe-VC) [3].

Vancomycin resistance among enterococci is mostly due to the spread of Tn*1546* (*vanA* genotype) and Tn*1549* (*vanB* genotype), which are generally identified on plasmids and chromosome, respectively [3]. The few studies in which plasmids carrying Tn*1546* from human or animal isolates were characterized revealed they belong to plasmid families RepA_N (pheromone-responsive plasmids and derivatives of pRUM and pLG1), Inc18 and pHTβ [9–18] suggesting an apparent plasmid promiscuity of this transposon influencing its dissemination among enterococcal populations.

Recent analysis of enterococcal populations in the clinical setting depicts a rugged epidemiological profile, with successive waves of isolates causing infections, which belong to specific lineages of *E. faecium* [ST17, ST18 and ST78, previously considered within the same clonal complex (CC) 17], and *E. faecalis* (ST6, ST40) [19–21]. However, regional differences in the rates of VRE cannot be only explained by clonal replacement dynamics as suggested for other pathogens [22–24].

The aim of this study was to address the dynamics of vancomycin resistance among enterococci in Portugal, one of the developed countries with higher rates of both VREfm (21–23%) and VREfs (1.8–4.1%) (www.earss.rivm.nl; http://www.cddep.org/ResistanceMap/bug-drug/EFe-VC), and where VanA is prevalent over VanB [3,25–27], by analyzing the clonal and plasmid backgrounds influencing the spread and persistence of Tn*1546*. Our study suggests that clonalization, the local selection of distinct clonal variants giving rise to durable bacterial lineages, might result and be modified by the local spread and recombinatorial dynamics of mobile genetic elements, thus providing new clues about the local multi-hierarchical evolutionary biology of vancomycin resistance.

RESULTS

Local dynamic landscape drives the spread and fixation of vancomycin resistance in Portuguese hospitals

We have determined that the enterococcal population from the Portuguese hospitals is formed by an ensemble of MLST/PFGE clones. Efm isolates fit in three out of six phylogenomic groups recently established by using Bayesian Analysis of Population Structure (BAPS), namely BAPS groups 2, 3 and 5 [19] (Figure 1). Most of the isolates cluster into the predominant BAPS group 3 [subgroup 3–3 comprising main human lineages ST18 (ST18 and ST132) and ST17 (ST16); and subgroup 3–1 comprising ST280], and the BAPS group 2 (including ST80 and ST656/ST78 lineage, ST5/CC5, ST190/CC9), which have been previously associated with isolates from humans and both animals and humans, respectively [10,19,25,28–30][10]. A number of clones cluster in the small Efm BAPS group 5 (ST366, ST367, ST369), which seems to comprise mosaic genomes [19]. Isolates of Efs belong to ST6/CC2, ST30, ST55, ST117, and ST159 lineages although, to the date of this publication, Efs population has not been clustered in different BAPS groups. Among all them, isolates within ST18 Efm and ST6 Efs lineages were predominant, in line with the intra- and interhospital spread of particular highly transmissible Efm and Efs clones recovered in Portuguese hospitals since the late 90s [22,25,31,32]. While ST6 Efs was widely disseminated in all hospitals analyzed in this country [26], specific Efm lineages were overrepresented in Coimbra (ST18) and Oporto (ST132, a single locus variant, SLV, of ST18). Strains belonging to ST18 (showing PFGE types H70 and H78), ST132 (PFGE type H88) and ST280 (with PFGE types 71 and H100) were spread in different hospitals (Figure 1 and Figure 2).

It is worthwhile to note the possible relatedness between isolates of different STs (Figure 1 and 2). They include some isolates linked to BAPS 3–3 subgroup as ST18, ST80, ST125, ST132, ST368, ST369, all SLVs of each other, with PFGE patterns differing in less than 8 bands difference. Similarly, strains identified as ST280 and ST391, both linked to BAPS group 3–1, showed related PFGE patterns despite being trilocus variants (≤ 8 bands difference).

vanA-Tn*1546* is located on highly transferable mosaic plasmids involving narrow host pRUM and pAD1 derivatives

The plasmid content of the isolates studied appears in Figure 2. Efm isolates carried a variable number of plasmids (n=1–6) which contained specific sequences of different families including rolling-circle plasmids (RCR) related to pRI1 and small theta plasmids related to pCIZ2, RepA_N (pRUM-like, pLG1), pHTβ (present in all ST132 isolates), and Inc18 (pRE25 and pEF1-related). All Efs contained RCR plasmids and pheromone responsive-plasmids.

vanA-Tn*1546* was located on plasmids ranging from 30 to 150 kb, successfully transferred by conjugation in 95% (n=71/75) of Efm and 97% (n=28/29) of Efs, with a variable frequency (10^{-1}–10^{-8}).

Transferable plasmids were identified as members of pRUM and Inc18 families or were mosaic plasmids of pRUM, Inc18 and pheromone plasmids (see sections below). Although some of these mosaic plasmids were detected in both Efm and Efs hosts, species-specific plasmid variants were predominant.

We have classified the enterococcal plasmids according to the content in rep/rel/TA systems, and RFLP profiles (Table 1, Figure 2). For the better interpretation of the results, we should keep in mind that members of the most common plasmid families classified in this and other studies as Inc18-like (pRE25, pIP501, pVEF1, pVEF2, pVEF3, pIP816, pEF1, pWZ909) or pRUM-like (pRUM, p5753cB, pS177) exhibit a high degree of *modular dissociability* or propensity for independent variation and shuffling, and may contain multiple replicons or be devoid of conjugation systems, thus making it very difficult to establish an accurate classification and to trace the origin of certain elements [9,35–42]. See Clewell *et al.* for a comprehensive updated revision of enterococcal plasmids [9]. In the following sections we will describe vancomycin resistant plasmids of Efm and Efs.

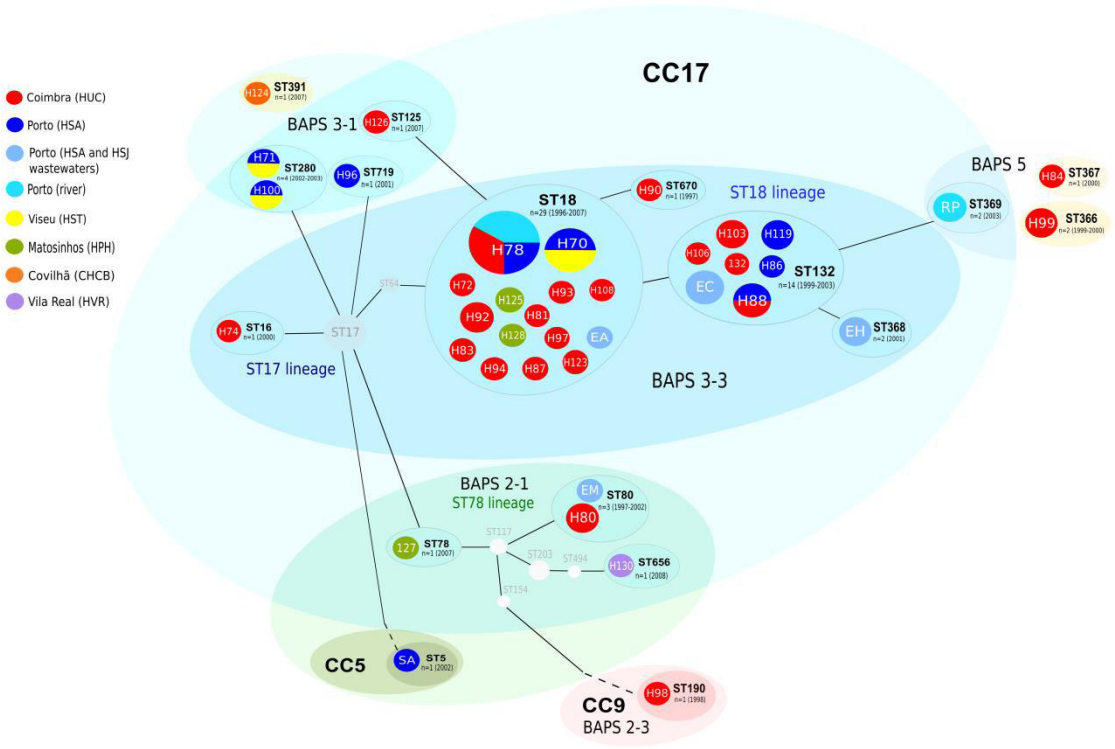

Figure 1. Population of vancomycin resistant *E. faecium*.

Abbreviations: ST, sequence types; CC, clonal complex; BAPS, Bayesian Analysis of Population Structure; HUC, Hospital Universitário de Coimbra; HSA, Hospital Santo António; HSJ, Hospital São João; HST, Hospital São Teotónio; HPH, Hospital Pedro Hispano; CHCB, Centro Hospitalar da Cova da Beira; HVR, Hospital S. Pedro. A colored circle represents each PFGE type (white numbers/letters; H for hospital, SW for sewage, R for river and S for swine clones) and each PFGE type is associated with the corresponding sequence type (STs are represented in black letter and in colored elipses grouping different PFGE types) and BAPS group (in colored elipses grouping different STs). The size of the colored circles corresponds to the number of isolates. CC17 (in light blue), CC5 (in light green), CC9 (in light red) and the singletons ST366, ST367 and ST391 (light yellow) are represented according to the eBURST algorithm (download on 26th January 2012) with black lines joining single locus variants (SLV). STs that were not identified in this study are represented as light grey nodes to link the sequence types identified in this study accordingly to eBURST. ST18 strains (H70, H78, H87, H93, H108, H125) and most ST132 strains (H86, H88, H106, SWC) were clonally related by PFGE (<7 bands difference). Remarkable relationships among PFGE banding patterns of strains belonging to different STs were observed (H125/ST18 and H126/ST125; H124/ST391 and H71/ST280, SWM/ST80 and H86/H88/H106/H119/SWC/ST132, and isolates SWA/ST18 and SWC/ST132 (<8 bands difference). This figure drawn up was performed in the "Open Source vector graphics editor Inkscape" (version Inkscape-0.48.2–1).

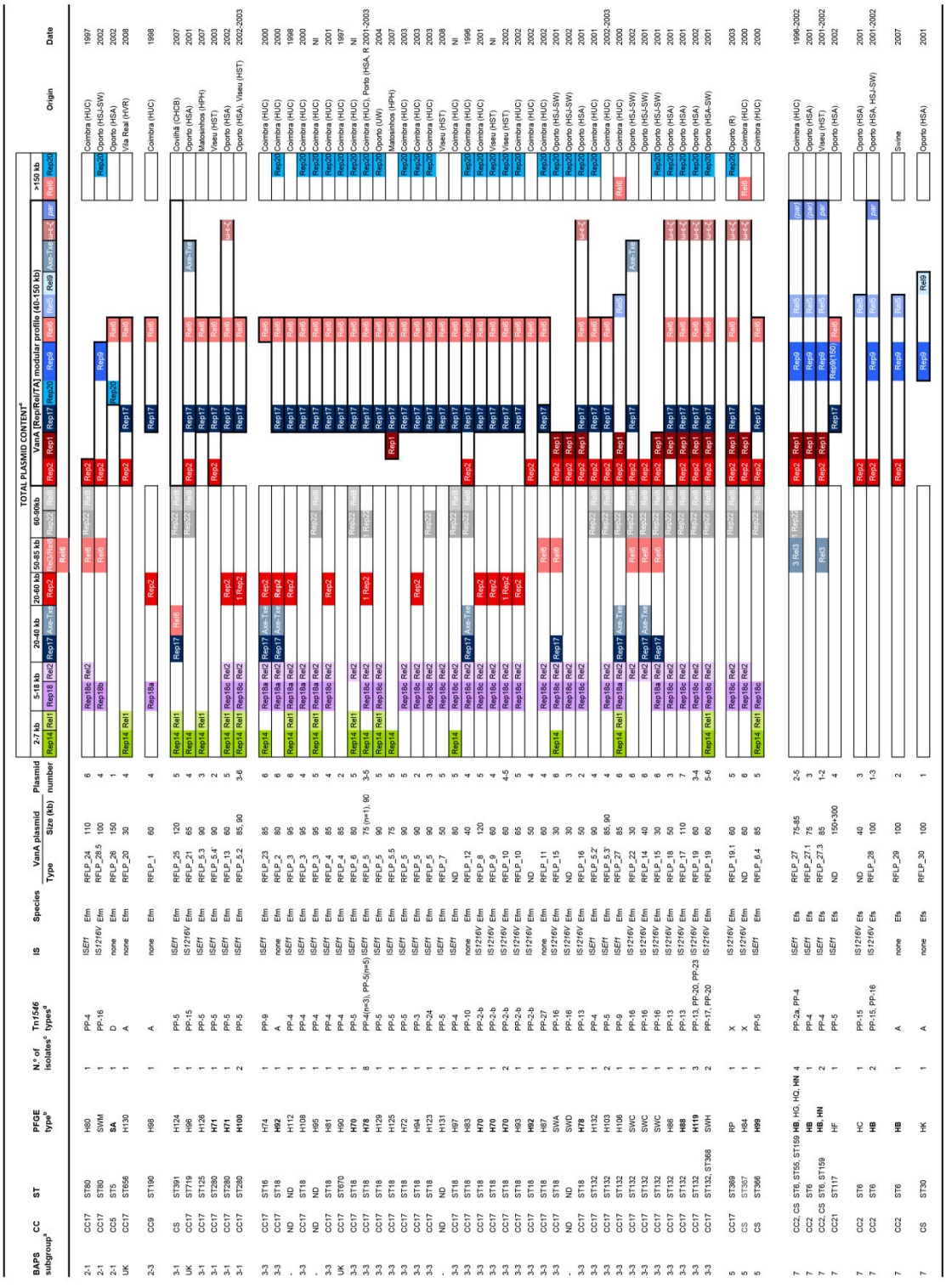

Figure 2. Clonal and plasmid diversity among VREfm and VREFs from Portugal.

Abbreviations: IS, insertion sequence; Efm, *Enterococcus faecium*; Efs, *Enterococcus faecalis*; kb, kilobases; BAPS, Bayesian Analysis of Population Structure; ST, sequence type; CC, clonal complex; rep (replicases); rel (relaxases); TA (toxin-antitoxin system); HUC, Hospital Universitário de Coimbra; HSA, Hospital Santo António; HSJ, Hospital São João; HST, Hospital São Teotónio; HPH, Hospital Pedro Hispano; CHCB, Centro Hospitalar da Cova da Beira; HVR, Hospital S. Pedro; SW, sewage wastewaters; UW, urban wastewaters; R, river; ND, not determined; NI, not identified; UK, unknown. [a]The distribution of the different isolates is shown by BAPS subgroups as described [19]. [b]PFGE types shown in bold represented widespread clones in Portuguese hospitals and/or aquatic surroundings over years. [c]Most Efm isolates expressed resistance to vancomycin, teicoplanin, erythromycin,

ampicillin, ciprofloxacin (92–100%) and to a lesser extent to high levels of kanamycin (65%), gentamicin (41%), streptomycin and tetracycline (28% each). While *acm* was identified in different CC17 and non-CC17 lineages (76%), *esp* was detected in CC17 isolates (35%, ST132 and its SLVs ST368, ST369) and *hyl* was sporadically found (9%, ST18, ST125, ST132, SLVs of each other, and ST280 isolates) [25]. Efs isolates (mostly ST6) showed resistance to vancomycin, teicoplanin, erythromycin, ciprofloxacin, high levels of gentamicin and kanamycin (82–100%), tetracycline and chloramphenicol (65% each) and high levels of streptomycin (46%), and mostly contained *gelE* and *agg* (>90%), *cyl* (82%) and *esp* (46%) [26]. [d]Tn*1546* designation is based on the results obtained by a PCR assay described by Woodford *et al.* consisting on the amplification of overlapped fragments covering the whole Tn*1546* [33]. Fragments of unexpected length were further analysed by sequencing (this study) [27]. [e]The total rep/rel/TA content of isolates is represented according to its location on plasmids of different size ranges. Rep (normal cells), rel (cells with dots) and TA (cells with diagonal stripes) genes belonging to the same plasmid are represented with the same color and that belonging to the same plasmid family with the same range of colors. The content of VanA plasmids including rep, rel, and TA genes is indicated according to the plasmid type in which they were identified, as well as by the numeric nomenclature used by Jensen *et al*. [34] for replicases (rep_1, rep_2, rep_9, rep_{14}, rep_{17}, rep_{18a}), given new and consistent designations to replicases non described in reference 34 (rep_{18b}, rep_{18c}, $rep_{20,}$ rep_{22}). Relaxases were designated per numerical order as designed by M. V. Francia (unpublished data). Rolling-Circle plasmids are represented in green ($rep_{14/pRI1\text{-}like}$, $rel_{1/pRI1}$), small-theta replicating plasmids in violet ($rep_{18a/pEF418}$, $rep_{18b/pB82}$, $rep_{18c/pCIZ2}$, $rel_{2/pCIZ2}$), Inc18-like plasmids in different red tones ($rep_{1/pIP501}$, $rep_{2/pRE25/pEF1}$, $rel_{6/pEF1}$, $TA_{Inc18\text{-}\omega\text{-}\varepsilon\text{-}\zeta}$), RepA_N plasmids in different blue tones, pRUM in dark blue ($rep_{17/pRUM}$, $rel_{3/pRUM}$, $TA_{pRUM\text{-}Axe\text{-}Txe}$), pLG1 in turquoise ($rep_{20/pLG1}$), pheromone-responsive plasmids in light blue ($rep_{9/pAD1}$, $rel_{5/pAD1}$, $rel_{9/pCF10}$, par_{pAD1}), and pHTβ/pMG1 plasmids in grey ($rep_{22/pHT\beta}$, $rel_{8/pHT\beta}$). Rep families are named Rep-"n" where "n" indicates the number assigned to different rep-families according to Jensen *et al*. [34]. The name of the most representative plasmid of the family is also represented for a better follow-up of the results (e.g. $rep_{17/pRUM}$, rep17 from pRUM and related plasmids p5753cB and pS177; $rep_{1/pIP501}$ rep1 linked to Inc18 plasmids as pIP501, pIP816 and pRE25; $rep_{9/pAD1}$, rep9 linked to pCF10, pAD1, pTEF1, pTEF2, pBEE99, pMG2200; $rep_{14/pRI1\text{-}like}$, rep14 associated with RCR plasmids pEFNP1, pJS42 and/or pRI1; $rep_{18a/pEF418}$, rep18 from pEF418; and $rep_{22/pHT\beta}$, rep of both pHTβ and pMG1 plasmids). We further specified the name of different plasmids associated with a given group if necessary. For example, it results helpful for Inc18 family given the number of plasmids containing the same rep gene. These plasmids are increasingly identified among isolates of different origins (e.g. $rep_{2/pRE25/pEF1}$ for designing rep2, as rep and rel modules of pEF1, a plasmid originally identified in olives [35], seems to be widely present in all Efm clinical isolates).Sequencing identified the different variants within these families (see text). Rep_{18b}, rep_{18c} and rep_{20} were not included in Jensen's scheme [34] and the numbers were assigned in this paper following that numeration ($rep_{18b/pB82}$, rep from pB82; $rep_{18c/pCIZ2}$, rep from pCIZ2; $rep_{20/pLG1}$, rep from pLG1). Rel genes were arbitrarily designated with numbers corresponding to different plasmid types [9] (Francia *et al*, unpublished data): Rel_1, pJS42, pRI1; Rel_2, rel from p200B, pCIZ2 and/or pB82 plasmids; Rel_3, pRUM; Rel_5, rel from pAD1, pTEF1, pAM373 and the pathogenicity island of V583; Rel_6, pEF1; Rel_8, pHTβ and pMG1; Rel_9, pCF10. Toxin-antitoxin systems included Axe-Txe from pRUM, ω-ε-ζ from Inc18 plasmids and *par* from pAD1. Genes hybridizing in the same band as *vanA* plasmids appear in bold rectangles

vanA plasmids of *E. faecium*

They were classified in two broad groups according to the plasmid replication modules and the background epidemiological context, i) pRUM-like variants ($Rep_{17.2/pRUM\text{-}like}$+ $Rel_{6/pEF1}$±$Rep_{1/pIP501}$± $Rep_{2/pRE25/pEF1}$/TA_{Inc18}), ii) mosaics of Inc18-pRUM-like ($Rep_{2/pRE25/pEF1}$ ± $Rep_{17.2/pRUM}$/$TA_{Axe\text{-}Txe}$). Highly transmissible pAD1-Inc18 mosaic plasmids from major Efs clones were also identified among Efm but they will be described in the next section.

i. ***pRUM derivatives*** ($Rep_{17.2/pRUM\text{-}like}$+$Rel_{6/pEF1}$) of variable size (30–120 kb) were detected since the mid 90 s from a diversity of clonal backgrounds. pRUM plasmids showing different *Cla*I-digested DNA RFLP patterns were identified carrying a whole copy of Tn*1546* (RFLP_1, RFLP_2, RFLP_20, 30–80 kb), IS*1216::*Tn*1546* (RFLP_8–12, 40–120 kb) or IS*Ef1::*Tn*1546* (RFLP_3–7, RFLP_13, 50–95 kb). Despite the heterogeneity of plasmid profiles, RFLP_3–6 or RFLP_8–10 shared a variable number of common bands that suggest a relationship among them (see Table 1 and Figure 3 for details about relationships among plasmids). pRUM-like plasmids exhibiting distinct RFLP profiles and carrying different transposon variants were isolated in early and recent isolates of different clonal backgrounds (Figure 2). They include ST190, carrying a 60 kb plasmid RFLP_1 type; ST670 carrying a 85 kb exhibiting a RFLP_4 plasmid type; ST656 carrying a 30 kb plasmid designated as RFLP_20, and ST18, ST132, ST280, carrying different transposon variants. These results suggest multiple independent acquisitions of pRUM-like plasmids and further rearrangements with other elements, some plasmid variants being efficiently

transferred among a diversity of different clones. It is of interest to highlight that epidemic ST18 PFGE types H83 (1996) and H92 (2000) harboured two pRUM-like plasmids. One was the $rep_{17.2/pRUM\text{-}like}$::$rel_{6/pEF1}$ vancomycin resistant plasmid showing RFLP_2 and RFLP_12 and the other was a 25kb carrying a $rep_{17.1/pRUM}$ gene and a copy of the Axe-Txe toxin-antitoxin system ($Rep_{17.1/pRUM}$+$TA_{Axe\text{-}Txe}$) identical to the pRUM derivatives described to date (pRUM, p5753cB and pS177) (GenBank accession number GQ900487; Figure 2) [12,40,41] and other vancomycin resistant plasmids circulating at international level (Freitas *et al*., unpublished data). Diversification in the Rep sequences of these pRUM-like plasmids (homology of 96% at nucleotide level and 95% at protein level) might have resulted in the compatibility with similar (but not identical) plasmids in the same clonal background along extended periods of time.

ii. ***Inc18 plasmids and mosaic Inc18-pRUM plasmids.*** Clonally related ST132 and ST18 Efm isolates from Oporto contained Inc18 plasmids ($Rep_{1/pIP501}$±$Rep_{2/pRE25/pEF1}$, RFLP_14–15) or mosaic plasmids of Inc18 and pRUM ($Rep_{2/pRE25/pEF1}$+$Rep_{1/pIP501}$+TA_{Inc18}+$Rep_{17.2/pRUM\text{-}like}$+$Rel_{pEF1}$, RFLP_16–19), all carrying IS*1216*-Tn*1546* variants. Plasmids showing RFLP types 16–19 were highly similar (5 bands/12 bands in common), RFLP_19 being persistently recovered from clonally related ST132, ST368 and ST369 isolates, collected from hospitalized patients of HSA nearby sewage plant and the river Douro from 2001 to 2003. This RFLP_19 has been also identified in a VREfm isolate recovered from swine in 2007 (Tn*1546* type "PP-31", RFLP_19.1), highlighting the remarkable stability of particular VanA Inc18 plasmids in ensembles of related clones able to spread in different hosts [10]. A diversity of Tn*1546*::IS*1216* variants (PP-13, PP-17, PP-20, PP-23, PP-31 and X) which differed in the number of IS*1216* copies, the presence of insertions identified as short regions of Inc18-like plasmids or duplicated Tn*1546* sequence fragments in different orientations, were identified among related plasmids showing the RFLP_19 pattern (Table 1, Figure 4). These results illustrate the possibility of efficient intraclonal and intraplasmid diversification of Tn*1546*::IS*1216* variants. Acquisition of a *vanA*-Inc18 ($Rep_{2/pRE25/pEF1}$+$Rep_{1/pIP501}$+ω-ε-ζ) plasmid carrying a Tn*1546*::IS*1216* "variant", predominant among poultry from Europe [43] by Portuguese strains containing VanA-pRUM ($Rep_{17.2/pRUM\text{-}like}$+$Rel_{6/pEF1}$) plasmids cannot be excluded. Recombination between pRUM::Tn*1546* and Inc18::Tn*1546* would explain duplicated Tn*1546* regions.

iii. ***Megaplasmids.*** Tn*1546* type "D" was located on a megaplasmid carrying $Rep_{20/pLG1}$ and $Rel_{6/pEF1}$ from isolates of a CC5 Efm clone spreading among swine and humans of different continents. This transposon has been previously associated with isolates from swine which frequently exhibit the G8234T mutation. The variable size (150–190 kb) of *vanA* megaplasmids linked to CC5 lineage has been previously reported [10].

Table 1. Plasmids identified in this study.

RFLP type	VanA modular profile	Size	No. isolates	Tn*1546*	PFGE type	City	Year
RFLP_1	$Rep_{17.2}$::Rel_6	60	1	A	ST190_H98	Coimbra	1998
RFLP_2	$Rep_{17.2}$::Rel_6	80	1	A	ST18_H92	Coimbra	2000
RFLP_8 [c]	$Rep_{17.2}$::Rel_6	120	1	PP2b	ST18_H70	Coimbra	2001
RFLP_9 [c]	$Rep_{17.2}$::Rel_6	60	1	PP2b	ST18_H70	Viseu	NI
RFLP_10 [c]	$Rep_{17.2}$::Rel_6	60	3	PP2b	ST18_H70, H93	Coimbra, Viseu	2002
RFLP_11	$Rep_{17.2}$::Rel_6	60	1	PP27	ST18_H87	Coimbra	2002
RFLP_3 [b]	$Rep_{17.2}$::Rel_6	95	3	PP4	ST18_H108	Coimbra	1998–2000-NI
RFLP_4 [b]	$Rep_{17.2}$::Rel_6	85	2	PP4	ST670_H90; ST18_H81	Coimbra	1997–2001
RFLP_7	$Rep_{17.2}$::Rel_6	50	1	PP5	NI	Viseu	2008
RFLP_6 [b]	$Rep_{17.2}$::Rel_6	80	1	PP5	ST18_H70	Coimbra	NI
RFLP_5 [b]	$Rep_{17.2}$::Rel_6	90	12	PP3, PP4, PP5, PP24	ST18_H78, H72, H94, H123, H129	Coimbra, Porto, Matosinhos	2001–2007
RFLP_5.2[b]	$Rep_{17.2}$::Rel_6	90	2	PP5	ST280_ H100	Porto, Viseu	2002–2003
RFLP_5.3[b]	$Rep_{17.2}$::Rel_6	90	1	PP5	ST125_H126	Matosinhos	2007
RFLP_5.3′[b]	$Rep_{17.2}$::Rep_2:: Rel_6	85	2	PP5	ST132_H103	Coimbra	2002–2003
RFLP_5.2′[b]	$Rep_{17.2}$::Rep_2:: Rel_6	90	1	PP4	ST132_H132	Coimbra	2001
RFLP_5.4′[b]	$Rep_{17.2}$::Rep_2:: Rel_6	90	1	PP5	ST280_ H71	Viseu	2003
RFLP_5.5[b]	$Rep_{17.2}$::Rep_2:: Rel_6	75	1	PP5	ST18_H125	Matosinhos	2007
RFLP_6.4	$Rep_{17.2}$::Rep_2:: Rel_6	85	1	PP5	ST366_H99	Coimbra	2000
RFLP_20	$Rep_{17.2}$::Rep_2:: Rel_6	30	1	A	ST656_H130	Vila Real	2008
RFLP_12 [a]	$Rep_{17.2}$::Rep_2:: Rel_6	40	1	PP10	ST18_H83	Coimbra	1996
RFLP_13 [b]	$Rep_{17.2}$:: Rel_6::TA_{Inc18}[a]	60	1	PP5	ST280_ H71	Oporto	2002
RFLP_18	$Rep_{17.2}$:: Rep_1:: Rel_6:: TA_{Inc18}	50	1	PP13	ST132_H86	Oporto	2001
RFLP_16	$Rep_{17.2}$:: Rep_1:: Rep_2:: Rel_6::TA_{Inc18}	50	1	PP13	ST18_H78	Oporto	2001
RFLP_17	$Rep_{17.2}$:: Rep_1:: Rep_2:: Rel_6::TA_{Inc18}	110	1	PP13	ST132_H88	Oporto	2001
RFLP_19	$Rep_{17.2}$:: Rep_1:: Rep_2:: Rel_6::TA_{Inc18}	60	3	PP13, PP20, PP23	ST132_H119	Oporto	2002
RFLP_19	$Rep_{17.2}$:: Rep_1:: Rep_2:: Rel_6::TA_{Inc18}	60	2	PP17, PP20	ST368_SWH	Oporto	2001
RFLP_19.1	$Rep_{17.2}$:: Rep_1:: Rep_2:: Rel_6::TA_{Inc18}	60	1	X	ST369_RP	Oporto	2003
RFLP_21	$Rep_{17.2}$:: Rep_2:: Rel_6:: TA_{pRUM}	65	1	PP15	ST719_H96	Oporto	2001
RFLP_22	$Rep_{17.2}$:: Rep_2:: Rel_6:: TA_{pRUM}	30	1	PP16	ST132_SWC	Oporto	2002
RFPL_27[d]	Rep_9: Rep_2: Rep_1:: Rel_5::TA_{pAD1}	75–85	4	PP2a, PP4	ST6_HB, ST55_HG, ST159_HN	Coimbra	1996–2002
RFPL_27.3[d]	Rep_9: Rep_2: Rep_1:: Rel_5::TA_{pAD1}	85	2	PP4	ST6_HB, ST159_HN	Viseu	2001–2002
RFPL_27.1[d]	Rep_9: Rep_1:: Rel_5:: TA_{pAD1}	75	1	PP4	ST6_HB	Oporto	2001
RFPL_27[d]	Rep_9: Rep_2: Rep_1:: Rel_5	85	1	PP9	ST132_H106	Coimbra	2000
RFPL_28.5	Rep_9: Rep_2	100	1	PP16	ST80_SWM	Oporto	2002
RFPL_28	Rep_9: Rep_2: Rel_5:: TA_{pAD1}	100	2	PP15, PP16	ST6_HB	Oporto	2001–2002
RFLP_29	Rep_9: Rep_2: Rel_5	100	1	A	ST6_HB	Swine	2007
RFLP_30	Rep_9: Rel_9	100	1	A	ST30_HK	Oporto	2001
RFLP_24	Rep_2	110	1	PP4	ST80_H80	Coimbra	1997
RFLP_14	Rep_2	40	1	PP16	ST132_SWC	Oporto	2002
RFLP_15	Rep_1:: Rep_2	30	2	PP16	ST18_SWA, ST132_SWC	Oporto	2001
RFLP_23	Rel_6	85	1	PP9	ST16_H74	Coimbra	2000
RFLP_26	Rep_{20}:: Rel_6	150	1	D	ST5_SA	Oporto	2002
RFLP_25	–	120	1	PP5	ST391_H124	Covilhã	2007

Abbreviations: RFLP, restriction fragment length polymorphism; ST, sequence type; NI, not identified.

[a]Plasmid type RFLP_12 ($Rep_{17.1pRUM\text{-}like}$+$Rep_2$+$Rel_{pEF1}$) contains a partial sequence of the replication gene of the RCR plasmid pEFNP1 (GenBank accession number AB038522), suggesting the integration of this RCR plasmid on the mobile element carrying Tn*1546* involving truncation of the $rep_{14/pRI1/pEFNP1}$.

[b]Plasmid types RFLP_3, _4, _5 _6 and _13 ($Rep_{17.1}$+$Rel_{6/pEF1}$ and eventually containing $Rep_{1/pIP501}$ or $Rep_{2/pRE25/pEF1}$ or TA_{Inc18}) shared common bands and were identified in the same or different clonal backgrounds in different cities for extended periods of time.

[c]Plasmids types RFLP 8, _9 and _10 also shared a variable number of common bands.

[d]Plasmids showing patterns related to RFLP_27 (75-85kb; $rep_{9/pAD1}$+rel_{pAD1}+$rep_{1/pIP501}$+*par* and/or $rep_{2/pRE25/pEF1}$) initially recovered from the widespread ST6-CC2 *Efs* clone in Coimbra in 1996 and other *Efs* (ST55 and ST159) and *Efm* clones contained similar IS*Ef1*-Tn*1546* variants (PP-2a, PP-4, PP-9). Other highly related mosaic Inc18-pAD1-related plasmids carrying IS*1216*-Tn*1546* were recovered from ST6 VREfs and ST80 VREfm isolates (type ″II_{Efs}″, $rep_{9/pAD1}$+rel_{pAD1}+par_{pAD1}+$rep_{2/pRE25/pEF1}$ *versus* type ″II_{Efm}″, $rep_{9/pAD1}$+$rep_{2/pRE25/pEF1}$).

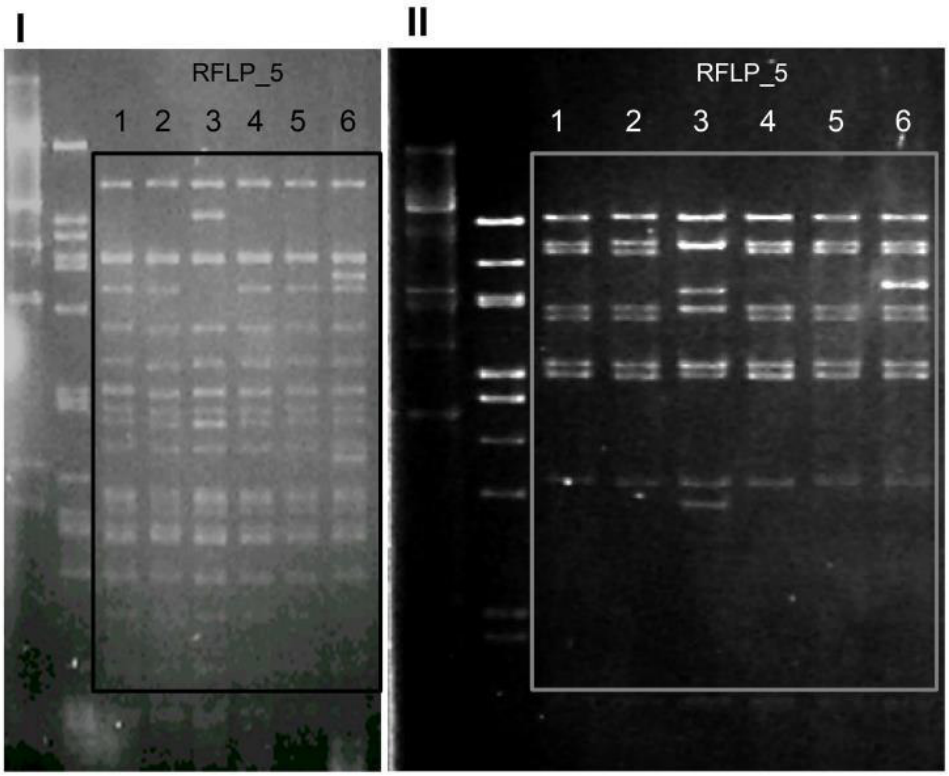

Figure 3. Restriction fragment length polymorphism patterns of plasmids showing RFLP_5 profiles after digestion with ClaI (I) and EcoRI (II) restriction enzymes (New England Biolabs Inc, UK).

Lane 1, RFLP_5 (PFGE H78, ST18 Efm,); lane 2, RFLP_5 (PFGE H72, ST18 Efm); lane 3, RFLP 5.2 (PFGE H100, ST280 Efm), lane 4, RFLP 5 (PFGE H78, ST18 Efm); lane 5, RFLP_5 (PFGE H78, ST18 Efm); lane 6, RFLP_5.2' (PFGE H132, ST132 Efm).

E. faecalis vanA plasmids

The *vanA* Efs plasmids were Inc18-pheromone-responsive mosaics, further classified in four main types on the basis of their RFLP patterns (RFLP_27–30), rep-rel/TA content, and replicase sequences. These plasmids have been documented in different Portuguese hospitals since the mid 90s [26].

Plasmids showing highly related patterns designated as RFLP_27 (carrying IS*Ef1*-Tn*1546)* or RFLP_28 (carrying IS*1216*-Tn*1546*) were recovered from both Efs (ST6, ST55, ST159) and Efm (ST80, ST132). However, despite the similarity of their RFLP patterns, they differed in the rep/rel/TA content and transposon variant content (Table 1). Conversely, the finding of an ST117 Efs isolate from Oporto with two different *vanA* plasmids of 150 kb and 300 kb indicates acquisition and further recombination of widespread pRUM-*vanA* plasmids from Efm with narrow host pheromone responsive plasmids of Efs.

The observed differences in transposon variants and plasmid modules reflect frequent rearrangements during transfer of plasmids between Efs and/or Efm clonal backgrounds and also highlight the connectivity of these enterococcal populations resulting in the acquisition and generation of plasmids with enhanced host range.

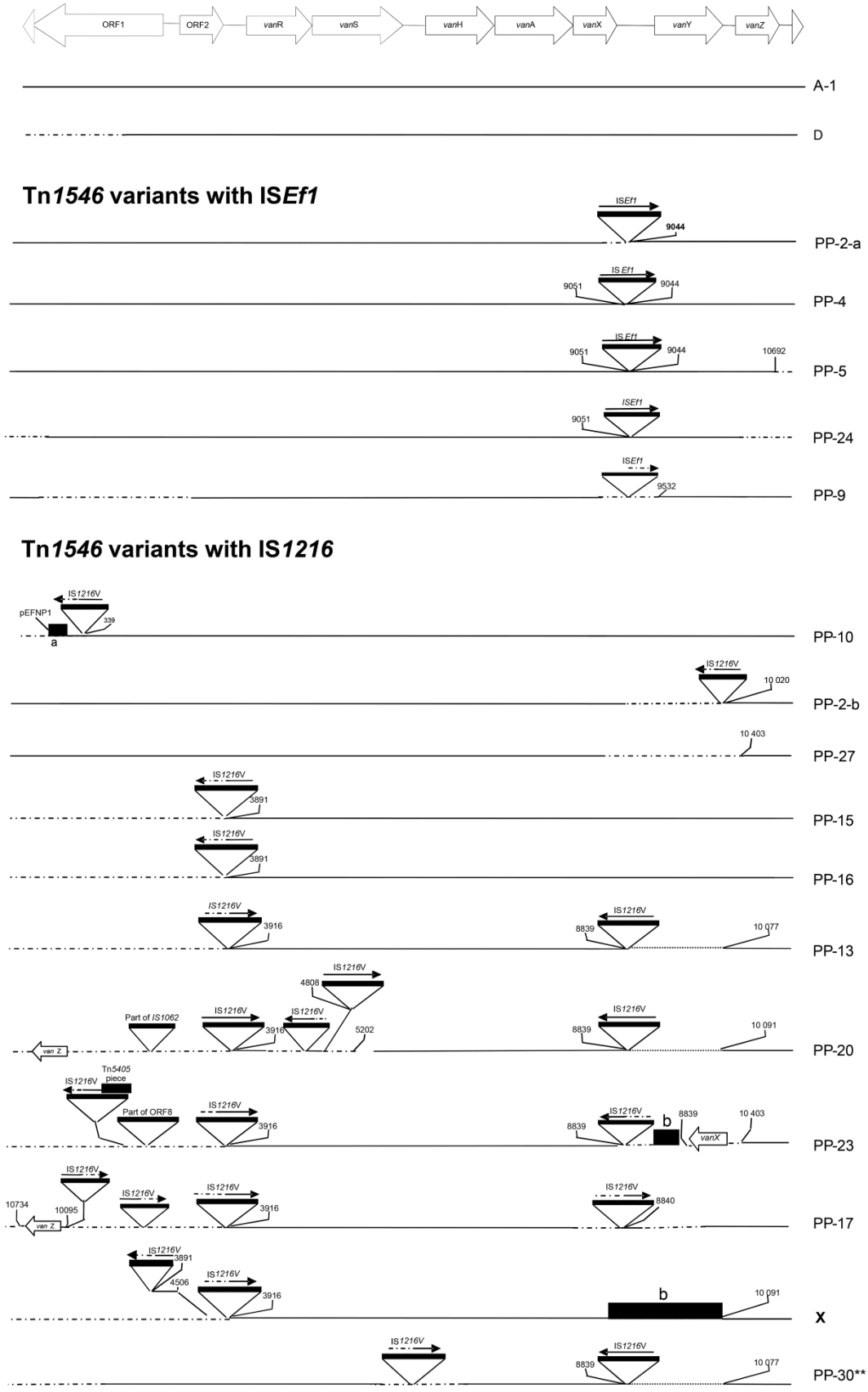

Figure 4. Genetic maps of Tn*1546* variants.

Tn*1546* variants are represented as previously described by Novais *et al*. [27] although grouped differently and specific types have been further explored (PP10, PP30): Tn*1546* prototype A corresponds to the original sequence described by Arthur *et al*. [44] and D corresponds to Tn*1546* variants from animals. Tn*1546* variants with IS*Ef1* within *vanX-vanY* intergenic region (PP2a, PP4, PP5,

PP9, PP24) and Tn*1546* variants with IS*1216* insertions at different positions (PP10, PP2b, PP13, PP15, PP16, PP17, PP20, PP23, PP27, PP30, X) are represented. The positions of genes and open reading frames and the direction of transcription are depicted with open arrows. IS elements are represented by triangles; other sequences are designated by rectangles. DNA insertions are represented highlighting the first nucleotide upstream and downstream from the insertion sites whenever known. Deletions are indicated by dots and discontinuous lines indicate sequences that were not characterized. (a) DNA sequence with homology to ORF3 (unknown protein product) and ORF1 (replication protein) of pEFNP1 plasmid (GenBank accession number AB038522). (b) DNA sequence with no match to any sequence available in GenBank. (*) PP23 was identified in an isolate susceptible to teicoplanin; this variant contained an insertion in the *vanY* gene that would affect the transcription ofvanZ and it might explain the susceptibility to this glycopeptide as previously reported [27]. (**) PP30 was identified in an ST78 isolate susceptible to both glycopeptides (MIC against vancomycin and teicoplanin of 4 mg/L) carrying *vanA*-Tn*1546*. This variant contained alterations within the *vanS-vanH* intergenic region (an IS*1216* insertion), which is involved in the expression and regulation of the resistance to vancomycin, and it constitutes the first description of a *vanA* isolate phenotypically susceptible to vancomycin in Portugal.

Fixation of *vanA*-Tn*1546* variants is associated with plasmid connectivity

Tn*1546* backbones were classified in three main groups corresponding to Tn*1546* with no insertion sequences (“type A” and “type D”) and variants containing IS*Ef1* (5 types) or IS*1216* (11 types) at different locations of the Tn*1546* backbone (Figure 4). Variants with a single copy of IS*Ef1* within the *vanX-vanY* region at nt 9044 were located on early (1996–1997) Efm plasmids identified as Inc18 and pRUM lacking Axe-Txe, and also on early (1996) Efs Inc18-pAD1 mosaics. Some of them were isolated from strains for more than one decade, which can be explained by their successful long-term recovered clonal and plasmid backgrounds.

Variants containing IS*1216* were mostly located on Inc18 plasmids or on mosaic plasmids Inc18-pRUM or Inc18-pAD1. Most variants contained the IS*1216* at 8839nt of the transposon (PP13, PP17, PP20, PP23, PP30) similarly to other Tn*1546* variants previously described in Europe [45]. Some of them also harboured different insertions corresponding to unknown sequences (X, PP23) or RCR plasmid sequences (PP10) [46] suggesting frequent recombination between acquired genes/plasmids and housekeeping Efm and Efs plasmids (Figure 4). Tn*1546* type D was specifically linked to megaplasmids from CC5 Efm from swine of different continents (Figure 2, Figure 4).

The presence of early plasmids carrying Tn*1546* belonging to different families suggests independent acquisitions of the transposon by pRUM and Inc18 plasmids, which would have been acquired by diverse Efm and Efs populations. Local fixation would be influenced by connectivity of plasmid and population backgrounds enabling further evolvability of transposon variants.

DISCUSSION

This paper shows the local dynamics of Tn*1546-vanA* among *Enterococci* is shaped by horizontal genetic transfer of pRUM and Inc18 plasmids and by recombination-driven evolution of them within and between Efs and Efm clones. The clonal diversity reported in this study has also been observed in areas where the spread of VRE has been documented [47]. Recent retrospective analysis of enterococcal populations suggests that the temporal evolution of the population biology of *Enterococci* is driven by a succession of epidemic waves of enterococcal human specific lineages, Efm ST78 and Efs ST6 emerging in the last decade at global scale similarly to that reported for other pathogens [19,23,24]. In Portugal, the population structure of VRE analysed in this study comprises isolates of main human Efm lineages,

ST18 (ST18, ST132) being much more abundant than ST17 (represented by a single isolate of early ST16 lineage) [31], or ST78 (represented by sporadic ST80 and ST656, the first one linked to early VRE outbreaks) [25,29]. It is worthwhile highlighting the recent detection of isolates of another Efm lineage in hospitals of the Oporto area (http://www.mlst.net) as ST117 Efm (ST78 lineage), which would reflect the increasing trend of isolates belonging to the ST78 lineage at international level. However, regional differences in the rates of VRE cannot be fully explained by clonal replacement dynamics since similar enterococcal clones appear widely distributed in areas with high and low rates of VRE (Tedim AP *et al.*, unpublished data). Instead, local conditions, including type and density of hosts, antibiotic usage, and transmission facilities, may influence regional differences in the proportions of VRE, as suggested by mathematical modelling studies on local trends of antibiotic resistance [48,49]. Clones can locally evolve by variation, drift and short-distance migration, leading to changes in colonization ability, pathogenicity or even host range, the fittest clonal variants being able to facilitate the spread of antibiotic resistance [23,50–53]. The observed clonal heterogeneity of the predominant ST18 lineage which comprises particular ST18 and ST132 strains widespread in different cities, highlights the role of certain efficiently transmissible clones in the dissemination of antibiotic resistance. Successful clones can eventually be able to disseminate at international level as strains of ST6 Efs or ST280 Efm within main Efm human lineages driving or contributing the spread of different traits as Tn*1546* or Tn*1549 [54]*. One remarkable fact is the similarity among PFGE patterns of isolates with different STs. Given the high content of plasmids and transposons of the isolates studied, and the frequent rearrangements identified among Efm and/or Efs isolates [21], chromosomal transfer cannot be discarded. Recent phylogenomic analysis based on the degree of admixture among a diversity of isolates studied suggests that recombination is restricted to isolates within specific BAPS groups [19]. Most plasmids coding for vancomycin resistance are found in similar clonal backgrounds. This observation suggests that recombination does occur within isolates of similar BAPS groups as recently described [19]. However, the observed mosaicism and enhanced host range of particular plasmid variants indicates the existence of an unexpectedly high degree of connectivity between phylogenetically distant enterococcal populations and/or in bacterial genetic exchange communities integrating enterococci.

Broad host and narrow host plasmids carrying vancomycin resistance would have a high "*betweenness centrality",* which is a pivotal index in network theory useful for measuring the load placed on the given node in the network as well as the node's importance to the network than just connectivity [55]. A recent *in silico* network analysis of all plasmid sequences available at the GenBank databases confirms very high *betweenness* values for some Inc18 plasmids as pVEF3 (an Inc18 derivative highly spread among Efm from animals in Europe) [13,39], and also for a pheromone-responsive plasmid pTEF1 (a plasmid recovered from ST6_Efs strain V583, highly related to the ST6 described in this work) [56] (unpublished data). Other plasmids with a high degree of *modular dissociability*, would be pRUM-like elements, which may enhance their complexity resulting in new configurations with enhanced *betweenness*. It is tempting to suggest that plasmid variability has contributed to intra-clonal diversification both in Efm and Efs, giving rise to a local wealth of clonal variants able to fully explore the

local adaptive landscape. In fact, this and other studies demonstrate that selected variants of Inc18, pAD1, and pRUM plasmids can determine differences in the dynamics of VRE in different areas, further influencing the plasmid host range and the selection of specific clones within human adapted lineages. Examples of widespread plasmid variants of Inc18 or pRUM plasmids coding for vancomycin resistance have been reported recently. They included Inc18 widespread among Efm poultry isolates from Europe [13] or among Efs clinical isolates from the USA, the last one being able to transfer Tn*1546* to *S. aureus* [15]; and mosaics of pRUM variants containing Axe-Txe and Inc18 from humans in different continents (Freitas AR *et al*. unpublished data). The identification of chimeric pRUM-Inc18 plasmids containing rep/rel/TA of Inc18 sequences and Tn*1546* variants widely observed in poultry, hospitals and hospital sewage in the Oporto area reflects genetic exchanges between enterococci from different origins and highlights the need to enforce barriers to avoid the spread of multi-drug resistance human pathogens to the environment and *vice-versa*.

In this scenario, the genetic context of Tn*1546* seems to greatly influence the evolvability of the transposon and explains the high diversity of variants found in this and other studies [1,27,45,57]. The frequent presence of insertions in the backbone of Tn*1546* and the abundance of IS*1216* and IS*Ef1* in enterococcal genomes [9,58] makes homoplasic evolution of Tn*1546* in different backgrounds possible. However, other IS (IS*1251*, IS*1542*, IS*1476*, IS*19* and IS*1485*) linked to different plasmid and clonal backgrounds [9,42] have been identified at different sites of Tn*1546,* thus suggesting that chance and selection are responsible to differences in variants collected in different areas. The widespread of Inc18 plasmids with a common origin in Europe [13,59] indicates local fixation of Tn*1546* influenced by a founder effect and further connectivity of plasmid and population backgrounds enabling further evolvability of transposon variants as reported in this study.

Our results suggest that VRE spread is facilitated by selected clones of different lineages through strong interactive processes of clonalization and plasmid diversification that might occur at local scales. Despite the maintenance of significant gene flow, a sympatric, or more probably, parapatric bacterial clonalization process (when diverging populations share a common or neighbouring environment), might contribute to the formation of temporary genetic mosaics and the preservation of ecologically important genomic traits [60]. Such micro-evolutionary process will result in an array of clonal complexes forming a population structure able to exploit the local spatio-temporal patch heterogeneities [61]. Note that exploitation of connected microenvironments should accelerate evolution of antibiotic resistance [62]. The expected result of such a successful population structure is the local persistence of antibiotic resistant clones, and eventually the local fixation [63] of vancomycin-resistance [49].

In summary, this study highlights the relevance of studying the local microecology of genes, elements, lineages and populations to decipher the robustness of the trans-hierarchical networks connecting these evolutionary elements in order to describe and predict the local evolvability of vancomycin-resistance

[64]. Traditional surveillance studies are *one-off cross* sectional surveys focused on single traits as epidemic strains, genes or mobile genetic elements over limited periods of time which only gives *one shot* view that precludes addressing the long-term dynamics of antibiotic resistance. The more comprehensive approach described in this study is needed for understanding in depth the evolution of complexity in multi-hierarchical systems as those involved in the spread of antibiotic resistance among the populations of bacterial human pathogens.

MATERIALS AND METHODS

Bacterial strains and epidemiological background

One hundred four VRE clinical isolates carrying Tn*1546* from different regions of Portugal, 75 VREfm and 29 VREfs, were analyzed in this study. They included: i) clinical isolates from hospitals of Coimbra (Hospital Universitário de Coimbra, HUC), Oporto (Hospital Santo António, HSA), Viseu (Hospital de São Teotónio, HST); Matosinhos (Hospital Pedro Hispano, HPH), Vila Real (Hospital S. Pedro, HVR) and Covilhã (Centro Hospitalar da Cova da Beira, CHCB) located in Northern and Central Portugal (62 Efm and 26 Efs; 1996–2008); ii) isolates from waste waters of hospitals (HSA and Hospital de São João, HSJ) (10 Efm and 3 Efs), and iii) isolates from the estuary of the River Douro (3 Efm) recovered in the Oporto area during 2001–2003. Part of the isolates analyzed in this work corresponds to strains from previous surveillance studies [25–27,65]; this paper constitutes the first description of isolates obtained during 2007 and 2008. Contemporary Portuguese VRE isolates of animal origin were used for comparative analysis of lateral transfer events [10].

Susceptibility against 15 antibiotics was determined by the agar dilution method following CLSI standard guidelines. Clonal relatedness was established by pulsed-field gel electrophoresis (PFGE), banding patterns were interpreted according to criteria previously suggested for long-term studies, and multilocus sequence typing (MLST) as described elsewhere (http://efaecium.mlst.net) [25,66–68].

The presence of putative virulence traits [collagen-binding adhesin (*acm*), enterococcal surface protein (*esp*), hyaluronidase ($hyl_{E.\ faecium}$), cytolysin/hemolysin (*cyl*), gelatinase (*gelE*) and aggregation substance (*agg*)] was searched by using PCR as described [69,70].

Genetic context of Tn*1546*

Characterization of Tn*1546* backbone was determined by amplification of overlapping transposon fragments and further sequencing of PCR products [27,33]. We have accomplished the analysis for the isolates not studied in previous surveys and have interpreted the resulting transposon diversity (this study) [27], under the light of the plasmid and clonal backgrounds identified in this geographical area.

Plasmid analysis

Isolates (n=62 Efm and n=13 Efs) representing the clonal diversity observed in both species were selected for plasmid characterization (Table 1, Figure 2). The content and size of plasmids from transconjugants obtained by filter mating were determined by using either the technique described by Barton *et al.* (plasmids >10 kb) or the alkaline lysis extraction method of Kado & Liu (plasmids <10 kb) [54,71,72]. Classification of *E. faecium* plasmids was based on the presence of specific modules for replication (rep-initiator proteins), mobilization (relaxases) and stability (toxin-antitoxin systems). *Relaxases* (rel) were sought by a multiplex-PCR-based relaxase typing method which differentiates relaxases of the MOB_Q, MOB_P, MOB_C and MOB_V families related to 27 known plasmids [9,73] (Francia MV, unpublished data). *Replication initiator proteins* (rep) were investigated by amplification of 24 replicons, which allows discriminating among DNA sequences from more than 100 published Gram-positive plasmids [9,34]. Designation of rep sequences pointed out the plasmid type in which they were initially identified, as well as the numeric nomenclature originally used by Jensen *et al*. (Figure 2's footnote) [34]. *Toxin-antitoxin systems* (TA) previously identified among streptococci and enterococci (Axe-Txe, ω-ε-ζ, *par, maz*EF) or Gram-negative bacteria (*rel*BE) were detected by PCR [74]. PCR products were sequenced in order to confirm the specificity of the method and to analyze similarities with other well-characterized plasmids. Genomic location of the Tn*1546* and the rel/rep/TA sequences was determined by hybridization of *vanA* and *rel/rep/TA* specific probes obtained by PCR from DNA from reference plasmids with *S*1 or *I-Ceu*I digested genomic DNA from representative strains [54,71]. Structural relationship between plasmids of similar size was established by comparison of their RFLP patterns obtained after digestion with different restriction enzymes (*Eco*RI, *Hind*III and *Cla*I; see Figure 3). Plasmid DNA was obtained by using a modified protocol based on the alkaline lysis method described by Handwerger *et al.* [75] consisting of increasing two-fold the volume of lysozyme, SDS/NaOH and acetate potassium solutions, extending the incubation period in potassium acetate solution for at least three hours, precipitating the supernatant obtained after extraction with phenol-chloroform using ethanol-acetate potassium solution (2:0.1 vol/vol) at 25°C for at least 2 hours, and resuspending final DNA pellets in 30 µl of water for further enzyme digestion analysis.

Molecular techniques

Southern blot DNA transfer and hybridization were performed by standard procedures [76]. The *vanA* and rep/rel/TA/bac probes used in the hybridization assays were generated by PCR using well known positive controls as template DNA. Labelling and detection were carried out using Gene Images Alkphos Direct Labelling system kit, following the manufacturer's instructions (Amersham GB/GE Healthcare Life Sciences UK Limited). PFGE was performed as described previously [77] using the following conditions: switch time of 5 s to 25 s for 6 h, followed by 30 s to 45 s for 18 h (*S1* nuclease); 5 s to 30 s for 22 h, 14°C, and 6 V/cm2 (*I-Ceu*I) and 1 s to 20 s for 26 h, 14°C, and 6 V/cm2 (*Sma*I).

Plasmid sequences

Analysis of nucleotide and amino acid sequences revealed two types of sequences amplified with primers used for identification of $rep_{17/pRUM}$. They were 100% (designated as $Rep_{17.1/pRUM}$) or 97% (96% identity at amino acid level; designated as $Rep_{17.2/pRUM\text{-}like}$) homologous to that of RepA_pRUM (GenBank accession number AF507977). Most $Rep_{1/pIP501}$ amino acid sequences were 98%–100% identical to RepE_pIP816, a member of the Inc18 family (GenBank accession number AM932524), and to a lesser degree to pRE25, pTEF1 or pSM19035; and $Rep_{2/pRE25/pEF1}$ showed 96%–100% amino acid identity to that of pEF1 (GenBank acc. no. DQ198088). Sequences identified as $Rel_{6/pEF1}$ showed 98%–100% homology to orf34_pEF1. Relaxases of the *E. faecalis* pheromone-responsive plasmids identified in this study displayed a high homology with those of known enterococcal pheromone plasmids pAD1, pAM373 and pTEF1 (orf57_pAD1, GenBank acc. no. AAL59457; EFA0025_pTEF1, GenBank AE016833; and EP0019_pAM373, GenBank acc. no. NC_002630). That of plasmid showing RFLP_27 showed a 67–84% homology with the above mentioned pheromone enterococcal plasmids but 94% identity with a MobC relaxase (annotated as a hypothetical protein) from a vancomycin-resistant *S. aureus* strain (GenBank acc. no. EIK35827).

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REFERENCES

1. Bonten MJ, Willems R, Weinstein RA. *Vancomycin-resistant enterococci: why are they here, and where do they come from?* Lancet Infect Dis. 2001;1: 314–25.

2. Murray BE. *Vancomycin-resistant enterococcal infections*. N Engl J Med. 2000;342: 710–21.

3. Werner G, Coque TM, Hammerum AM, Hope R, Hryniewicz W, Johnson A, et al. *Emergence and spread of vancomycin resistance among enterococci in Europe*. Euro Surveill. 2008;13.

4. De Lencastre H, Brown AE, Chung M, Armstrong D, Tomasz A. *Role of transposon Tn5482 in the epidemiology of vancomycin-resistant Enterococcus faecium in the pediatric oncology unit of a New York City Hospital.* Microb Drug Resist. 1999;5: 113–29.

5. Martone WJ. *Spread of vancomycin-resistant enterococci: why did it happen in the United States?* Infect Control Hosp Epidemiol. 1998;19: 539–45.

6. Thal L, Donabedian S, Robinson-Dunn B, Chow JW, Dembry L, Clewell DB, et al. *Molecular analysis of glycopeptide-resistant Enterococcus faecium isolates collected from Michigan hospitals over a 6-year period*. J Clin Microbiol. 1998;36: 3303–8.

7. Kawalec M, Gniadkowski M, Hryniewicz W. *Outbreak of Vancomycin-Resistant Enterococci in a Hospital in Gdansk, Poland, due to Horizontal Transfer of Different Tn1546-Like Transposon Variants and Clonal Spread of Several Strains*. J Clin Microbiol. 2000;38: 3317–3322.

8. Stampone L, Del Grosso M, Boccia D, Pantosti A. *Clonal spread of a vancomycin-resistant Enterococcus faecium strain among bloodstream-infecting isolates in Italy.* J Clin Microbiol. 2005;43: 1575–80. doi:10.1128/JCM.43.4.1575-1580.2005

9. Clewell DB, Weaver KE, Coque TM, Dunny GM, Francia MV, Hayes F. *Extrachromosomal and Mobile Elements in Enterococci: Transmission, Maintenance, and Epidemiology*. *In*: Gilmore MS, Clewell DB, Ike Y, Shankar N, editors. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Boston: Massachusetts Eye and Ear Infirmary; 2014.

10. Freitas AR, Coque TM, Novais C, Hammerum AM, Lester CH, Zervos MJ, et al. *Human and swine hosts share vancomycin-resistant Enterococcus faecium CC17 and CC5 and Enterococcus faecalis CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids.* J Clin Microbiol. 2011;49: 925–31. doi:10.1128/JCM.01750-10

11. Laverde Gomez JA, van Schaik W, Freitas AR, Coque TM, Weaver KE, Francia MV, et al. *A multiresistance megaplasmid pLG1 bearing a hylEfm genomic island in hospital Enterococcus faecium isolates.* Int J Med Microbiol. 2011;301: 165–75. doi:10.1016/j.ijmm.2010.08.015

12. Rosvoll TCS, Pedersen T, Sletvold H, Johnsen PJ, Sollid JE, Simonsen GS, et al. *PCR-based plasmid typing in Enterococcus faecium strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHTbeta-related replicons associated with glycopeptide resistance and stabilizing toxin-antitoxin systems*. FEMS Immunol Med Microbiol. 2010;58: 254–68. doi:10.1111/j.1574-695X.2009.00633.x

13. Sletvold H, Johnsen PJ, Wikmark O-G, Simonsen GS, Sundsfjord A, Nielsen KM. *Tn1546 is part of a larger plasmid-encoded genetic unit horizontally disseminated among clonal Enterococcus faecium lineages*. J Antimicrob Chemother. 2010;65: 1894–906. doi:10.1093/jac/dkq219

14. Tomita H, Pierson C, Lim SK, Clewell DB, Ike Y. *Possible connection between a widely disseminated conjugative gentamicin resistance (pMG1-like) plasmid and the emergence of vancomycin resistance in Enterococcus faecium.* J Clin Microbiol. 2002;40: 3326–33.

15. Zhu W, Murray PR, Huskins WC, Jernigan JA, McDonald LC, Clark NC, et al. *Dissemination of an Enterococcus Inc18-Like vanA plasmid associated with vancomycin-resistant Staphylococcus aureus.* Antimicrob Agents Chemother. 2010;54: 4314–20. doi:10.1128/AAC.00185-10

16. Valdezate S, Miranda C, Navarro A, Freitas AR, Cabrera JJ, Carrasco G, et al. *Clonal outbreak of ST17 multi-drug-resistant Enterococcus faecium harbouring an Inc18-like::Tn1546 plasmid in a haemo-oncology ward of a Spanish hospital.* J Antimicrob Chemother. 2012;67: 832–6. doi:10.1093/jac/dkr545

17. Garcia-Migura L, Sanchez-Valenzuela AJ, Jensen LB. *Presence of glycopeptide-encoding plasmids in enterococcal isolates from food and humans in Denmark.* Foodborne Pathog Dis. 2011;8: 1191–7. doi:10.1089/fpd.2011.0897

18. Qu T-T, Yang Q, Shen P, Wei Z-Q, Yu Y-S. *Novel vancomycin-resistance transposon, plasmid replicon types, and virulence factors of vancomycin-resistant Enterococci in Zhejiang, China*. Microb Drug Resist. 2012;18: 183–8. doi:10.1089/mdr.2011.0140

19. Willems RJL, Top J, van Schaik W, Leavis H, Bonten M, Sirén J, et al. *Restricted gene flow among hospital subpopulations of Enterococcus faecium.* MBio. 2012;3: e00151–12.

20. McBride SM, Fischetti VA, Leblanc DJ, Moellering RC, Gilmore MS. *Genetic diversity among Enterococcus faecalis*. PLoS One. 2007;2: e582.

21. Manson JM, Hancock LE, Gilmore MS. *Mechanism of chromosomal transfer of Enterococcus faecalis pathogenicity island, capsule, antimicrobial resistance, and other traits*. Proc Natl Acad Sci U S A. 2010;107: 12269–7422. Leavis HL, Bonten MJM, Willems RJL. *Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance*. Curr Opin Microbiol. 2006;9: 454–60.

23. Wyllie D, Paul J, Crook D. Waves of trouble: MRSA strain dynamics and assessment of the impact of infection control. J Antimicrob Chemother. 2011;66: 2685–8. doi:10.1093/jac/dkr392

24. Chambers HF, Deleo FR. Waves of resistance: Staphylococcus aureus in the antibiotic era. Nat Rev Microbiol. 2009;7: 629–41.

25. Freitas AR, Novais C, Ruiz-Garbajosa P, Coque TM, Peixe L. *Dispersion of multi-drug-resistant Enterococcus faecium isolates belonging to major clonal complexes in different Portuguese settings*. Appl Environ Microbiol. 2009;75: 4904–8.

26. Freitas AR, Novais C, Ruiz-Garbajosa P, Coque TM, Peixe L. *Clonal expansion within clonal complex 2 and spread of vancomycin-resistant plasmids among different genetic lineages of Enterococcus faecalis from Portugal*. J Antimicrob Chemother. 2009;63: 1104–11.

27. Novais C, Freitas AR, Sousa JC, Baquero F, Coque TM, Peixe L V. *Diversity of Tn1546 and its role in the dissemination of vancomycin-resistant enterococci in Portugal.* Antimicrob Agents Chemother. 2008;52: 1001–8.

28. Willems RJL, Hanage WP, Bessen DE, Feil EJ. *Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance*. FEMS Microbiol Rev. 2011;35: 872–900. doi:10.1111/j.1574-6976.2011.00284.x

29. Mato R, Almeida F, Pires R, Rodrigues P, Ferreira T, Santos-Sanches I. *Assessment of high-level gentamicin and glycopeptide-resistant Enterococcus faecalis and E. faecium clonal structure in a Portuguese hospital over a 3-year period*. Eur J Clin Microbiol Infect Dis. 2009;28: 855–859.

30. Araújo C, Torres C, Gonçalves A, Carneiro C, López M, Radhouani H, et al. *Genetic detection and multilocus sequence typing of vanA-containing Enterococcus strains from mullets fish (Liza ramada).* Microb Drug Resist. 2011;17: 357–61.

31. Galloway-Peña JR, Nallapareddy SR, Arias CA, Eliopoulos GM, Murray BE. *Analysis of clonality and antibiotic resistance among early clinical isolates of Enterococcus faecium in the United States*. J Infect Dis. 2009;200: 1566–73. doi:10.1086/644790

32. Willems RJ, van Schaik W. *Transition of Enterococcus faecium from commensal organism to nosocomial pathogen.* Future Microbiol. 2009;4: 1125–35.

33. Woodford N, Adebiyi AM, Palepou MF, Cookson BD*. Diversity of VanA glycopeptide resistance elements in enterococci from humans and nonhuman sources*. Antimicrob Agents Chemother. 1998;42: 502–8.

34. Jensen LB, Garcia-Migura L, Valenzuela AJS, Løhr M, Hasman H, Aarestrup FM. *A classification system for plasmids from enterococci and other Gram-positive bacteria.* J Microbiol Methods. 2010;80: 25–43.

35. Ruiz-Barba JL, Floriano B, Maldonado-Barragán A, Jiménez-Díaz R. *Molecular analysis of the 21-kb bacteriocin-encoding plasmid pEF1 from Enterococcus faecium 6T1a.* Plasmid. 2007;57: 175–81.

36. Weaver KE, Kwong SM, Firth N, Francia MV. *The RepA_N replicons of Gram-positive bacteria: a family of broadly distributed but narrow host range plasmids*. Plasmid. 2009;61: 94–109.

37. Schwarz F V, Perreten V, Teuber M. *Sequence of the 50-kb conjugative multiresistance plasmid pRE25 from Enterococcus faecalis RE25*. Plasmid. 2001;46: 170–87.

38. Sletvold H, Johnsen PJ, Simonsen GS, Aasnaes B, Sundsfjord A, Nielsen KM. *Comparative DNA analysis of two vanA plasmids from Enterococcus faecium strains isolated from poultry and a poultry farmer in Norway.* Antimicrob Agents Chemother. 2007;51: 736–9.

39. Sletvold H, Johnsen PJ, Hamre I, Simonsen GS, Sundsfjord A, Nielsen KM. *Complete sequence of Enterococcus faecium pVEF3 and the detection of an omega-epsilon-zeta toxin-antitoxin module and an ABC transporter.* Plasmid. 2008;60: 75–85.

40. Grady R, Hayes F. *Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multi-drug-resistant, clinical isolate of Enterococcus faecium*. Mol Microbiol. 2003;47: 1419–32.

41. Halvorsen EM, Williams JJ, Bhimani AJ, Billings EA, Hergenrother PJ. *Txe, an endoribonuclease of the enterococcal Axe-Txe toxin-antitoxin system, cleaves mRNA and inhibits protein synthesis*. Microbiology. 2010;157: 387–397.

42. Coque TM, Freitas AR, Novais C, Peixe L, Baquero F. *Mobile Genetic Elements and Lateral Genetic Transfer in Enterococci*. *In*: Semedo-Lemsaddek T, Barreto-Crespo MT, Tenreiro R, editors*. Enterococcus and Safety*. Nova Science Publishers; 2012. pp. 59–100.

43. Sørum M, Johnsen PJ, Aasnes B, Rosvoll T, Kruse H, Sundsfjord A, et al. *Prevalence, persistence, and molecular characterization of glycopeptide-resistant enterococci in Norwegian poultry and poultry farmers 3 to 8 years after the ban on avoparcin*. Appl Environ Microbiol. 2006;72: 516–21.

44. Arthur M, Molinas C, Depardieu F, Courvalin P. *Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in Enterococcus faecium BM4147*. J Bacteriol. 1993;175: 117–27.

45. Schouten MA, Willems RJ, Kraak WA, Top J, Hoogkamp-Korstanje JA, Voss A. *Molecular analysis of Tn1546-like elements in vancomycin-resistant enterococci isolated from patients in Europe shows geographic transposon type clustering*. Antimicrob Agents Chemother. 2001;45: 986–9.

46. Garcia-Migura L, Hasman H, Jensen LB. *Presence of pRI1: a small cryptic mobilizable plasmid isolated from Enterococcus faecium of human and animal origin*. Curr Microbiol. 2009;58: 95–100.

47. Zheng B, Tomita H, Xiao YH, Wang S, Li Y, Ike Y. *Molecular characterization of vancomycin-resistant enterococcus faecium isolates from mainland China*. J Clin Microbiol. 2007;45: 2813–8.

48. McCormick AW, Whitney CG, Farley MM, Lynfield R, Harrison LH, Bennett NM, et al. *Geographic diversity and temporal trends of antimicrobial resistance in Streptococcus pneumoniae in the United States*. Nat Med. 2003;9: 424–30.

49. Johnsen PJ, Townsend JP, Bøhn T, Simonsen GS, Sundsfjord A, Nielsen KM. *Factors affecting the reversal of antimicrobial-drug resistance*. Lancet Infect Dis. 2009;9: 357–64.

50. Price LB, Stegger M, Hasman H, Aziz M, Larsen J, Andersen PS, et al. *Staphylococcus aureus CC398: host adaptation and emergence of methicillin resistance in livestock.* MBio. 2012;3: e00305–11.

51. Robinson DA, Kearns AM, Holmes A, Morrison D, Grundmann H, Edwards G, et al. *Re-emergence of early pandemic Staphylococcus aureus as a community-acquired meticillin-resistant clone*. Lancet. 2005;365: 1256–1258. doi:10.1016/S0140-6736(05)74814-5

52. De Regt MJA, van Schaik W, van Luit-Asbroek M, Dekker HAT, van Duijkeren E, Koning CJM, et al. *Hospital and community ampicillin-resistant Enterococcus faecium are evolutionarily closely linked but have diversified through niche adaptation.* Ahmed N, editor. PLoS One. 2012;7: e30319.

53. Li M, Du X, Villaruz AE, Diep BA, Wang D, Song Y, et al. *MRSA epidemic linked to a quickly spreading colonization and virulence determinant*. Nat Med. 2012;18: 816–9.

54. Freitas AR, Tedim AP, Novais C, Ruiz-Garbajosa P, Werner G, Laverde-Gomez JA, et al. *Global spread of the hyl(Efm) colonization-virulence gene in megaplasmids of the Enterococcus faecium CC17 polyclonal subcluster*. Antimicrob Agents Chemother. 2010;54: 2660–5.

55. Freeman LC. *A set of measures of centrality based on betweenness*. Sociometry. 1977;40: 35–41.

56. Tamminen M, Virta M, Fani R, Fondi M. *Large-scale analysis of plasmid relationships through gene-sharing networks.* Mol Biol Evol. 2012;29: 1225–40.

57. Willems RJ, Top J, van den Braak N, van Belkum A, Mevius DJ, Hendriks G, et al. Molecular diversity and evolutionary relationships of Tn1546-like elements in enterococci from humans and animals. Antimicrob Agents Chemother. 1999;43: 483–91.

58. Paulsen IT. *Role of Mobile DNA in the Evolution of Vancomycin-Resistant Enterococcus faecalis*. Science. 2003;299: 2071–2074.

59. Garcia-Migura L, Hasman H, Svendsen C, Jensen LB. *Relevance of hot spots in the evolution and transmission of Tn1546 in glycopeptide-resistant Enterococcus faecium (GREF) from broiler origin*. J Antimicrob Chemother. 2008;62: 681–7.

60. Via S. *Natural selection in action during speciation*. Proc Natl Acad Sci U S A. 2009;106 Suppl : 9939–46.

61. Levin SA, Paine RT. *Disturbance, patch formation, and community structure*. Proc Natl Acad Sci U S A. 1974;71: 2744–7.

62. Zhang Q, Lambert G, Liao D, Kim H, Robin K, Tung C, et al. *Acceleration of emergence of bacterial antibiotic resistance in connected microenvironments*. Science. 2011;333: 1764–7.

63. Aguilée R, Claessen D, Lambert A. *Allele fixation in a dynamic metapopulation: founder effects vs refuge effects.* Theor Popul Biol. 2009;76: 105–17. doi:10.1016/j.tpb.2009.05.003

64. Baquero F, Tedim AP, Coque TM. A*ntibiotic resistance shaping multi-level population biology of bacteria*. Front Microbiol. 2013;4: 15.

65. Novais C, Coque TM, Ferreira H, Sousa JC, Peixe L. *Environmental contamination with vancomycin-resistant enterococci from hospital sewage in Portugal*. Appl Environ Microbiol. 2005;71: 3364–8. doi:10.1128/AEM.71.6.3364-3368.2005

66. Novais C, Sousa JC, Coque TM, Peixe L V. *Molecular characterization of glycopeptide-resistant Enterococcus faecium isolates from Portuguese hospitals*. Antimicrob Agents Chemother. 2005;49: 3073–9. doi:10.1128/AAC.49.7.3073-3079.2005

67. Tenover FC, Arbeit RD, Goering R V, Mickelsen PA, Murray BE, Persing DH, et al. *Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing*. J Clin Microbiol. 1995;33: 2233–9.

68. Morrison D, Woodford N, Barrett SP, Sisson P, Cookson BD. *DNA banding pattern polymorphism in vancomycin-resistant Enterococcus faecium and criteria for defining strains*. J Clin Microbiol. 1999;37: 1084–91.

69. Nallapareddy SR, Weinstock GM, Murray BE. *Clinical isolates of Enterococcus faecium exhibit strain-specific collagen binding mediated by Acm, a new member of the MSCRAMM family*. Mol Microbiol. 2003;47: 1733–47.

70. Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, et al. *Development of a multiplex PCR for the detection of asa1, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among European hospital isolates of Enterococcus faecium*. J Clin Microbiol. 2004;42: 4473–9.

71. Barton BM, Harding GP, Zuccarelli AJ. *A general method for detecting and sizing large plasmids. Anal Biochem*. 1995;226: 235–40.

72. Kado CI, Liu ST. *Rapid procedure for detection and isolation of large and small plasmids*. J Bacteriol. 1981;145: 1365–73.

73. Garcillán-Barcia MP, Francia MV, de la Cruz F. *The diversity of conjugative relaxases and its application in plasmid classification*. FEMS Microbiol Rev. 2009;33: 657–87.

74. Moritz EM, Hergenrother PJ. *Toxin-antitoxin systems are ubiquitous and plasmid-encoded in vancomycin-resistant enterococci*. Proc Natl Acad Sci U S A. 2007;104: 311–6.

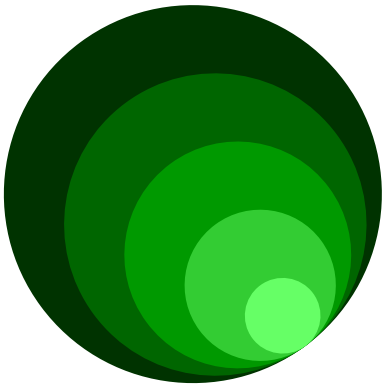
75. Handwerger S, Skoble J. *Identification of chromosomal mobile element conferring high-level vancomycin resistance in Enterococcus faecium*. Antimicrob Agents Chemother. 1995;39: 2446–53.

76. Sambrook J, Frisch E, Maniatis T. *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.

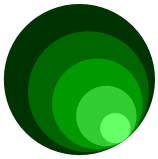
77. Kaufmann ME. *Pulsed-Field Gel Electrophoresis*. *In*: Woodford N, Johnson A, editors. *Molecular Bacteriology: protocols and clinical applications*. Totowa, NJ: Humana Press: Inc; 1998. pp. 17–31.

I don't pretend we have all the answers. But the questions are certainly worth thinking about.

Arthur C. Clarke



Chapter 6



Co-diversification of transferable ampicillin-resistant PBP5 and *Enterococcus faecium* phylogenomic groups

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Authors contribution:

C.N., L.P. and T.M.C. design the study. C.N., A.R.F., A.P.R. M.H.A, E.S. and R.E. performed wet lab experiments and participated in the analysis of the data. A.P.T. and V.F.L. performed bioinformatic analysis. F.B., A.P.R. and L.P. provided expertise, participated in the analysis of data, and in the revision of the manuscript. C.N., A.P.T and T.M.C. performed the analysis of data and wrote the manuscript. All authors approved the final version of the manuscript.

Frontiers in Microbiology *in review*

ABSTRACT

Ampicillin-resistance is probably a major cause of the recent dramatic increase of a cluster of human adapted *Enterococcus faecium* lineages (ST17, ST18 and ST78) in hospital-based infections. Different levels of ampicillin-susceptibility were associated with changes in the *pbp5* gene, leading to protein variants (here designated as PBP5 C-types) with diverse degrees of reduction in penicillin affinity. The analyses of 78 *E. faecium* strains, as well as published *E. faecium* genomes, suggests that the diversity of *pbp5* mirrors the phylogenomic diversification of *E. faecium*. The presence of identical PBP5 C-types as well as similar *pbp5* genetic milieu in different *E. faecium* lineages and clones from quite different geographical and environmental origin was also documented and would indicate their horizontal gene transfer among *E. faecium* populations. This was supported by experimental assays showing transfer of large (≈180-280 kb) chromosomal genetic platforms containing *pbp5* alleles, *ponA* (transglycosilase) and other metabolic and adaptive features, from *E. faecium* donor isolates to suitable *E. faecium* recipient strains (10^{-11}-10^{-12} UFC/recipient). Mutation profile analysis of PBP5 from available genomes and strains from this study suggests that the spread of PBP5 C-types might have occurred even in the absence of a significant ampicillin-resistance phenotype, but changes would have been facilitated by ampicillin exposure and, eventually, by compensatory evolution. In summary, genetic platforms containing polymorphic *pbp5* sequences were able to be stably maintained in particular *E. faecium* lineages, but were also able to be transferred among *E. faecium* clones of different origins, emphasizing the growing risk of further spread of ampicillin-resistance in this nosocomial pathogen.

INTRODUCTION

Infections caused by *Enterococcus faecium* have increasingly been reported since the early 1980s' (Top et al., 2007). Currently, most clinical isolates of *E. faecium* are ampicillin resistant (AmpR) which are stably maintained for long periods in the hospital setting, serving as substrate for the acquisition of genetic elements including transposons coding for resistance to vancomycin (Arias and Murray, 2012; Wagenvoort et al., 2015). Multidrug resistant *E. faecium* isolates frequently cause infections associated with treatment failure and high mortality rates (Arias et al., 2010; Centers for Disease Control and Prevention, 2013)

E. faecium is intrinsically resistant to cephalosporins and exhibits reduced susceptibility to penicillins due to the low affinity class B penicillin binding protein (PBP) 5, a transpeptidase that requires the participation of class A PBPs with glycosyltransferase activity (PonA, PbpF) to synthesize the cell wall in the presence of cephalosporins (Rice et al., 2009; Williamson et al., 1983). Acquired high level of AmpR in *E. faecium* was initially linked to either the enhanced production of PBP5 or polymorphisms in the beta subunit of this protein (Fontana et al., 1996). Soon after, it was demonstrated that single point mutations (Sifaoui et al., 2001), the levels of *pbp5* mRNA expression (Belhaj et al., 2016), or the quantities of relative *pbp5* transcripts were frequently strain specific and did not necessarily correlate with differences in the MIC values (Fontana et al., 1996; Rice et al., 2001, 2004; Rybkine et al., 1998; Zorzi et al., 1996). Further analysis of the PBP5 sequences of *E. faecium* isolates with different ampicillin susceptibility levels (0,5->128 mg/L) revealed that the highest variability of PBP5 sequences occurred in 21 specific positions of the protein and suggested that a sequential acquisition of mutations could have resulted in the progressive tolerance to ampicillin from the early 1980s onwards (Galloway-Peña et al., 2011; Pietta et al., 2014). Additional AmpR mechanisms described in *E. faecium* included mutations in genes encoding other species-specific proteins that participate in the cell wall synthesis as D,D-carboxypeptidases (Ddcp and DdcY), L,D-transpeptidases (Ldt_{fm}), glycosiltransferases (PgtA) and acetylmuramoyl-L-alanine amidase (LytG) resulting in slightly increased MICs values for ampicillin (Kristich et al., 2014; Rice et al., 2007; Zhang et al., 2012), even in the absence of PBP5 (Sacco et al., 2014). Also, the occurrence of β-lactamases has been documented in *E. faecium,* although β-lactamase producers still remain rare in this species (Coudron et al., 1992; Hendrickx et al., 2013; Klare et al., 1992; Zhang et al., 2012). A particularly relevant observation was the transferability of a large 60kb chromosomal region comprising the *pbp5* gene and a transposon that confers resistance to glycopeptides (*vanB2*-CTn*5386*) (Carias et al., 1998; Rice et al., 2005b).

Most AmpR *E. faecium* isolates belong to one of the two phylogenomic groups or sub-populations, namely "clade A" or hospital-associated clade, which mainly comprises isolates from hospitalized patients (Galloway-Peña et al., 2012; Lebreton et al., 2013; Palmer et al., 2012). Clonal groups within a subgroup of "clade A", namely "clade A1", are enriched in mobile genetic elements and have enhanced ability to colonize and persist in human hosts due to the presence of adhesins and specific metabolic

traits (Freitas et al., 2010a, 2010b; Heikens et al., 2007; Top et al., 2008; Willems et al., 2005; Zhang et al., 2012). In contrast, "clade B", the community-associated *E. faecium* subpopulation, mostly comprises ampicillin susceptible (AmpS) isolates from healthy non-hospitalized persons (Lebreton et al., 2013; Tedim et al., 2015). The increasing detection of AmpR among isolates from hosts and environments not associated with the hospital setting (Gonçalves et al., 2011; Novais et al., 2013; de Regt et al., 2012; Santos et al., 2013; Tremblay et al., 2013) is of concern, as the acquisition of transferable genes encoding AmpR might facilitate its further spread into other AmpS *E. faecium* populations or to other less frequently recovered enterococcal species for which AmpR has been exceptionally documented (Raze et al., 1998).

Available knowledge about the diversity of AmpR genotypes in *E. faecium* is fractionated, and mainly focused on the PBP5 polymorphisms in a limited number of strains (Galloway-Peña et al., 2011). Similarly, data related to *pbp5* transferability describe conjugative processes mediated by Tn*916*-like elements (CTn*5382* or the interaction of CTn*916* and CTn*5386*) in a few *E. faecium* strains (Carias et al., 1998; Rice et al., 2005a, 2005b). Here, we report a comprehensive analysis of PBP5 diversity and evaluate *pbp5* transfer ability among diverse *E. faecium* lineages to enhance the understanding of the impact of resistance to penicillins in the evolvability of this species. By comparative genomics of the transferable chromosomal regions containing *pbp5* in available *E. faecium* genomes, we document a relationship between the diversity of *pbp5* and the differentiation of clonal lineages, which suggests that AmpR should be considered from a population biology perspective.

MATERIAL AND METHODS

Bacterial strains.

Seventy-eight AmpR *E. faecium* isolates from a collection of 1,700 enterococci recovered in Portugal from the last decade were analyzed (Novais et al., 2005a, 2005b, 2005c, 2006, 2013). They were selected on the basis of their resistance phenotype to ampicillin (≥16mg/L) and other antibiotics, origin (39 from different patients admitted in five hospitals from different cities, 18 from swine feces and piggeries, 4 from retail poultry carcasses, 2 from healthy human feces and 15 from hospital wastewater) and date of isolation. Antibiotic susceptibility to ampicillin and another 10 antibiotics of different classes was evaluated by disk diffusion and/or agar dilution method (Clinical and Laboratory Standards Institute, 2012). ß-lactamase production was tested in AmpR *E. faecium* isolates by the nitrocefin test (5µl were directly placed in bacteria growing around the ampicillin disc) and amplification of *bla*Z by PCR using primers based on the GenBank sequence no. M25257.1 (blaZF-3'-TTGCCTATGCTTCGACTTCA-5', blaZR-3'-AGTGAAACCGCCAAGAGTGT-5'). Clonal relatedness was established by Pulsed-Field Gel Electrophoresis (PFGE) and Multilocus Sequence Typing (MLST) as previously described (Freitas et al., 2013; Homan et al., 2002; Tenover et al., 1995) (http://pubmlst.org/). Population genetic analysis was performed using BAPS software as previously described (Tedim et al., 2015; Willems et al., 2012).

Transferability of ampicillin resistance.

Filter mating assays were performed in Brain Heart Infusion (BHI) at 37ºC overnight, using a donor/recipient ratio of 1:1 (1-3 experiments per isolate) using *E. faecium* GE1 as the recipient. Those donor strains transferring *pbp5* to *E. faecium* GE1 were also included in additional filter mating assays using *E. faecium* BM4105RF and *E. faecium* 64/4 using the same conditions. These laboratory recipient strains differed in the susceptibility against ampicillin and the presence of *pbp5*: GE1 (Δ*pbp5*; MIC_{Amp}<0.016mg/L; tetracycline, rifampicin and fusidic acid resistant; ST515/BAPS 2.3b), BM4105RF (*pbp*5; rifampicin and fusidic acid resistant; MIC_{Amp}=0.5mg/L; ST172/BAPS 1.3) and 64/3 (*pbp*5; rifampicin and fusidic acid resistant; MIC_{Amp}=1mg/L; ST21/BAPS 2.3a). Transconjugants were selected in BHI agar supplemented with antibiotics (ampicillin-10mg/L, fusidic acid-25mg/L; rifampicin-30mg/L) and incubated for 24-96h at 37ºC. Transconjugants were confirmed by comparison of their antibiotic resistance phenotype and PFGE profile with those of the wild type and the recipient strains. Conjugation frequency was calculated as the number of transconjugants by *E. faecium* GE1 recipient cell. Stability of acquired *pbp5* platforms after serial daily passages (x30) on antibiotic free BHI agar was evaluated in both wild type strains and transconjugants. Colonies from each passage were inoculated on plates containing the same agar medium and tested for antibiotic susceptibility by disc diffusion (Clinical and Laboratory Standards Institute, 2012).

Characterization of the region conferring AmpR.

Genomes of the donor (AmpR *E. faecium* clinical isolate HPH-2), the recipient strain (AmpS *E. faecium* GE1) and the resulting GE1 transconjugant (AmpR *E. faecium* TC_GEHPH2.1) were sequenced. Total DNA from these strains was extracted from 2mL overnight cultures using PureElute™ Bacterial Genomic Kit (EdgeBio, Gaithersburg, MD, USA) and DNA concentration was measured with a Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA). A total of 250-350ng of genomic DNA was sequenced using the Illumina MiSeq platform. MiSeq reagent kit was applied to prepare a library of Pair end DNA fragments according the manufacturer instructions (Illumina, San Diego, USA). Sequencing was carried out using a standard 2 x 71 base protocol (300-400 bp insert size) in a Genome Analyzer IIx (Illumina, San Diego, CA) at the sequencing facility of the University of Newcastle (United Kingdom). The main statistics for the three sequence datasets (number of reads and coverage) analyzed are shown in Table S1. Assembly of sequence data was done using Newbler software (454 life sciences, Roche, Connecticut, USA). Table S2 shows the final assembly results.

Prediction of ORFs was performed using GeneMark 3.05 (Besemer et al., 2001) and gene annotation was carried out by the Best Blast Hit approach using the UniRef100 database (Table S3). The characterization of the transferred region containing the *pbp5* gene was done based on a gene-by-gene comparison approach using *blastn* [genes present in transconjugant (TCGEHPH2.1) and donor (HPH2) strain but not in recipient (GE1)]. The results are summarized in supplementary Table S4 where the presence/absence of each transconjugant gene in the donor and/or receptor is shown. The region characterized as

"transferred region" was compared against the genome of the *E. faecium* strain DO (RefSeq Accession: NC_017960) using Easyfig 1.2 software (Sullivan et al., 2011). The functional annotation of such transferred region was performed according to that one of *E. faecium* DO using the KEGG database (http://www.genome.jp/kegg/genome.html). Besides *E. faecium* DO, the "transferred region" was also aligned against fully sequenced and closed genomes of *E. faecium* Aus0004, *E. faecium* NRRL B-2354 and *E. faecium* Aus0085 strains (RefSeq accessions numbers: NC_017022, NC_020207 and NC_021994, respectively) using Mauve (Darling et al., 2004).

Analysis of the *pbp5* genetic milieu.

An 8-10kb region comprising *pbp5* of 15 transconjugants and 21 wild type strains (Table 1) was characterized by an overlapping PCR assay. PCR conditions were adapted according to amplicon size (<3kb/>3kb): 0.5/1mM of each primer, 2/2.5mM $MgCl_2$, 1x of reaction buffer, 0.2/0.4 mM of each deoxynucleoside triphosphate, 1,25U GoTaq® Flexi DNA Polymerase [Promega Corporation, Madison, USA)]/2.5U Takara LA Taq polymerase (Takara™ Bio Inc., Shiga, Japan). The amplification program was 25 cycles of 30s at 94ºC, 30s at 55ºC, 30s at 72ºC; 1 cycle 10 min at 72ºC (for fragments <3kb) or 35 cycles of 30s at 96ºC, 1 min at 55ºC, 7 min at 72ºC; 1 cycle 10 min at 72ºC (for fragments >3kb). Differences between regions were established by comparing the RFLP patterns of the corresponding amplicons after digestion with *Dde*I or *Apa*I, which were further sequenced. Genomic location of *pbp5* was identified by hybridization of I-*Ceu*I and *Sma*I -digested genomic DNA using 23S rDNA and/or *pbp5* probes (Freitas et al., 2013; Liu et al., 1993). Labeling and detection were carried out using the Gene Images Alkphos direct labeling system kit following the manufacturer's instructions (Amersham GB/GE Healthcare Life Sciences UK limited). The presumptive presence of integrative conjugative elements previously associated with transference of AmpR was searched by analyzing the presence of integrases and excisionases of CTn*916* and CTn*5386* as well as the non-integrase left region of CTn*5382,* as described (Carias et al., 1998; Novais et al., 2009). The transferred 8-10kb regions carrying *pbp5* characterized in this study were compared with those of all *E. faecium* available genomes at the National Center for Biotechnology Information (NCBI) database (Table S5).

Phylogenetic analysis of PBP5.

Comparative analysis of all PBP5 protein sequences available in the GenBank database with those identified in this study was performed using the searching basic local sequence alignment (nBLAST) tool (Altschul et al., 1997) and the ClustalW2 algorithm for multiple sequence alignment (Goujon et al., 2010; Larkin et al., 2007) which are provided by the NCBI and the European Bioinformatic Centre (EBI), respectively. The PBP5 amino acid sequences were designated by a "C" followed by a number (Table S6). Phylogenetic analysis of all PBP5 sequences was performed using a Neighbor-Joining algorithm with bootstrap analysis based in 1000 permutations and a cut-off of ≥ 70% through MEGA 5.05 software (www.megasoftware.net).

Table 1. Epidemiological background, *pbp5* gene characterization and antibiotic resistance among *E. faecium* strains and transconjugants this study.

Isolate type (isolate name)	*pbp5* genetic environment *type (pbp5-fstW)* (RFLP_*Dde* I)	Sequencing	PBP5 alleles	PFGE-*SmaI* *pbp5* hybridization (kb)	Epidemiological background ST	BAPS	PFGE[a]	Date	Source/ sample	MIC to Amp (mg/L)	Resistance to other antibiotics
Rc (*Efc* GE1)	-	-	-	-	515	2.3b	A			≤0.016	TET, RIF, FA
Rc (*Efc* BM4105RF)	A	I		290	172	1.3	B			0.5	RIF, FA
Rc (*Efc* 64/3)	B	I		250	21	2.3a	C			1	RIF, FA
***E. faecium* with *pbp* 5 transfer**											
WT (H323)	C	I[b]		210	280	3.1	100	2002	H(C)/urine	128	VAN, TEC, ERY, CIP, GEN, STR
TC*Efm* GE1 (GEH323.3)	C	I	C12	210						32	VAN, TEC, TET, ERY, RIF, FA
WT (HPH2)	C	I[b]		210	125	3.1	126	2007	H(A)/urine	128	VAN, TEC, ERY, CIP, NIT
TC*Efm* GE1 (GEHPH2.1)	C	I	C20	210						64	VAN, TEC, TET, ERY, RIF, FA
TC*Efm* 64/3 (64HPH2.1)		ND		170+250						8	RIF, FA
WT(70411)	D	II[b]		210	670	3.3a	90	1997	H(B)/urine	32	VAN, TEC, TET, ERY, Q/D, STR
TC*Efm* GE1 (GE70411.2)	D	II	C4	210						32	VAN, TEC, TET, ERY, RIF, FA
TC*Efm* BM4105RF (BM70411.5)	D	II	C4	170+200						8	VAN, TEC, TET, ERY, RIF, FA
WT (E4)	D	II[b]		210	132	3.3a	Z	2001	HS(E)/water	128	TET, ERY, CIP, GEN, STR
TC*Efm* GE1 (GEE4.1)	D	II	C19	210						16	TET, RIF, FA
WT (28798)	D	III[b]		210	132	3.3a	88	1999	H(B)/blood	64	VAN, TEC, TET, ERY, QD, CIP, GEN, STR
TC*Efm* GE1 (GE28798.1)	D	III	C8	210						32	VAN, TEC, TET, ERY, STR, RIF, FA
WT (E233)	D	III[b]		210	132	3.3a	88.1	2002	HS(E)/water	64	ERY, CIP, GEN, STR
TC*Efm* GE1 (GEE233.1)	D	III		210						32	TET, RIF, FA
WT (E169)	D	III[b]		210	132	3.3a	88.2	2001	HS(C)/water	64	ERY, CIP, GEN, STR
TC*Efm* GE1 (GEE169.3)	D	III		210						32	TET, RIF, FA
TC*Efm* BM4105RF (BME169.3)		ND		200+240						8	RIF, FA
WT (E49)	D	III[b]		210	132	3.3a	C3	2001	HS(E)/water	128	VAN, TEC, ERY, CIP, STR
TC*Efm* GE1 (GEE49.1)	D	III	C8	210						32	VAN, TEC, TET, RIF, FA
WT (H207)	ND	ND	C4	210	280	3.1	150	2002	H(C)/exsudate	32	TET, ERY, CIP, GEN, STR
TC*Efm* GE1 (GEH207.1)	ND	unknown[c]		180						32	TET, RIF, FA
TC*Efm* BM4105RF (BMH207.3)	ND	ND		210						8	RIF, FA
WT (SN71, SN133)	C	I[b]		200	393	2.1a	SN208	2006	PG/n=4-waste lagoon, animal faeces	64	TET, ERY, STR, NIT
TC*Efm* GE1 (GESN71.1; GESN133.1)	C	I	C9	200						64	TET, RIF, FA
TC*Efm* BM4105RF (BMSN71.1)	C	I		200						8	RIF, FA
TC*Efm* 64/3 (64SN71.1)	C	I		210						64	RIF, FA
WT (VD79C1)	D	II[b]		200	18	3.3a	59	2001	HH/faeces	128	VAN, TEC, TET, ERY, CIP, GEN
TC*Efm* GE1 (GEVD79C1.5)	D	II	C7	210						128	VAN, TEC, TET, ERY, GEN, RIF, FA
TC*Efm* BM4105RF (BMVD79C1.6)	D	II	C7	200						128	VAN, TEC, RIF, FA

Table 1. Epidemiological background, *pbp5* gene characterization and antibiotic resistance among *E. faecium* strains and transconjugants this study (cont.)

Isolate type (isolate name)	*pbp5* genetic environment type (*pbp5-fstW*)		PBP5 alleles	PFGE-*SmaI* *pbp5* hybridization (kb)	Epidemiological background					MIC to Amp (mg/L)	Resistance to other antibiotics
	(RFLP_*Dde* I)	Sequencing			ST	BAPS	PFGE[a]	Date	Source/ sample		
Representative *E. faecium* without *pbp* 5 transfer											
WT (529940)	ND	II	C6	ND	16	3.3a	74	2000	H(B)/urine	>64	VAN, TEC, TET, ERY, STR
WT (H352)	ND	II	C4	ND	280	3.1	71	2000	H(D)/sputum	>32	VAN, TEC, ERY, CIP, GEN
WT (HPH6)	ND	II	C5	ND	18	3.3a	128	2007	H(A)/pus	>32	VAN, TEC, ERY, CIP, NIT
WT (E197)	ND	III	C8	ND	368	3.3a	H4	2001	HS(C)/water	>32	VAN, ERY, CIP, GEN, STR
WT (SN446)	ND	III	C8	ND	132	3.3a	H119.5	2007	PG/ water	>32	VAN, TEC, ERY, GEN
WT (H196)	ND	V	C1	ND	390	3.1	129	2002	H(D)/unknown	>32	TET, ERY, STR
WT (C373)	ND	I	C11	ND	148	2.1b	36.2	2001	HH/faeces	>32	TET, ERY, CIP, GEN, STR, NIT
WT (SE97F1)	ND	I	C10	ND	*pur* K6	ND	ND	2001	P/skin	>32	TET, ERY, CIP, STR
WT (164306)	ND	V	C2	ND	190	2.3a	98	1998	H(B)/urine	32	VAN, TEC, TET, ERY, CIP, STR
WT (SN194)	ND	V	C3	ND	264	2.3a	ND	2006	PG/residual water	32	TET, CIP, CHL, STR

Comparative analysis of the phylogenetic trees of *pbp5* sequences and core genomes was performed to analyze the congruence between the *pbp5* and the rest of the genome. The core genome phylogeny was built using 233 *E. faecium* (Table S5) draft genome sequences downloaded from NCBI Trace Database (last updated on February 2014). ORF prediction was performed for each strain by GeneMark (Besemer et al., 2001). The core genome was defined as genes present in all strains, non-repeated and with at least 80% of identity and 80% of coverage. The core genome search was performed by CD-HIT (Li and Godzik, 2006) and parsed by homemade Perl script. The core genome alignment is the result of the concatenation of all individual gene alignment. The multiple alignment of each core gene was performed by MAFFT (Katoh and Toh, 2010). The phylogenetic tree was built by FastTree2 (Price et al., 2010). Tree comparison was carried out by *cophyloplot* command of APE (Analyses of Phylogenetics and Evolution) (Paradis et al., 2004) from R software.

RESULTS

Transferability of AmpR (*pbp5*).

Twelve clinical isolates (n=12/78; 15%; Table 1) were able to transfer a region containing *pbp5* to *E. faecium* strain GE1 at a frequency of 10^{-11} to 10^{-12} CFU/mL per recipient. Five of them were also able to transfer the *pbp5* (AmpR) region to *E. faecium* BM4105RF and two, to the *E. faecium* 64/3. All donor strains belonged to major human lineages associated with clinical isolates causing hospital infections, namely BAPS subgroups 3.3a (ST670, ST132, ST280), 3.1 (ST280, ST125), and 2.1a (ST393). β-lactamase production was not detected in any of the isolates.

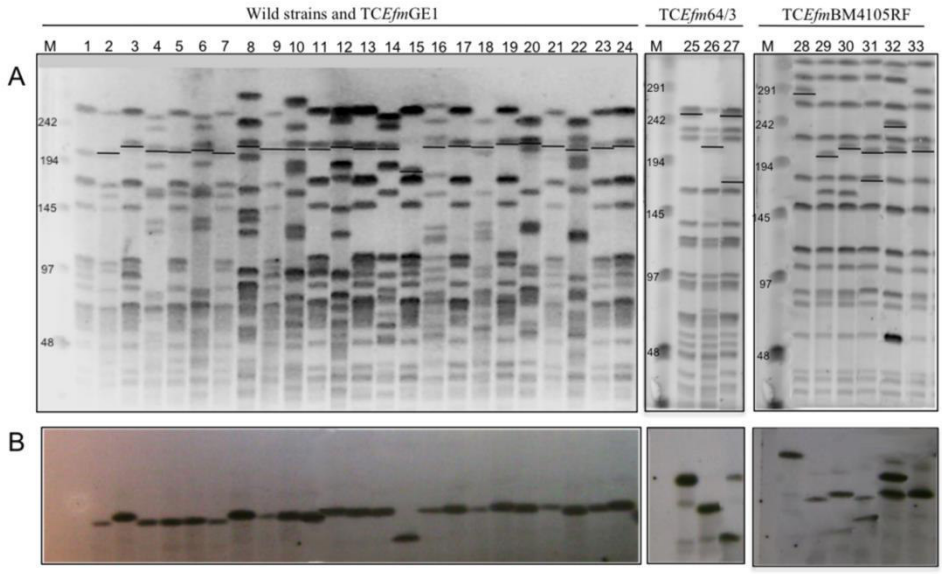


Figure 1. Clonality and hybridization assays with *pbp5* probes of wild type, recipients and transconjugants strains.

A-PFGE SmaI digested DNA of recipient strains (1-*E. faecium* GE1, 25-*E. faecium* 64/3, 28-*E. faecium* BM4105RF), wild type (2-VD79C1, 4-SN71, 6-SN133, 8-HPH2, 10-H323, 12-70411, 14-H207, 16-E49, 18-E233, 20-E169, 22-E4) and transconjugants (3-GEVD79C1.5, 5-GESN71.1, 7-GESN133.1, 9-GEHPH2.1, 11-GEH323.3, 13-GE70411.2, 15-GE207.1, 17-GEE49.1, 19-GEE233.1, 21-GEE169.3, 23-GE28798.1, 24-GEE4.1, 26- 64SN71.1, 27- 64HPH2.1, 29- BMSN71.1, 30- BMH207.3, 31- BM70411.5, 32- BME169.3, 33- BMVD79C1.6). **B**- Hybridization assays with a *pbp*5 probe (primers P1 and P2-Figure 4). M- Low Range PGE Marker, kb (New England, BioLabs). **Abbreviations**: TC*Efm*- Transconjugant *E. faecium*

All transconjugants had similar PFGE profiles to recipient strains (Figure 1), were resistant to rifampicin and fusidic acid and exhibited ampicillin MICs=8-128mg/L lower than their corresponding wild types in some cases (8mg/L or 16 vs 32mg/L to >256mg/L). Besides the AmpR phenotype, some transconjugants also exhibited resistance to vancomycin (n=8), teicoplanin (n=8), erythromycin (n=6), tetracycline (n=1; corresponding only to non-*E. faecium* GE1 transconjugants; as GE1 strain is resistant to this antibiotic), streptomycin (n=1) and/or gentamicin (n=1). Transference of the *pbp5* platform either alone or with plasmids that carry genes encoding resistance to different antibiotics only occurred when plates were supplemented with ampicillin (data not shown).

The *pbp5* gene of AmpR isolates is located on large and transferable chromosomal platforms containing metabolic traits.

Hybridization of *pbp5* and 23S rDNA probes was observed in the same high band of digested I-*Ceu*I DNA, thus indicating its chromosomal location. Further hybridization of *Sma*I-digested genomic DNA with the *pbp5* probe showed signals in fragments of ~210kb in all but one GE1 transconjugants, for which the *pbp5* was detected in a band of ~180kb (Figure 1). Hybridization of the same probe with fragments of *Sma*I-digested genomic DNA of *E. faecium* BM4105RF and 64/3 transconjugants of different size suggests the occurrence of independent transfer events that seem to occur in a particular region of the genome based on the similarity of PFGE patterns of the transconjugants obtained using different donors (Figure 1). The donor strains lack integrases/excisionases of Tn*916*-like transposons (Tn*916*, Tn*5386*, Tn*5382*) suggesting a different mechanism of movement than that previously reported (Rice et al., 2005a, 2005b).

Sequencing of the recipient (*E. faecium* strain GE1), the donor (*E. faecium* HPH2) and the transconjugant (*E. faecium* strain TCGEHPH2.1) allowed us to identify 7 contigs containing genes present in the donor and its transconjugant but absent in the recipient strain. They were considered to correspond to the "transferred region", which comprised the *pbp5* gene and had a size of approximately 280kb, slightly larger than that inferred from the *Sma*I-PFGE gels. A more detailed analysis of the contigs revealed that two of them (contg00158 and contig00068), particularly contig00158, contained genes present in the recipient and transconjugants but not in the donor strain, which could be explained either by the partial transfer of the platform and/or by post-transfer recombination events (Figure 2). The comparative analysis of the genomes sequenced in this work with the four closed *E. faecium* genomes (DO, NRRL B-2354, Aus0004, Aus0085 strains) available at the NCBI database at the time of writing this manuscript, revealed a common region among our donor/transconjugant strains and the four genomes of 153kb containing the *pbp5* gene, which is absent in the recipient *E. faecium* GE1 genome (Figure 3). The remaining region of the transferable platform was variably present at different chromosomal positions in different strains (Figure 3).

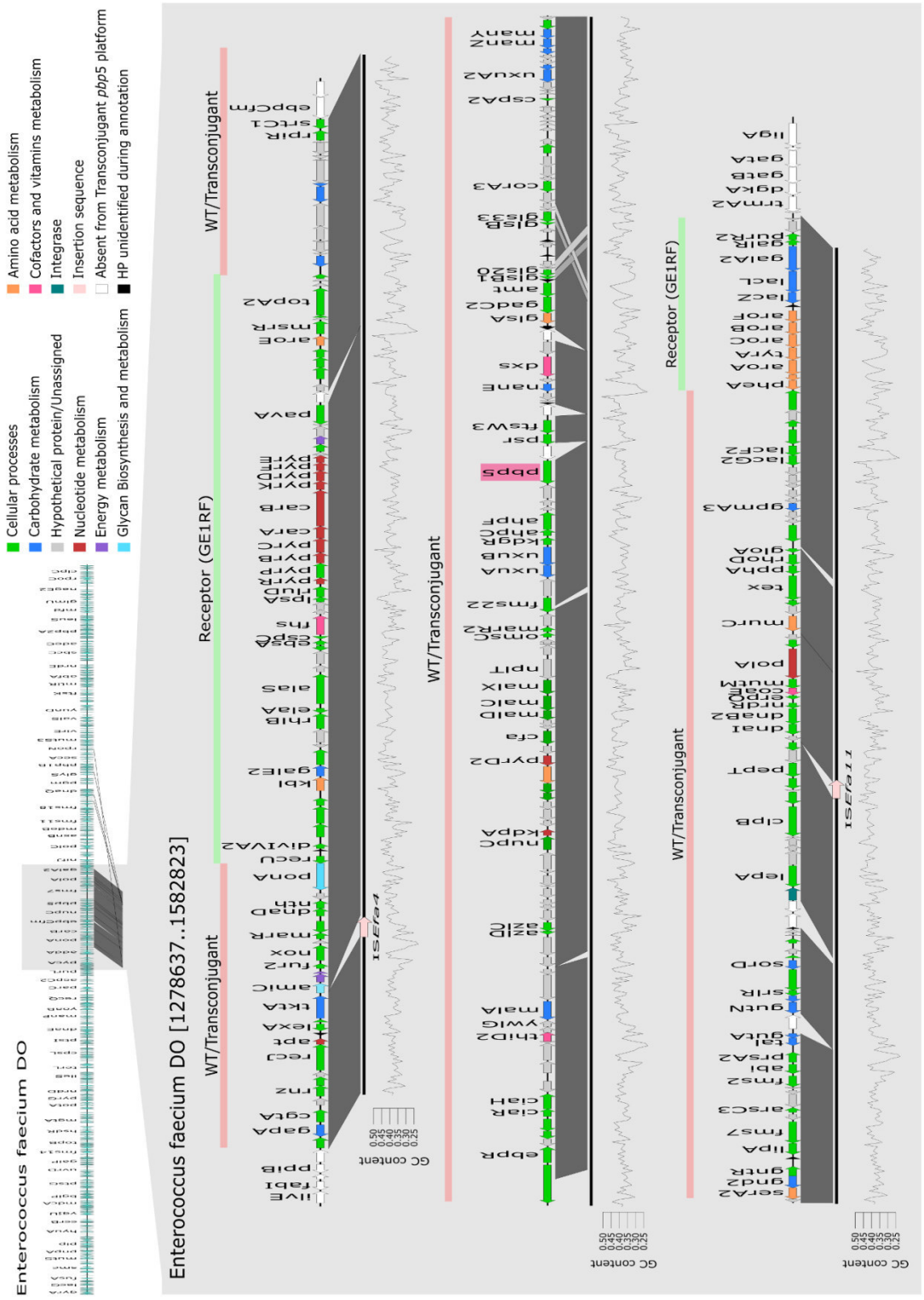


Figure 2. Representation of the transferable chromosomal region containing *pbp5*.

Mapping and annotation (using KEGG database) of transferable region of transconjugant TCGEHPH2.1 (represented by black line) using *E. faecium* DO as reference genome. Lines above *E. faecium* DO genetic structure represent the transferable genetic platform observed in wild type and transconjugant isolates (pink) and genomic regions of *E. faecium* GE1 recipient and transconjugant (green). GC content was calculated using seqinr in Rstudio. The window used to calculate the GC content was 200bp, represented in the figure by each vertices of the graph. **Abbreviations:** WT, wild type.

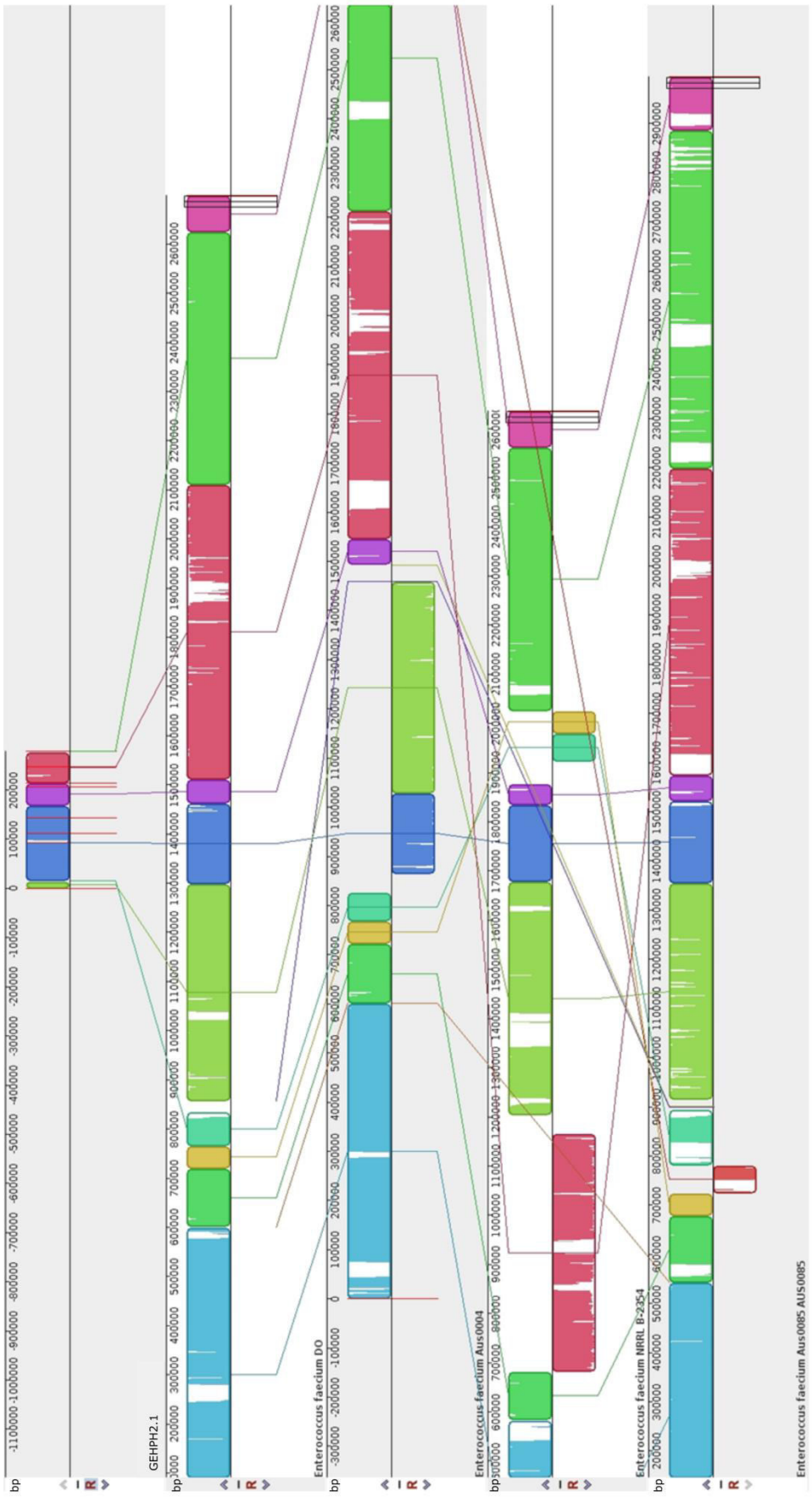


Figure 3. Comparison by MAUVE of the transferable region containing *pbp5* (TCGEHPH2.1) with the four close *E. faecium* genomes (DO, Aus 0004, Aus0085 and NRRL B-2354), present in GenBank database.

Each color block represents a genome region present in at least two of the sequences analyzed. These similar blocks can be identified by their color. Blank regions within the block represent point mutations or even regions that are absent in the same color blocks of the other sequences.

Figure 2 shows a detailed characterization of the region transferred containing ORFs with a G+C content ranging from 24 to 41%. Beside *pbp5*-related resistance to β-lactams, genes contained in this transferred region are involved in different cell functions such as transport of some amino acids and carbohydrates, redox processes and survival under stressful conditions in the intestinal environment (e.g. acid and bile tolerance). Among genes related to β-lactam resistance we detected a gene encoding a bifunctional class A PBP with a transpeptidase and transglycosylase activity named *ponA*, which is involved in the synthesis of the peptidoglycan, allowing the survival of the cell in the presence of cell wall inhibitory compounds (Rice et al., 2009). A copy of the CiaRH operon, a two component and cell envelope stress response, a system that was restricted to the genus *Streptococcus* to date, was also detected. In this species, the CiaRH operon is weakly induced by prolonged incubation with sub-lethal amounts of cell wall active antibiotics and it seems to mediate resistance to lysis induced by these compounds. The CiaRH-dependent regulation has been said to influence the virulence of *Streptococcus pneumoniae* but its role in enterococci has not yet been elucidated (Jordan et al., 2008; Krawczyk-Balska and Markiewicz, 2016). The transferable chromosomal region containing *pbp5* in *Enterococcus* has also genes with presumed influence on the microbe-host interaction. Among them, some involved in the transport and use of different substrates generated by the host, including three phosphotransferase systems (PTS, namely glucitol/sorbitol, L-ascorbate and mannose/fructose/sorbose), the ABC transport and transformation system of maltodextrines (*malACDX*) and the N-acetylmannosamine-6-phosphate epimerase (NanE; part of a pathway that allows the usage of sialic acids, major components of glycoproteins, gangliosides, and other sialoglycoconjugates). Other metabolic genes in the platform could be involved in tolerance to intestinal acid environment, including glyceraldehyde-3-phosphate dehydrogenase, ATP synthase subunit α, NADH dehydrogenase, glutaminase and genes encoded enzymes that favored the production of ammonia from glutamine, and deamination or transport of branched/nitrogenated amino acids. The region also contains five stress response proteins including two belonging to the Csp (cold shock proteins) system (CspA and CspC) and the small chaperone Hsp20, and Gls33, which are also present in *E. faecium* genomes available in the GenBank databases. They have been involved in stress response to salts, pH and ethanol exposure in *Clostridium*; Hsp20 is an small chaperone protein involved in the survival at different abiotic stress conditions as heat (55ºC) and salt (5mM) in *Bifidobacterium longum* (Khaskheli et al., 2015).

The large and transferable chromosomal platforms exhibit hotspots for insertions.

The occurrence of different insertion sequences (Figure 4) in the boundaries of the *pbp5* gene and the presence of a >3027 bp *psr-pbp5* fragment in *E. hirae* (99.9% identical to that found on *E. faecium*) suggest the occurrence of hot spots that could facilitate recombination of certain regions. To test this possibility we further analyzed the 8-10Kb genetic context of *pbp5* genes from 21 wild type and 15 transconjugants isolates included in this study as well as in available enterococcal genomes. RFLP amplicons corresponding to *ftsW* to *pbp5* region as well as *pbp5* to ion efflux gene showed an identical pattern both in donor and transconjugants, but were different from the same regions of *pbp5* carrying

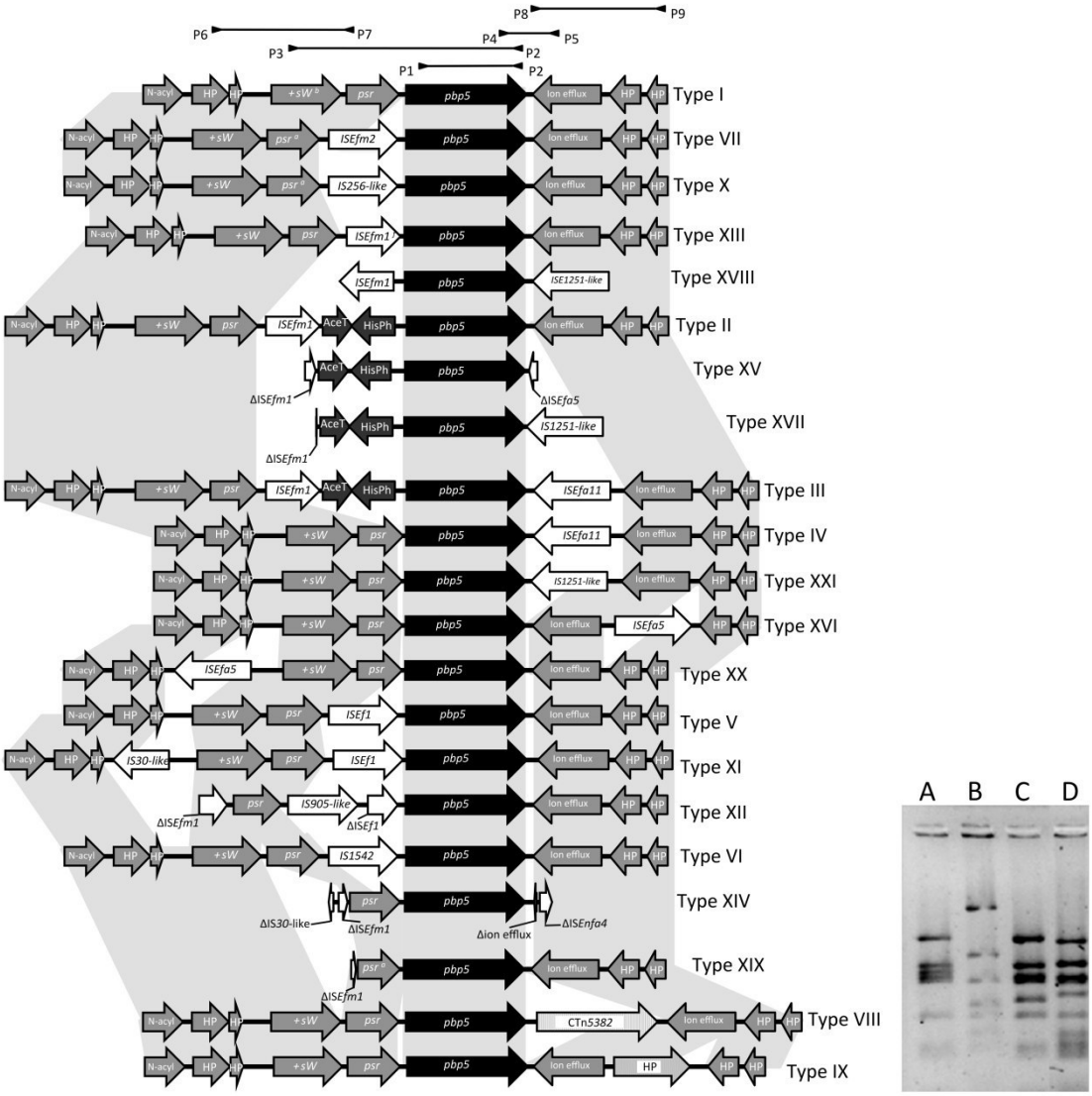
recipient strains *E. faecium* BM4105RF and 64/3 (Figure 4). Sequencing of fragments representing distinct RFLP patterns and comparative analysis with similar regions of GenBank available genomes, revealed 21 variants of such 8-10kb-chromosomal fragment (designed by roman numerals), which differed in the number and type of insertions sequences (Figure 4). Three variants (types I, II, III) correspond to transferable platforms described in this study (Figure 4; Table 1).

The predominant 8-10kb fragment identified in both AmpR and AmpS *E. faecium* analyzed in this study did not contain indels and was considered the prototype chromosomal region arbitrarily named Type I (Table S5, Figure 4, Figure 5). The *pbp5* or other genes (e.g. *psr*, *ftsw*, ion efflux genes) were flanked by one or two insertions sequences of the IS*256,* IS*L3* of IS*30* families (IS*Ef1*, IS*Efm1*, IS*Efm2*, IS*1542*, IS*256*-like, IS*Efa11*, or IS*1251*-like) in isolates carrying types II to XXI, with the exception of type VIII that had CTn*5382* (*vanB2*) inserted just after *pbp5* gene. The identifiable boundaries of ISs detected in most platforms as well as the common nucleotide positions at which insertions occurred, suggested recent acquisition events at the platform hot spots (Figure 4). Also, some types can represent evolved platforms from others by insertion of additional IS or occurrence of recombination events. That could be the case for example of types III, XIV, XV and XVII evolving from type II or XI and XII from type V, respectively. Despite of predominance of type I, some types seem to be more associated with specific hosts as the case of type V to pigs and type II/II-like, III or type XIV-like to the clinical setting. Epidemiological distribution of isolates appears in Table S5.

Diversity of PBP5 sequences reflects the phylogenomic diversification of *E. faecium.*

We identified 75 PBP5 protein variants (Table S5 and S6) corresponding to AmpR and AmpS strains which comprise 20 of the previously described variants C4, C7, C9, C11, C15-C18, C21, C24, C46-C48, C50, C51, C61, C63, C65, C67, C71 (Galloway-Peña et al., 2011; López et al., 2009; Rybkine et al., 1998). The other 55 variants were firstly detected in this study either in strains from Portugal (n=11) or from available genomes at NCBI database (n=44). The C4, C7, C8, C9, C12, C19, and C20 sequences are linked to isolates able to transfer a region containing *pbp5* (Table 1 and S6, Figure 5), which belong mostly to ST18, its SLV ST125, and the ST280 and ST670 (BAPS 3.3a, 3.1 and 2.1a, respectively) (Table 1).

Figure 5 shows the phylogenetic tree constructed with all known PBP5 protein sequences (this study and those available at GenBank databases). The tree is split in two major clades arbitrarily named B and A, mirroring the clades associated with populations of non-hospitalized persons and hospital isolates respectively, in agreement with what was previously inferred from phylogenomic studies of *E. faecium* (Lebreton et al., 2013). Clade B comprises PBP5 of AmpS isolates (PBP5-S) mainly belonging to BAPS 1.2 and 1.5. Some are similar to the prototype PBP5-S C46 sequence from *E. faecium* BM4107 strain (Sifaoui et al., 2001) but most of them also exhibit mutations at positions T25A, S39T and D644N, which are also common to PBP5 sequences of clade A isolates (Table S6). Two strains, isolated in 1964 and 2006, further showed changes at S27G+T324A and S27G, respectively, such mutations corresponding to the



Primer	Sequence (3´-5')	Amplicon size (bp) considering *pbp5* genetic environment type			
		I	II	III	V
P1:pbp5/1	GCACGGCAAAAATCGAACAGG	1983	1983	1983	1983
P2:pbp5/2	TGCTCGTTTGGTTGCTGAATCATC	4315	6496	6496	6496
P3:ftsW/1	AGGCTGTTGCCCAGAAAC				
P4:pbp5/2inv1	GATGATTCAGCAACCAAACGAGCA	326	326	1948	326
P5:EFLKRinv/2	CGTGGAGAAATGGCTTTGAT				
P6:NacylFinv/1	TTCACGAAAGACGCAGACAC	1819	1819	1819	1819
P7:ftsWRinv2	TCGGCTTGCAATAAACGAT				
P8:EFLKR/1	ATCAAAGCCATTTCTCCACG	2030	2030	2030	2030
P9:HPIIEFLK/2	CGGGTTATTCCCAAACAGAA				

Figure 4. Characterization of *E. faecium pbp*5 genetic environment by PCR and sequencing.

The Roman numbers I, II, III, and V represent the different genetic platforms detected in *E. faecium* from this study. The numbers IV, VI to XXI were detected in available genomes from GenBank database. The different types were named according to diversity of insertions sequences, genomic fragments or conjugative transposons within genes or intergenic regions. Mutations or recombinations within genes or intergenic regions were not considered for type classification. The Table indicates the primers used (designed for this study; P1/P2 described by Dahl et al, 2000) and the size of PCR amplicons from genetic environment of types I-III and V. The A, B, C and D lines of the bottom right side figure represent RFLP patterns of amplicon P3-P2 of mobile platforms I (pattern C) and II/III (pattern D) of isolates included in this study, when digested with DdeI restriction enzyme. The patterns A and B correspond to the amplicons of the recipient strains *E. faecium* BM4105RF and 64/3, respectively. [a] These gene has an extra stop codon within its sequence. **Abbreviations:** N-acyl, (N-acyl-glucosamine-6-phosphate-2-epimerase); HP (hypothetical protein); *ftsW* (cell cycle protein); *psr*, (*pbp5* synthesis repressor); *pbp5* (gene encoding penicillin binding protein 5); AceT (acetiltransferase); HisPh (Histidinol Phosphate Phosphatase).

PBP5-R consensus sequence (Pietta et al., 2014). The type I chromosomal region above mentioned was observed in all available isolates from this group (Figure 5; Table S5).

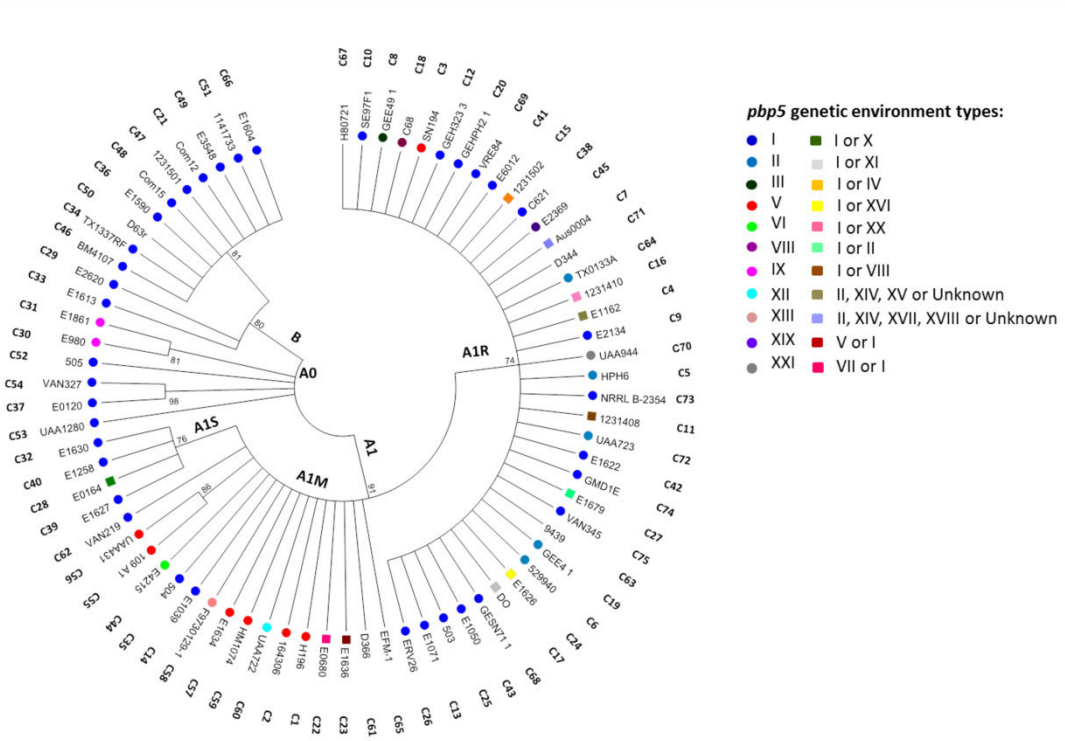


Figure 5. Phylogenetic analysis of PBP5 protein sequences from *E. faecium* isolates of this study and available in Genbank database (until February 2014).

The Maximum Likelihood tree was obtained using Mega 7 based on the JTT method. The tree with the highest log likelihood (-2000.3736) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The analysis involved 75 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 445 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. Bootstrap values are indicated and are based in 1000 permutations. The cut-off used was ≥ 70%. "C" followed by a number represent PBP5 amino acid combinations described in Table S6. Only one *E. faecium* isolate carrying representative PBP5 sequences (each type of "C") was included in the tree. The *pbp5* genetic environments types described in Figure 4 are indicated by colored circles and, when more than one type is present in isolates carrying the same "C", by colored squares. If no symbol is present, the genetic environment was undetermined. **Abbreviations:** NA, not applicable

Clade A includes PBP5 variants grouped in two main clusters arbitrarily designed here A0 and A1, with most isolates sharing mutations at positions V24A, S27G, E100Q, K144Q, T172A, T324A, N496K, A499T and E525D (7 of the 21 positions used to establish sequence diversity of this protein) (Pietta et al., 2014). The Clade A0, represented by C30, C31, C37, C52, C53 and C54 PBP5 sequences, corresponds to AmpS isolates of different BAPS groups (BAPS 1.2, BAPS 2.3a and BAPS 3.3b), hosts (animals and humans), countries and were collected from 1995 to 2001 (Figure 5, Table S6). Most isolates carrying *pbp5* within a type I fragment. Sequences within the Clade A1 were divided in three subclusters that parallels *E. faecium* populations of BAPS groups 2 and 3 (Tedim et al., 2015; Willems et al., 2012). They were designated as A1S, comprising a subset of PBP5-AmpS variants (mainly associated with human and animal isolates of BAPS subgroup 2.1b); A1M, comprising a subset of PBP5-AmpS and PBP5-AmpR (mostly associated with animal isolates of diverse BAPS subgroups 2.1b, 2.3a, 2.3b, 3.1, 3.2 and 3.3b);

and A1R, including almost all PBP5-AmpR (mostly associated with the clinical setting and BAPS subgroups 3.1, 3.3a and 2.1a, with the latter two only observed in this group).

All isolates from Clades A1S, A1M and A1R share mutations R34Q, G66E, L177I and A216S but also present some differences. Mutations S39N and A401S plus A499I were specific for subclades A1S and A1M, respectively. The last two polymorphisms were previously documented in two AmpR strains (Pietta et al., 2014) but this study suggests that they are fixed in certain populations despite they were not previously considered within the PBP5 relevant changes. One of the A1M strains had also variations at specific positions linked to the A1R group (A68T, E85D, M485T, V586L, E629V and P667S). Of note is the PBP5 variant C23 within this cluster, which is here overrepresented (n=44).

PBP5 variants within the A1R group exhibited six mutations (A68T, E85D, S204G, 466´S/D, M485A/T, E629V, P667S) predominant in this group, some of them located at the active site of the protein (466´S/466´D, M485A/T) and at the end of a turn between the β1 and β2 strands (E629V; P667S) (Fontana et al., 1996; Rice et al., 2004). It is of note that some AmpS isolates (BAPS subgroups 3.1, 2.3a and 3.2) with PBP5 clustering in the A1R subgroup (including the PBP5 of the recipient *E. faecium* 64/3) lack the mutation M485A and E629V, which suggest that such mutations might be necessary for AmpR phenotype, as reported (Rice et al., 2004). Changes A68T, E85D and S204G were shared by AmpS and AmpR isolates of A1R group (Table S6). Some strains exhibited the particular pattern of mutations Q408H, A558T, G582S, K632Q and, eventually, V462A, N546T and P642L. They have not previously been associated with AmpR. The *pbp5* genetic environment type I was distributed in all clade A1, although the subgroups A1M and A1R included isolates with the most types variety. Of note, most isolates carrying types V and XI grouped in A1M and all carrying the similar types II, III, XIV, XV and XVII grouping in A1R (Figure 5, Table S5).

We also analyzed, in available genomes, other genes that have previously been associated with AmpR (*ddc*P, *ddc*Y, *ldt*Efm, *pgt*A, *lytG*) and considered representative protein sequence per UniRef100 available at Uniprot using as reference *E. faecium* strain Aus0004. Single-locus phylogenetic tree of PBP5 was congruent with those of proteins codified by *ldt*Efm and *ddcY* but was non-congruent with those of *ddcP, pgtA* and *lytG* (data not shown).

Analysis of the topology of the phylogenetic tree of the available *E. faecium* core genomes and the phylogenetic trees of *E. faecium pbp5* genes analyzed in this study revealed branches in both trees with the same and different topologies. Such discrepancies further suggested that the *pbp5* gene could be transferred within *E. faecium* populations (Figure 6) potentially explaining the occurrence of the same PBP5 variants in isolates of different clonal lineages and phylogenomic groups containing different PBP5 variants (Figure 5, Table S5). As examples, isolates of BAPS 1.2 (PBP5 variants of clades A0 and B), BAPS 4 (clades A1R and B) or BAPS 7 (clades A1S, A1R and B).

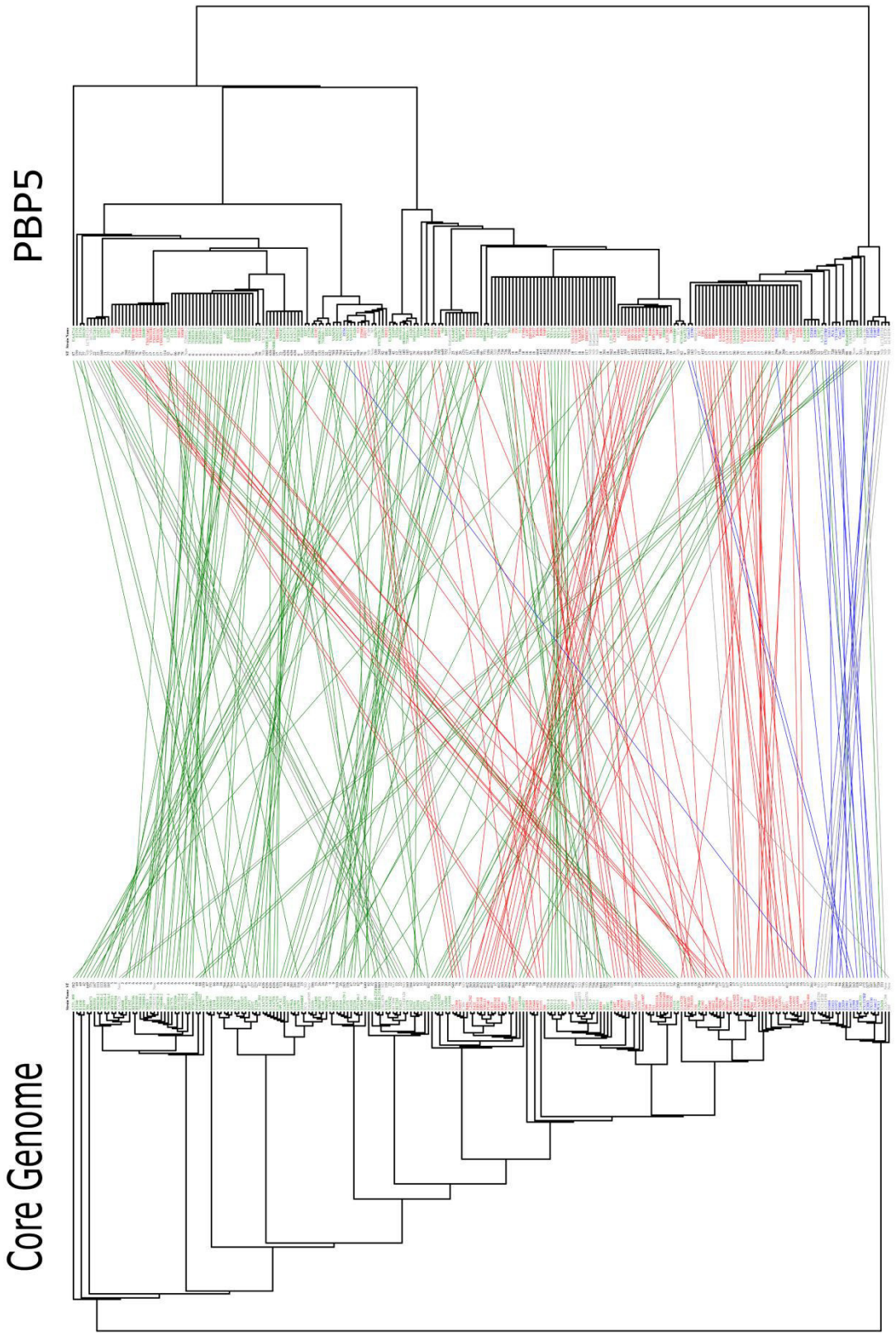


Figure 6. Comparison between 233 *E. faecium* Core Genome phylogeny and *pbp5* phylogeny.

Strains in blue belong to Clade B, strains in red belong to Clade A1, strains in green belong to Clade A2. The edges join the core genome and the corresponding *pbp5* of each strain. Both trees were made by ML using GTR-CAT. Tress and edges was made with APE packages. The result of Mantel test (r=-0.03 and significance of 0.761) show the rearrangement of the *pbp5* in comparison with the core genome. The Mantel test (APE package) was made using the distance matrix calculated from nucleotide alignments.

Ampicillin resistance was maintained without selective pressure.

AmpR was maintained in the wild type strains and respective *E. faecium* GE1 transconjugants over 200 generations in antibiotic free BHI indicating that the acquired genetic platforms containing *pbp5* may persist in absence of selective pressure in different genetic backgrounds.

DISCUSSION

This study documents the transferability of *pbp5* within a large chromosomal region not associated with CTn (Tn*5282*, Tn*916*, Tn*5386*) previously described to be involved with this event and shows a trend between the PBP5 diversity and the phylogeny of this species.

The evolution of the PBP5 remains elusive despite of its role in the resistance to cephalosporins and penicillins in *E. faecium* and the emergence of AmpR *E. faecium* on a global level. This PBP is a member of the Mec family, which comprises proteins with natural low affinity for β-lactams as PBP2r (*mecA*) of *Staphylococcus aureus*, and shows a 99% identity with PBP3r of *Enterococcus hirae* (suggesting a common origin) (Hiramatsu et al., 2013; Raze et al., 1998). Hiramatsu *et al.* hypothesized that proteins of the Mec family such as MecA (PBP2r) would have been essential for the survival of ancestral members of staphylococci in the presence of ß-lactam antibiotics produced by fungi and Actinobacteria and further lost in the Devonian period with the emergence of mammals (more than 400 million of years ago) coincidental with the separation and adaptation of staphylococcal species to different hosts (Hiramatsu et al., 2013). It is tempting to suggest a similar scenario for the PBP5 of *E. faecium*. This species has traditionally been classified within the enterococcal groups established by Carvalho *et al*. (Carvalho et al., 1998) based on metabolic profiles, namely “group II” restricted to humans and only comprising *E. faecalis* and *E. faecium*. Some *E. faecium* variants linked to the “group III” (associated with water and plants; also grouping *Enterococcus hirae*) could be the origin of *pbp5* and *psr* genes (G+C content of 38.9% compatible with an enterococcal origin). Based on the apparent universal presence of *pbp5* gene in *E. faecium* populations, we could speculate that acquisition might have predated the evolutionary split among mammals, reptiles, birds, and insects, with further multiple acquisition events to shape the contemporary platform if we consider the variable G+C content of this set of genes. However, the low number of non-mammals isolates in databases might mask a more recent acquisition.

The full characterization of the 153kb chromosomal region that contains not only the *pbp5* gene but also genes codifying for traits that might facilitate the survival in the gastrointestinal tract (e.g. resistance to stress by acids and bile; substrates produced by the host as maltodextrines, sialic acids) in almost all available *E. faecium* genomes suggest a contribution of this region to the adaptation to the mammalian intestine and persistence in abiotic environments. To date, only two *E. faecium* strains lacking *pbp5* have been documented, strains GE1 and D344S, which derived from AmpS and AmpR isolates, respectively. The D344S strain is susceptible to cephalosporin and ampicillin due to the spontaneous deletion of a 170 kb genome fragment that included *pbp5* and other *pbp* genes. This deletion occurred by the interaction

of CTn*5386* (a 60kb element that comprises *pbp5* and *vanB2*) with Tn*916* (Rice et al., 2007). The GE1 strain does not harbor Tn*916* and the causes for the loss of the region in which *pbp5* is located are unknown.

Polymorphisms in the PBP5 protein sequences allowed us to group the variants in clusters that parallel phylogenomic diversification of *E. faecium* (Lebreton et al., 2013). Lebreton *et al* suggested a model for evolvability of this enterococcal species consisting on a first split of "clade B" and "clade A" coincidental with human and animal insulation occurring 3000y ago, and a further split of "clade A" in subclades "A1" and "A2" after the introduction of antibiotics in the therapeutical arsenal in late 1940s (Lebreton et al., 2013). Two main clusters of PBP5 variants were also identified in this study, designated as "B" (associated with the *E. faecium* "clade B"), and "A" that further split in subgroups A0 (including only AmpS isolates) and A1 comprising three small groups linked to different *E. faecium* populations differing in the susceptibility to ampicillin; A1S (AmpS from healthy humans of different BAPS groups), A1M (AmpS and AmpR from humans and animals), and A1R (AmpR from clinical isolates). Although some authors have suggested sequential acquisition of amino acid changes (Galloway-Peña et al., 2011; Pietta et al., 2014), such diversification may also indicate different evolvability routes for AmpR in response to distinct selective pressures in different hosts similarly to what has been observed for different β-lactamase enzymes of Gram negative organisms (Novais et al., 2010). Nonetheless, transfer events could also have contributed to the diversification of the PBP5-A1 group.

The transfer of platforms carrying *pbp5* gene in *E. faecium* resulting from the interaction between transposons as Tn*5382* or Tn*916* plus Tn*5386* (Rice et al., 2005a) was demonstrated for a few isolates. This was not the case of any of the strains included in our study lacking Tn*916*-like elements, thus opening the possibility of the involvement of different mechanisms responsible for intercellular transfer or mobilization (Manson et al., 2010). It is of note that disparate topologies of the phylogenetic trees of *E. faecium* core genomes and *E. faecium* PBP5 would further reflect how frequent the transference of *pbp5* genetic platforms under natural circumstances may occur. However, such horizontal gene transfer (HGT) events preferentially occur or are fixed in diverse populations prone to mutation (the subclade A1 that is characterized by a higher mutation rate that clade B) that would further facilitate their clonal expansion under ampicillin selective pressure, reflecting the "*ex unibus plurum*" evolutionary dynamics (Baquero, 2011). Heterogeneity of populations that colonize humans, designated as "clouds" elsewhere (Stanczak-Mrozek et al., 2015), leads to a possible global adaptive benefit for certain clones and finally for the overall species, that is enhanced by HGT.

The progressive increase in the number of isolates with reduced susceptibility to ampicillin paralleled the number of infections caused by *E. faecium* throughout the last decades (Grayson et al., 1991; National Nosocomial Infections Surveillance System, 2004; Treitman et al., 2005). While AmpR isolates recovered from humans are in low numbers and are always recovered under selective pressure, AmpR from pet animals have frequently been detected and are often associated with PBP5 variants commonly

found in these hosts (de Regt et al., 2012; Tedim et al., 2015). The specific causes associated with this AmpR-*E.faecium* emergence in different hosts remains unknown. Besides the increase in intestinal *E. faecium* population size following selective events in humans and the possible transmission events between animals and humans (Sánchez-Díaz et al., 2015; Ubeda et al., 2010), other factors could be responsible for *bloomings* or a more frequent representation of AmpR populations in the normal flora of another non-human hosts (Stecher et al., 2013).

The necessary contribution of different genes for the expression of AmpR phenotype is suggested by the lower MIC values of the transconjugants in comparison with the wild type strains in this and in previous studies (Rice et al., 2005b; Zhang et al., 2012). The transferable genetic region identified here contained the *pbp5* and *ponA* but also other genes (*ciaRH* operon) previously associated with β-lactam resistance in *Streptococcus pneumoniae*, but still unexplored in enterococci (Guenzi et al., 1994). Outside this region, only ldt_{Efm} and *ddcY* showed a similar non-congruent topology with that of *pbp5* indicating that these genes are also under accelerated evolution, which could explain eventual AmpR phenotypes with a lack of correlation with PBP5 sequences in some cases (Zhang et al., 2012). The variable ampicillin-susceptibility phenotypes observed in different transconjugants even when using the same recipient strain suggested either a partial transfer of the platform or occurrence of recombination events leading to variation in MICs, which seem to occur frequently in commensal bacteria (Levine et al., 2016).

In summary, the characterization of a chromosomal region containing the *pbp5* carrying different adaptive traits suggests its possible involvement in the adaptation of *E. faecium* to the gastrointestinal tract of mammals and the evolution of this species. The apparent frequent transfer events of an adaptive chromosomal region among “clouds” of closely related populations, indicates the relevance of bacterial shifts in the evolution of pathogenicity and antibiotic resistance. Such adaptations probably reflect changes in patients` demographics and medical strategies and interventions, within the paradigm of the “Hamiltonian medicine” (Fraser et al., 2005) and highlight the increasing need for evolutionary biology be aligned with medical challenges (Nesse et al., 2010).

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NUCLEOTIDE SEQUENCE GENBANK ACCESSION NUMBERS.

The sequences corresponding to representative *pbp5* genetic platforms (Types I-IV) were assigned Genbank accession numbers JN208885, JN208888, JN208884 and JN208886, respectively. PBP5 amino acid were analyzed and designated by a "C" followed by a number (Table S6). New sequences correspond to Genbank accession numbers JN208883 (C6 amino acid combination), JN208889 (C19 amino acid combination); JN208887 (C8 amino acid combination); JN208886 (C3 amino acid combination), JN208882 (C2 amino acid combination), KC479673 (C1 amino acid combination), KC479675 (C5 amino acid combination), KC479676 (C10 amino acid combination) and KC479674 (C12 amino acid combination).

REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs*. Nucleic Acids Res. 25, 3389–402.

Arias, C. A., Contreras, G. A., and Murray, B. E. (2010). Managem*ent of multidrug-resistant enterococcal infections*. Clin. Microbiol. Infect. 16, 555–562.

Arias, C. A., and Murray, B. E. (2012). *The rise of the Enterococcus: beyond vancomycin resistance.* Nat. Rev. Microbiol. 10, 266–78. doi:10.1038/nrmicro2761.

Baquero, F. (2011). *The 2010 Garrod Lecture: the dimensions of evolution in antibiotic resistance: ex unibus plurum et ex pluribus unum*. J. Antimicrob. Chemother. 66, 1659–72.

Belhaj, M., Boutiba-Ben Boubaker, I., and Slim, A. (2016). *Penicillin-Binding Protein 5 Sequence Alteration and Levels of plp5 mRNA Expression in Clinical Isolates of Enterococcus faecium with Different Levels of Ampicillin Resistance.* Microb. Drug Resist. 22, 202–10.

Besemer, J., Lomsadze, A., and Borodovsky, M. (2001). *GeneMarkS: a self-training method for prediction of gene starts in microbial genomes. Implications for finding sequence motifs in regulatory regions*. Nucleic Acids Res. 29, 2607–18.

Carias, L. L., Rudin, S. D., Donskey, C. J., and Rice, L. B. (1998). *Genetic linkage and cotransfer of a novel, vanB-containing transposon (Tn5382) and a low-affinity penicillin-binding protein 5 gene in a clinical vancomycin-resistant Enterococcus faecium isolate*. J. Bacteriol. 180, 4426–34.

Carvalho, M. G., Teixeira, L. M., and Facklam, R. R. (1998). *Use of tests for acidification of methyl-alpha-D-glucopyranoside and susceptibility to efrotomycin for differentiation of strains of Enterococcus and some related genera*. J. Clin. Microbiol. 36, 1584–7.

Centers for Disease Control and Prevention (2013). *Antibiotic Resistance Threats in the United States*.

Clinical and Laboratory Standards Institute (2012). *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement M100-S22*. CLSI, Wayne, PA, USA.

Coudron, P. E., Markowitz, S. M., and Wong, E. S. (1992). *Isolation of a beta-lactamase-producing, aminoglycoside-resistant strain of Enterococcus faecium*. Antimicrob. Agents Chemother. 36, 1125–6.

Darling, A. C. E., Mau, B., Blattner, F. R., and Perna, N. T. (2004). *Mauve: multiple alignment of conserved genomic sequence with rearrangements*. Genome Res. 14, 1394–403.

Fontana, R., Ligozzi, M., Pittaluga, F., and Satta, G. (1996). *Intrinsic penicillin resistance in enterococci*. Microb. Drug Resist. 2, 209–13.

Fraser, C., Hanage, W. P., and Spratt, B. G. (2005). *Neutral microepidemic evolution of bacterial pathogens.* Proc. Natl. Acad. Sci. U. S. A. 102, 1968–73.

Freitas, A. R., Novais, C., Tedim, A. P., Francia, M. V. M. V., Baquero, F., Coque, T. M., et al. (2013). *Microevolutionary Events Involving Narrow Host Plasmid Influences Local Fixation of Vancomycin-Resistance in Enterococcus*. PLoS One, e60589. doi:10.1371/journal.pone.0060589.

Freitas, A. R., Tedim, A. P., Novais, C., Ruiz-Garbajosa, P., Werner, G., Laverde-Gomez, J. A., et al. (2010a). *Global spread of the hyl(Efm) colonization-virulence gene in megaplasmids of the Enterococcus faecium CC17 polyclonal subcluster*. Antimicrob. Agents Chemother. 54, 2660–5. doi:10.1128/AAC.00134-10.

Freitas, A. R., Tedim-Pedrosa, A., Francia, M. V., Jensen, L. B., Novais, C., Ruiz-Garbajosa, P., et al. (2010b). *Plasmid diversity among Enterococcus faecium belonging to the polyclonal sub-cluster CC17: their impact in the spread of the vancomycin resistance worldwide (1986-2009)*. *in* 3rd ASM Conference on Enterococci (Portland) (Whashington DC, USA), 18–19.

Galloway-Peña, J. R., Rice, L. B., and Murray, B. E. (2011). *Analysis of PBP5 of early U.S. isolates of Enterococcus faecium: sequence variation alone does not explain increasing ampicillin resistance over time*. Antimicrob. Agents Chemother. 55, 3272–7.

Galloway-Peña, J., Roh, J. H., Latorre, M., Qin, X., and Murray, B. E. (2012). *Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of Enterococcus faecium*. PLoS One 7, e30187.

Gonçalves, A., Igrejas, G., Radhouani, H., López, M., Guerra, A., Petrucci-Fonseca, F., et al. (2011). *Detection of vancomycin-resistant enterococci from faecal samples of Iberian wolf and Iberian lynx, including Enterococcus faecium strains of CC17 and the new singleton ST573.* Sci. Total Environ. 410-411, 266–8.

Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J., et al. (2010). *A new bioinformatics analysis tools framework at EMBL-EBI*. Nucleic Acids Res. 38, W695–9.

Grayson, M. L., Eliopoulos, G. M., Wennersten, C. B., Ruoff, K. L., De Girolami, P. C., Ferraro, M. J., et al. (1991). *Increasing resistance to beta-lactam antibiotics among clinical isolates of Enterococcus faecium: a 22-year review at one institution*. Antimicrob. Agents Chemother. 35, 2180–4.

Guenzi, E., Gasc, A. M., Sicard, M. A., and Hakenbeck, R. (1994). *A two-component signal-transducing system is involved in competence and penicillin susceptibility in laboratory mutants of Streptococcus pneumoniae*. Mol. Microbiol. 12, 505–15.

Heikens, E., Bonten, M. J. M., and Willems, R. J. L. (2007). *Enterococcal surface protein Esp is important for biofilm formation of Enterococcus faecium E1162*. J. Bacteriol. 189, 8233–40.

Hendrickx, A. P. A., van Schaik, W., and Willems, R. J. L. (2013). *The cell wall architecture of Enterococcus faecium: from resistance to pathogenesis.* Future Microbiol. 8, 993–1010.

Hiramatsu, K., Ito, T., Tsubakishita, S., Sasaki, T., Takeuchi, F., Morimoto, Y., et al. (2013). *Genomic Basis for Methicillin Resistance in Staphylococcus aureus.* Infect. Chemother. 45, 117.

Homan, W. L., Tribe, D., Poznanski, S., Li, M., Hogg, G., Spalburg, E., et al. (2002). *Multilocus sequence typing scheme for Enterococcus faecium*. J. Clin. Microbiol. 40, 1963–71.

Jordan, S., Hutchings, M. I., and Mascher, T. (2008). C*ell envelope stress response in Gram-positive bacteria.* FEMS Microbiol. Rev. 32, 107–46.

Katoh, K., and Toh, H. (2010). *Parallelization of the MAFFT multiple sequence alignment program*. Bioinformatics 26, 1899–900.

Khaskheli, G. B., Zuo, F., Yu, R., and Chen, S. (2015). *Overexpression of Small Heat Shock Protein Enhances Heat- and Salt-Stress Tolerance of Bifidobacterium longum NCC2705.* Curr. Microbiol. 71, 8–15.

Klare, I., Rodloff, A. C., Wagner, J., Witte, W., and Hakenbeck, R. (1992). *Overproduction of a penicillin-binding protein is not the only mechanism of penicillin resistance in Enterococcus faecium*. Antimicrob. Agents Chemother. 36, 783–7.

Krawczyk-Balska, A., and Markiewicz, Z. (2016). *The intrinsic cephalosporin resistome of Listeria monocytogenes in the context of stress response, gene regulation, pathogenesis and therapeutics*. J. Appl. Microbiol. 120, 251–65.

Kristich, C., Rice, L., and Arias, C. (2014). "*Enterococcal Infection—Treatment and Antibiotic Resistance*," *in Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*, eds. M. S. Gilmore, D. B. Clewell, Y. Ike, and N. Shankar (Boston: Massachusetts Eye and Ear Infirmary).

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., et al. (2007). *Clustal W and Clustal X version 2.0*. Bioinformatics 23, 2947–8.

Lebreton, F., van Schaik, W., McGuire, A. M., Godfrey, P., Griggs, A., Mazumdar, V., et al. (2013). *Emergence of epidemic multidrug-resistant Enterococcus faecium from animal and commensal strains*. MBio 4 e00534-13.

Levine, M. M., Stinear, T., Holt, K. E., Robins-Browne, R. M., Ingle, D. J., Kuzevski, A., et al. (2016). *In silico serotyping of E. coli from short read data identifies limited novel O-loci but extensive diversity of O:H serotype combinations within and between pathogenic lineages*. Microb. Genomics.

Li, W., and Godzik, A. (2006). *Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences*. Bioinformatics 22, 1658–9.

Liu, S. L., Hessel, A., and Sanderson, K. E. (1993). *Genomic mapping with I-Ceu I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in Salmonella spp., Escherichia coli, and other bacteria*. Proc. Natl. Acad. Sci. U. S. A. 90, 6874–8.

López, M., Sáenz, Y., Rojo-Bezares, B., Martínez, S., del Campo, R., Ruiz-Larrea, F., et al. (2009). *Detection of vanA and vanB2-containing enterococci from food samples in Spain, including Enterococcus faecium strains of CC17 and the new singleton ST425*. Int. J. Food Microbiol. 133, 172–8.

Manson, J. M., Hancock, L. E., and Gilmore, M. S. (2010). *Mechanism of chromosomal transfer of Enterococcus faecalis pathogenicity island, capsule, antimicrobial resistance, and other traits*. Proc. Natl. Acad. Sci. U. S. A. 107, 12269–74.

National Nosocomial Infections Surveillance System (2004). *National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004, issued October 2004*. Am. J. Infect. Control 32, 470–85.

Nesse, R. M., Bergstrom, C. T., Ellison, P. T., Flier, J. S., Gluckman, P., Govindaraju, D. R., et al. (2010). *Evolution in health and medicine Sackler colloquium: Making evolutionary biology a basic science for medicine.* Proc. Natl. Acad. Sci. U. S. A. 107 Suppl , 1800–7.

Novais, A., Comas, I., Baquero, F., Cantón, R., Coque, T. M., Moya, A., et al. (2010). *Evolutionary trajectories of beta-lactamase CTX-M-1 cluster enzymes: predicting antibiotic resistance.* PLoS Pathog. 6, e1000735.

Novais, C., Coque, T. M., Costa, M. J., Sousa, J. C., Baquero, F., and Peixe, L. V (2005a). *High occurrence and persistence of antibiotic-resistant enterococci in poultry food samples in Portugal.* J. Antimicrob. Chemother. 56, 1139–43.

Novais, C., Coque, T. M., Ferreira, H., Sousa, J. C., and Peixe, L. (2005b). *Environmental contamination with vancomycin-resistant enterococci from hospital sewage in Portugal*. Appl. Environ. Microbiol. 71, 3364–8.

Novais, C., Coque, T. M., Sousa, J. C., and Peixe, L. V (2006). *Antimicrobial resistance among faecal enterococci from healthy individuals in Portugal.* Clin. Microbiol. Infect. 12, 1131–4.

Novais, C., Freitas, A. R., Francia, M. V., Peixe, L., Baquero, F., and Coque, T. M. (2009). *Characterization of Gram-positive conjugative transposons among international epidemic vancomycin-resistant isolates of Enterococcus faecalis and Enterococcus faecium*. *in* 19th European Congress Clinical Microbiology Infectious Diseases (Helsinki, Finland: European Society of Clinical Microbiology and Infectious Diseases), 247.

Novais, C., Freitas, A. R., Silveira, E., Antunes, P., Silva, R., Coque, T. M., et al. (2013). *Spread of multidrug-resistant Enterococcus to animals and humans: an underestimated role for the pig farm environment*. J. Antimicrob. Chemother. 68, 2746–54.

Novais, C., Sousa, J. C., Coque, T. M., and Peixe, L. V (2005c). *Molecular characterization of glycopeptide-resistant Enterococcus faecium isolates from Portuguese hospitals*. Antimicrob. Agents Chemother. 49, 3073–9.

Palmer, K. L., Godfrey, P., Griggs, A., Kos, V. N., Zucker, J., Desjardins, C., et al. (2012). *Comparative genomics of enterococci: variation in Enterococcus faecalis, clade structure in E. faecium, and defining characteristics of E. gallinarum and E. casseliflavus.* MBio 3, e00318–11.

Paradis, E., Claude, J., and Strimmer, K. (2004). *APE: Analyses of Phylogenetics and Evolution in R language.* Bioinformatics 20, 289–290.

Pietta, E., Montealegre, M. C., Roh, J. H., Cocconcelli, P. S., and Murray, B. E. (2014). *Enterococcus faecium PBP5-S/R, the missing link between PBP5-S and PBP5-R*. Antimicrob. Agents Chemother. 58, 6978–81.

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010*). FastTree 2--approximately maximum-likelihood trees for large alignments*. PLoS One 5, e9490.

Raze, D., Dardenne, O., Hallut, S., Martinez-Bueno, M., Coyette, J., and Ghuysen, J. M. (1998). *The gene encoding the low-affinity penicillin-binding protein 3r in Enterococcus hirae S185R is borne on a plasmid carrying other antibiotic resistance determinants*. Antimicrob. Agents Chemother. 42, 534–9.

de Regt, M. J. A., van Schaik, W., van Luit-Asbroek, M., Dekker, H. A. T., van Duijkeren, E., Koning, C. J. M., et al. (2012). *Hospital and community ampicillin-resistant Enterococcus faecium are evolutionarily closely linked but have diversified through niche adaptation.* PLoS One 7, e30319.

Rice, L. B., Bellais, S., Carias, L. L., Hutton-Thomas, R., Bonomo, R. A., Caspers, P., et al. (2004). *Impact of specific pbp5 mutations on expression of beta-lactam resistance in Enterococcus faecium.* Antimicrob. Agents Chemother. 48, 3028–32.

Rice, L. B., Carias, L. L., Hutton-Thomas, R., and Rudin, S. (2007). *Interaction of related Tn916-like transposons: analysis of excision events promoted by Tn916 and Tn5386 integrases*. J. Bacteriol. 189, 3909–17. doi:10.1128/JB.00859-06.

Rice, L. B., Carias, L. L., Hutton-Thomas, R., Sifaoui, F., Gutmann, L., and Rudin, S. D. (2001). *Penicillin-binding protein 5 and expression of ampicillin resistance in Enterococcus faecium.* Antimicrob. Agents Chemother. 45, 1480–6.

Rice, L. B., Carias, L. L., Marshall, S., Rudin, S. D., and Hutton-Thomas, R. (2005a). *Tn5386, a novel Tn916-like mobile element in Enterococcus faecium D344R that interacts with Tn916 to yield a large genomic deletion.* J. Bacteriol. 187, 6668–77.

Rice, L. B., Carias, L. L., Rudin, S., Hutton, R., Marshall, S., Hassan, M., et al. (2009). *Role of class A penicillin-binding proteins in the expression of beta-lactam resistance in Enterococcus faecium.* J. Bacteriol. 191, 3649–56.

Rice, L. B., Carias, L. L., Rudin, S., Lakticová, V., Wood, A., and Hutton-Thomas, R. (2005b). *Enterococcus faecium low-affinity pbp5 is a transferable determinant*. Antimicrob. Agents Chemother. 49, 5007–12.

Rybkine, T., Mainardi, J. L., Sougakoff, W., Collatz, E., and Gutmann, L. (1998). *Penicillin-binding protein 5 sequence alterations in clinical isolates of Enterococcus faecium with different levels of beta-lactam resistance.* J. Infect. Dis. 178, 159–63.

Sacco, E., Cortes, M., Josseaume, N., Rice, L. B., Mainardi, J.-L., and Arthur, M. (2014). *Serine/threonine protein phosphatase-mediated control of the peptidoglycan cross-linking L,D-transpeptidase pathway in Enterococcus faecium.* MBio 5, e01446–14..

Sánchez-Díaz, A. M., Cuartero, C., Rodríguez, J. D., Lozano, S., Alonso, J. M., Rodríguez-Domínguez, M., et al. (2015). *The rise of ampicillin-resistant Enterococcus faecium high-risk clones as a frequent intestinal colonizer in oncohaematologic neutropenic patients on levofloxacin prophylaxis: a risk for bacteraemia?* Clin. Microbiol. Infect. 22:59.e1-8.

Santos, T., Silva, N., Igrejas, G., Rodrigues, P., Micael, J., Rodrigues, T., et al. (2013). *Dissemination of antibiotic resistant Enterococcus spp. and Escherichia coli from wild birds of Azores Archipelago*. Anaerobe 24, 25–31.

Sifaoui, F., Arthur, M., Rice, L., and Gutmann, L. (2001). *Role of penicillin-binding protein 5 in expression of ampicillin resistance and peptidoglycan structure in Enterococcus faecium*. Antimicrob. Agents Chemother. 45, 2594–7.

Stanczak-Mrozek, K. I., Manne, A., Knight, G. M., Gould, K., Witney, A. A., and Lindsay, J. A. (2015). *Within-host diversity of MRSA antimicrobial resistances*. J. Antimicrob. Chemother. 70, 2191–8.

Stecher, B., Maier, L., and Hardt, W.-D. (2013). *"Blooming" in the gut: how dysbiosis might contribute to pathogen evolution.* Nat. Rev. Microbiol. 11, 277–84.

Sullivan, M. J., Petty, N. K., and Beatson, S. A. (2011). *Easyfig: a genome comparison visualizer*. Bioinformatics 27, 1009–10.

Tedim, A. P., Ruiz-Garbajosa, P., Corander, J., Rodríguez, C. M., Cantón, R., Willems, R. J., et al. (2015). *Population Biology of Intestinal Enterococcus Isolates from Hospitalized and Nonhospitalized Individuals in Different Age Groups*. Appl. Environ. Microbiol. 81, 1820–1831.

Tenover, F. C., Arbeit, R. D., Goering, R. V, Mickelsen, P. A., Murray, B. E., Persing, D. H., et al. (1995). *Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing*. J. Clin. Microbiol. 33, 2233–9.

Top, J., Willems, R., Blok, H., de Regt, M., Jalink, K., Troelstra, A., et al. (2007). *Ecological replacement of Enterococcus faecalis by multiresistant clonal complex 17 Enterococcus faecium.* Clin. Microbiol. Infect. 13, 316–9.

Top, J., Willems, R., and Bonten, M. (2008). *Emergence of CC17 Enterococcus faecium: from commensal to hospital-adapted pathogen*. FEMS Immunol. Med. Microbiol. 52, 297–308.

Treitman, A. N., Yarnold, P. R., Warren, J., and Noskin, G. A. (2005). *Emerging incidence of Enterococcus faecium among hospital isolates (1993 to 2002).* J. Clin. Microbiol. 43, 462–3.

Tremblay, C.-L., Charlebois, A., Masson, L., and Archambault, M. (2013). *Characterization of hospital-associated lineages of ampicillin-resistant Enterococcus faecium from clinical cases in dogs and humans*. Front. Microbiol. 4, 245.

Ubeda, C., Taur, Y., Jenq, R. R., Equinda, M. J., Son, T., Samstein, M., et al. (2010). *Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans*. J. Clin. Invest. 120, 4332–41.

Wagenvoort, J. H. T., De Brauwer, E. I. G. B., Penders, R. J. R., van der Linden, C. J., Willems, R. J., Top, J., et al. (2015). *Environmental survival of vancomycin-sensitive ampicillin-resistant Enterococcus faecium (AREfm).* Eur. J. Clin. Microbiol. Infect. Dis. 34, 1901–3.

Willems, R. J. L., Top, J., van Santen, M., Robinson, D. A., Coque, T. M., Baquero, F., et al. (2005). *Global spread of vancomycin-resistant Enterococcus faecium from distinct nosocomial genetic complex.* Emerg. Infect. Dis. 11, 821–8.

Willems, R. J. L., Top, J., van Schaik, W., Leavis, H., Bonten, M., Sirén, J., et al. (2012). *Restricted gene flow among hospital subpopulations of Enterococcus faecium.* MBio 3, e00151–12.

Williamson, R., Calderwood, S. B., Moellering, R. C., and Tomasz, A. (1983). *Studies on the mechanism of intrinsic resistance to beta-lactam antibiotics in group D streptococci.* J. Gen. Microbiol. 129, 813–22.

Zhang, X., Paganelli, F. L., Bierschenk, D., Kuipers, A., Bonten, M. J. M., Willems, R. J. L., et al. (2012). *Genome-wide identification of ampicillin resistance determinants in Enterococcus faecium.* PLoS Genet. 8, e1002804.

Zorzi, W., Zhou, X. Y., Dardenne, O., Lamotte, J., Raze, D., Pierre, J., et al. (1996). *Structure of the low-affinity penicillin-binding protein 5 PBP5fm in wild-type and highly penicillin-resistant strains of Enterococcus faecium*. J. Bacteriol. 178, 4948–57.

SUPLEMENTARY MATERIAL

Table S1. Number of reads and coverage of the bacterial genomes analyzed

Genome	Number of sequenced reads (Genome coverage)	DNA bases (Genome coverage)
E. faecium GE1 (recipient strain)	1169494	416713283
E. faecium HPH2 (wild strain)	1120024	394271010
E. faecium TCHPH2-1(transconjugant)	1123087	414888338

Table S2. Statistics of the sequence datasets.

Bacterial sample	Aligned reads	Aligned bases	Number of contigs	N50 contig size
E. faecium GE1 (recipient strain)	1133856 (96.95%)	401958556 (96.46%)	313	7.4 K
E. faecium HPH2 (wild strain)	109511 (97.78%)	384306935 (97.47%)	421	3.7 K
E. faecium TCHPH2-1(transconjugant)	1082923 (96.42%)	397931731 (95.91%)	157	6.7 K

Table S3 - Best BLAST Hit annotation of E. faecium HPH2, GE1 and GEHPH2.1.

Efm GEHPH2.1	*Efm* HPH2	*Efm* GE1	Best BLAST Hit Annotation
contig00001|1	0	1	UniRef100_D4SNG0 Zeta toxin family protein n=7 Tax=Enterococcus faecium RepID=D4SNG0_ENTFC
contig00001|2	0	1	UniRef100_L2IE87 DJ-1/Pfp1 family protein n=5 Tax=Enterococcus faecium RepID=L2IE87_ENTFC
contig00001|3	0	1	UniRef100_K0ZNC5 Uncharacterized protein n=22 Tax=Enterococcus RepID=K0ZNC5_9ENTE
contig00001|4	0	1	UniRef100_D4QSL9 LD-carboxypeptidase superfamily n=39 Tax=Enterococcus RepID=D4QSL9_ENTFC
contig00001|5	1	1	UniRef100_H8L917 YbaK/prolyl-tRNA synthetase-associated domain protein n=146 Tax=Firmicutes RepID=H8L917_ENTFU
contig00001|6	0	1	UniRef100_H8L918 Uncharacterized protein n=199 Tax=Firmicutes RepID=H8L918_ENTFU
contig00001|7	0	1	UniRef100_D4SNF2 DNA binding domain, excisionase family protein n=67 Tax=Enterococcus RepID=D4SNF2_ENTFC
contig00001|8	0	1	UniRef100_H8L920 Sugar transport protein n=198 Tax=Firmicutes RepID=H8L920_ENTFU
contig00001|9	0	1	UniRef100_J6AI78 Glucose 1-dehydrogenase n=87 Tax=Enterococcus RepID=J6AI78_ENTFC
contig00001|10	0	1	UniRef100_H8L922 Riboflavin biosynthesis protein RibF n=130 Tax=Firmicutes RepID=H8L922_ENTFU
contig00001|11	0	1	UniRef100_J6RB01 tRNA pseudouridine synthase B n=58 Tax=Enterococcus faecium RepID=J6RB01_ENTFC
contig00001|12	0	1	UniRef100_S4F5X4 D-xylulose 5-phosphate/D-fructose 6-phosphate phosphoketolase n=3 Tax=Enterococcus faecium RepID=S4F5X4_ENTFC
contig00001|13	0	1	UniRef100_C9B4V5 Ribosome-binding factor A n=145 Tax=Enterococcus RepID=C9B4V5_ENTFC
contig00001|14	0	1	UniRef100_C9BZ75 Translation initiation factor IF-2 n=92 Tax=Enterococcus RepID=C9BZ75_ENTFC
contig00001|15	1	1	UniRef100_H8L928 50S ribosomal protein L7Ae n=203 Tax=Firmicutes RepID=H8L928_ENTFU
contig00001|16	1	1	UniRef100_H8L929 Cytosolic protein YlxR n=231 Tax=Firmicutes RepID=H8L929_ENTFU
contig00001|17	0	1	UniRef100_D4SNE1 Transcription termination factor NusA n=36 Tax=Enterococcus faecium RepID=D4SNE1_ENTFC
contig00001|18	1	1	UniRef100_H8L931 Ribosome maturation factor RimP n=124 Tax=Firmicutes RepID=H8L931_ENTFU
contig00001|19	0	1	UniRef100_D4SND9 DNA polymerase III polC-type n=36 Tax=Enterococcus faecium RepID=D4SND9_ENTFC
contig00001|20	0	1	UniRef100_D4SND8 Proline--tRNA ligase n=35 Tax=Enterococcus faecium RepID=D4SND8_ENTFC
contig00001|21	0	1	UniRef100_J6KDD3 RIP metalloprotease RseP n=29 Tax=Enterococcus faecium RepID=J6KDD3_ENTFC
contig00001|22	0	1	UniRef100_D4SND6 Phosphatidate cytidylyltransferase n=13 Tax=Enterococcus faecium RepID=D4SND6_ENTFC
contig00001|23	0	1	UniRef100_H8L936 Isoprenyl transferase n=194 Tax=Firmicutes RepID=H8L936_ENTFU
contig00001|24	1	1	UniRef100_H8L937 Ribosome-recycling factor n=227 Tax=Firmicutes RepID=H8L937_ENTFU
contig00001|25	1	1	UniRef100_S4E7P4 Uridylate kinase n=1 Tax=Enterococcus faecium SD3B-2 RepID=S4E7P4_ENTFC
contig00001|26	0	1	UniRef100_J6KDE7 Elongation factor Ts n=7 Tax=Enterococcus faecium RepID=J6KDE7_ENTFC
contig00001|27	1	1	UniRef100_E4I4V0 30S ribosomal protein S2 n=48 Tax=Enterococcus faecium RepID=E4I4V0_ENTFC
contig00001|28	1	1	UniRef100_H8L941 Arginine repressor n=179 Tax=Firmicutes RepID=H8L941_ENTFU
contig00001|29	0	1	UniRef100_S4EV59 2-aminoethylphosphonate--pyruvate transaminase domain protein n=5 Tax=Enterococcus faecium RepID=S4EV59_ENTFC
contig00001|30	0	1	UniRef100_D4SNC8 2-aminoethylphosphonate:pyruvate transaminase n=1 Tax=Enterococcus faecium E1039 RepID=D4SNC8_ENTFC
contig00001|31	0	1	UniRef100_D4SNC7 Phosphonoacetaldehyde hydrolase n=6 Tax=Enterococcus faecium RepID=D4SNC7_ENTFC
contig00001|32	0	1	UniRef100_D4SLK2 Arginine--tRNA ligase n=3 Tax=Enterococcus faecium RepID=D4SLK2_ENTFC
contig00001|33	0	1	UniRef100_H8L946 Carbamate kinase n=194 Tax=Firmicutes RepID=H8L946_ENTFU
contig00001|34	0	1	UniRef100_H8L947 Ornithine carbamoyltransferase n=231 Tax=Firmicutes RepID=H8L947_ENTFU
contig00001|35	0	1	UniRef100_H8L948 Arginine deiminase n=201 Tax=Firmicutes RepID=H8L948_ENTFU
contig00001|36	0	1	UniRef100_H8L949 Cyclic nucleotide-binding domain protein n=176 Tax=Enterococcus RepID=H8L949_ENTFU
contig00001|37	0	1	UniRef100_D4QQM9 tRNA N6-adenosine threonylcarbamoyltransferase n=78 Tax=Enterococcus RepID=D4QQM9_ENTFC
contig00001|38	0	0	UniRef100_D4QQN0 Ribosomal-protein-alanine acetyltransferase n=79 Tax=Enterococcus RepID=D4QQN0_ENTFC
contig00001|39	0	1	UniRef100_L2JDN6 Ribosomal-protein-alanine acetyltransferase n=84 Tax=Enterococcus RepID=L2JDN6_ENTFC
contig00001|40	0	1	UniRef100_S4F5Z1 Universal bacterial protein YeaZ n=30 Tax=Enterococcus faecium RepID=S4F5Z1_ENTFC
contig00001|41	0	1	UniRef100_L2IC58 LacI family transcriptional regulator n=5 Tax=Enterococcus faecium RepID=L2IC58_ENTFC
contig00001|42	0	1	UniRef100_H8L955 Galactose-1-phosphate uridylyltransferase n=145 Tax=Firmicutes RepID=H8L955_ENTFU
contig00001|43	0	1	UniRef100_S4EXE0 UDP-glucose 4-epimerase n=1 Tax=Enterococcus faecium OC2A-1 RepID=S4EXE0_ENTFC
contig00001|44	1	1	UniRef100_H8L957 Uncharacterized protein n=230 Tax=Firmicutes RepID=H8L957_ENTFU
contig00001|45	0	1	UniRef100_C9BQG2 FAD(NAD)-dependent oxidoreductase n=14 Tax=Firmicutes RepID=C9BQG2_ENTFC
contig00001|46	1	1	UniRef100_H8L959 Uncharacterized protein n=196 Tax=Firmicutes RepID=H8L959_ENTFU
contig00001|47	1	1	UniRef100_H8L9W4 Rhodanese family protein n=150 Tax=Firmicutes RepID=H8L9W4_ENTFU
contig00001|48	0	1	UniRef100_H8L9W5 Glucokinase n=152 Tax=Firmicutes RepID=H8L9W5_ENTFU
contig00001|49	1	1	UniRef100_H8L9W6 Uncharacterized protein n=233 Tax=Firmicutes RepID=H8L9W6_ENTFU
contig00001|50	1	1	UniRef100_S4FX86 Peptidase, S54 family n=6 Tax=Enterococcus faecium RepID=S4FX86_ENTFC
contig00001|51	1	1	UniRef100_H8L9W8 5-formyltetrahydrofolate cyclo-ligase family protein n=198 Tax=Firmicutes RepID=H8L9W8_ENTFU
contig00001|52	0	1	UniRef100_D3LGD5 Uncharacterized protein n=51 Tax=Enterococcus faecium RepID=D3LGD5_ENTFC
contig00001|53	0	1	UniRef100_C9BQH0 Iron-hydroxamate transporter permease subunit n=147 Tax=Firmicutes RepID=C9BQH0_ENTFC
contig00001|54	0	1	UniRef100_S4F5Z8 Ferrichrome ABC transporter, ATP-binding protein FhuC n=5 Tax=Enterococcus faecium RepID=S4F5Z8_ENTFC
contig00001|55	0	1	UniRef100_J6PNA8 Periplasmic binding protein n=13 Tax=Enterococcus faecium RepID=J6PNA8_ENTFC
contig00001|56	0	1	UniRef100_H8L9X3 Dihydrofolate reductase n=178 Tax=Firmicutes RepID=H8L9X3_ENTFU
contig00001|57	1	1	UniRef100_D0AG91 Uncharacterized protein n=156 Tax=Firmicutes RepID=D0AG91_ENTFC
contig00001|58	0	1	UniRef100_C9B4Q9 Asparagine synthetase n=104 Tax=Enterococcus RepID=C9B4Q9_ENTFC
contig00001|59	0	1	UniRef100_D0AG93 Uncharacterized protein n=46 Tax=Enterococcus faecium RepID=D0AG93_ENTFC
contig00001|60	1	1	UniRef100_G9SRP3 Putative uncharacterized protein n=1 Tax=Enterococcus faecium E4453 RepID=G9SRP3_ENTFC
contig00001|61	1	1	UniRef100_H8L9Y3 Transcriptional regulator, TetR family n=142 Tax=Firmicutes RepID=H8L9Y3_ENTFU
contig00001|62	0	1	UniRef100_L2HZF2 Uncharacterized protein n=4 Tax=Enterococcus faecium RepID=L2HZF2_ENTFC
contig00001|63	0	1	UniRef100_H8L9Y5 Uncharacterized protein n=150 Tax=Firmicutes RepID=H8L9Y5_ENTFU
contig00001|64	0	1	UniRef100_D0ADQ9 Uncharacterized protein n=62 Tax=Enterococcus RepID=D0ADQ9_ENTFC
contig00001|65	0	1	UniRef100_L2HZQ9 Carboxymuconolactone decarboxylase n=7 Tax=Enterococcus faecium RepID=L2HZQ9_ENTFC
contig00001|66	1	1	UniRef100_H8L9Y9 Uncharacterized protein n=180 Tax=Firmicutes RepID=H8L9Y9_ENTFU

Due to the size of the table it will be provided only in digital format.

Table S4 - Annotation of transferable region of *E. faecium* GEHPH2.1 using Best BLAST Hit and *E. faecium* DO as a reference strain

E. Faecium GEHPH2.1	*E. faecium* HPH2	*E. faecium* GE1	Best BLAST Hit Annotation	*E. faecium* DO Annotation	*E. faecium* DO GenBank accession no.	GC content (%)
contig00158|94	1	0	UniRef100_H8LEC1 X-Pro dipeptidyl-peptidase n=171 Tax=Firmicutes RepID=H8LEC1_ENTFU	S9 family peptidase (Ch) CDS 747	YP_006375905	39.9
contig00158|93	1	0	UniRef100_S4EMS2 Glyceraldehyde-3-phosphate dehydrogenase, type I n=41 Tax=Enterococcus RepID=S4EMS2_ENTFC	gapA (ch)	YP_006375906	40.5
contig00158|92	1	0	UniRef100_H8LEB9 GTPase obgn=138 Tax=Firmicutes RepID=H8LEB9_ENTFU	cgtA (Ch)	YP_006375907	45.1
contig00158|91	1	0	UniRef100_H8LEB8 Uncharacterized protein n=209 Tax=Firmicutes RepID=H8LEB8_ENTFU	HP (Ch) CDS 748	YP_006375908	40.1
contig00158|89	1	0	UniRef100_H8LEB7 Ribonuclease Z n=200 Tax=Firmicutes RepID=H8LEB7_ENTFU	rnz (Ch)	YP_006375909	40.6
contig00158|88	1	0	UniRef100_C9B5V5 Short chain dehydrogenase/reductase n=49 Tax=Enterococcus RepID=C9B5V5_ENTFC	Dehydrogenase (Ch) CDS 749	YP_006375910	41.4
contig00158|87	1	0	UniRef100_C9B5V4 Predicted protein n=55 Tax=Enterococcus faecium RepID=C9B5V4_ENTFC	HP (Ch) CDS 750	YP_006375911	38.3
contig00158|86	1	0	UniRef100_L2NQR6 Single-stranded-DNA-specific exonuclease RecJ n=2 Tax=Enterococcus faecium RepID=L2NQR6_ENTFC	recJ (Ch)	YP_006375912	40.8
contig00158|85	1	0	UniRef100_H8LEB3 Adenine phosphoribosyltransferase n=229 Tax=Firmicutes RepID=H8LEB3_ENTFU	apt (Ch)	YP_006375913	41.9
contig00158|84	1	0	UniRef100_E4J2D5 LexA repressor n=21 Tax=Enterococcus faecium RepID=E4J2D5_ENTFC	lexA (Ch)	YP_006375915	42.7
contig00158|83	1	0	UniRef100_H8LEB0 UPF0291 protein EFAU004_01010 n=178 Tax=Firmicutes RepID=H8LEB0_ENTFU	HP (Ch) CDS 752	YP_006375916	39.3
contig00158|82	1	0	UniRef100_J6Z718 Transketolase n=8 Tax=Enterococcus faecium RepID=J6Z718_ENTFC	tktA (Ch)	YP_006375917	42.5
contig00158|81	0	0	UniRef100_C9BZP1 Transposase n=119 Tax=Firmicutes RepID=C9BZP1_ENTFC	ISEfa4 (IS200/IS605 family)	YP_006377716	32.6
contig00158|79	0	0	UniRef100_L2MTX1 Uncharacterized protein n=1 Tax=Enterococcus faecium EnGen0025 RepID=L2MTX1_ENTFC	ΔamiC (Ch)	YP_006375918	41
contig00158|77	1	0	UniRef100_S5V6G7 Cysteine synthase A n=1 Tax=Enterococcus faecium Aus0085 RepID=S5V6G7_ENTFC	cysteine synthase A (Ch) (Low homology probably)	YP_006375920/19	41.1
contig00158|76	1	1	UniRef100_H8LEA5 Transcriptional regulator, Fur family n=220 Tax=Firmicutes RepID=H8LEA5_ENTFU	fur2 (Ch)	YP_006375921	37.6
contig00158|75	1	0	UniRef100_L2MTW4 NADH oxidase n=3 Tax=Enterococcus faecium RepID=L2MTW4_ENTFC	nox (Ch)	YP_006375922	38.7
contig00158|74	1	0	UniRef100_H8LEA3 Uncharacterized protein n=103 Tax=Firmicutes RepID=H8LEA3_ENTFU	HP (Ch) CDS 754	YP_006375923	35.7
contig00158|73	1	0	UniRef100_E4IAX4 Transcriptional regulator, MarR family n=34 Tax=Enterococcus faecium RepID=E4IAX4_ENTFC	marR (Ch)	YP_006375924	32.6
contig00158|72	1	0	UniRef100_H8LEA1 Major facilitator superfamily transporter n=99 Tax=Firmicutes RepID=H8LEA1_ENTFU	MFS transporter (Ch) CDS 755	YP_006375925	39.2
contig00158|71	1	0	UniRef100_H8LEA0 DnaD and phage-associated domain protein n=198 Tax=Firmicutes RepID=H8LEA0_ENTFU	dnaD (Ch)	YP_006375926	36.2
contig00158|70	1	0	UniRef100_H8LE99 Endonuclease III n=129 Tax=Firmicutes RepID=H8LE99_ENTFU	nth (h)	YP_006375927	41.9
contig00158|69	1	0	UniRef100_E4IAX0 Uncharacterized protein n=52 Tax=Enterococcus faecium RepID=E4IAX0_ENTFC	HP (Ch) CDS 756	YP_006375928	26.4
contig00158|68	1	0	UniRef100_H8LE97 Penicillin-binding protein 1A n=107 Tax=Firmicutes RepID=H8LE97_ENTFU	ponA	YP_006375929	42.1
contig00158|67	1	1	UniRef100_H8LE96 Holliday junction resolvase RecU n=141 Tax=Firmicutes RepID=H8LE96_ENTFU	recU (Ch)	YP_006375930	39.5
contig00158|65	1	1	UniRef100_J8ZL23 Cell cycle protein GpsB n=7 Tax=Enterococcus RepID=J8ZL23_ENTFC	divIVA2 (CH)	YP_006375932	36.1
contig00158|64	0	1	UniRef100_D4SK72 Methyltransferase n=3 Tax=Enterococcus faecium RepID=D4SK72_ENTFC	methyltransferase (Ch) CDS 758	YP_006375933	41
contig00158|63	0	1	UniRef100_H8LE92 Carboxypeptidase Taq metallopeptidase n=169 Tax=Firmicutes RepID=H8LE92_ENTFU	peptidase (Ch) CDS 759	YP_006375934	37
contig00158|62	0	1	UniRef100_H8LE91 HD domain-containing protein n=200 Tax=Firmicutes RepID=H8LE91_ENTFU	phosphohydrolase (Ch) CDS 760	YP_006375935	39.8
contig00158|61	0	1	UniRef100_J6EDE4 Putative 8-amino-7-oxononanoate synthase n=1 Tax=Enterococcus faecium ERV161 RepID=J6EDE4_ENTFC	kbl (Ch)	YP_006375936	39.2
contig00158|60	0	1	UniRef100_D4SK76 Epimerase/reductase, putative n=3 Tax=Enterococcus faecium RepID=D4SK76_ENTFC	galE2 (Ch)	YP_006375937	40.8
contig00158|59	0	1	UniRef100_S4EFC0 Putative serine/threonine exchanger SteT n=1 Tax=Enterococcus faecium SD1C-2 RepID=S4EFC0_ENTFC	APC transporter (Ch) CDS 761	YP_006375938	41.2
contig00158|58	0	1	UniRef100_L2HCD9 Uncharacterized protein n=12 Tax=Enterococcus faecium RepID=L2HCD9_ENTFC	MP (Ch) CDS 762	YP_006375939	35.8
contig00158|57	0	1	UniRef100_D4RG09 Permease n=23 Tax=Enterococcus faecium RepID=D4RG09_ENTFC	permease (vanZ family) (Ch) CDS 763	YP_006375940	36.9
contig00158|56	0	1	UniRef100_H8LE84 DEAD-box ATP dependent DNA helicase n=176 Tax=Firmicutes RepID=H8LE84_ENTFU	rhlB (Ch)	YP_006375941	38.1
contig00158|55	0	1	UniRef100_J6L2G7 Acetyltransferase, GNAT family n=101 Tax=Enterococcus RepID=J6L2G7_ENTFC	elaA (Ch)	YP_006375942	38.9
contig00158|53	0	1	UniRef100_D4SK82 Alanine--tRNA ligase n=5 Tax=Enterococcus faecium RepID=D4SK82_ENTFC	alaS (Ch)	YP_006375943	39.9
contig00158|52	0	1	UniRef100_H8LE81 Integral membrane protein n=195 Tax=Firmicutes RepID=H8LE81_ENTFU	MP (Ch) CDS 764	YP_006375944	40.9
contig00158|51	0	1	UniRef100_R3Y9F4 Cation diffusion facilitator family transporter n=2 Tax=Enterococcus faecium RepID=R3Y9F4_ENTFC	HP (Ch) CDS 765	YP_006375945	36.3
contig00158|50	0	1	UniRef100_C9B5R7 RNase H n=13 Tax=Enterococcus faecium RepID=C9B5R7_ENTFC	rnhA (Ch)	YP_006375946	37.2
contig00158|49	0	1	UniRef100_H8LE78 Uncharacterized protein n=201 Tax=Firmicutes RepID=H8LE78_ENTFU	ebsA (Ch)	YP_006375947	34
contig00158|48	1	1	UniRef100_D4QWT8 Cold-shock protein n=59 Tax=Enterococcus RepID=D4QWT8_ENTFC	cspC (Ch)	YP_006375948	38.7
contig00158|46	0	1	UniRef100_J6YWS9 Formate--tetrahydrofolate ligase n=1 Tax=Enterococcus faecium 509 RepID=J6YWS9_ENTFC	fhs (Ch)	YP_006375949	41.9
contig00158|45	1	0	UniRef100_C9B5R3 Predicted protein n=1 Tax=Enterococcus faecium 1,231,501 RepID=C9B5R3_ENTFC	HP (Ch) CDS 766	YP_006375950	36.4
contig00158|44	0	1	UniRef100_H8LE74 CBS domain pair protein n=195 Tax=Firmicutes RepID=H8LE74_ENTFU	HP (Ch) CDS 767	YP_006375951	38.3
contig00158|43	0	1	UniRef100_J6YFW6 Lipoprotein signal peptidase n=6 Tax=Enterococcus faecium RepID=J6YFW6_ENTFC	lpsA (Ch)	YP_006375952	36.1
contig00158|42	0	1	UniRef100_J7CZX4 Pseudouridine synthase n=3 Tax=Enterococcus faecium RepID=J7CZX4_ENTFC	rluD (Ch)	YP_006375953	41.6
contig00158|41	0	1	UniRef100_J6Z6Y5 Bifunctional protein PyrR n=1 Tax=Enterococcus faecium 504 RepID=J6Z6Y5_ENTFC	pyrR(Ch)	YP_006375954	40.1
contig00158|40	0	1	UniRef100_D4SK94 Uracil permease n=6 Tax=Enterococcus faecium RepID=D4SK94_ENTFC	pyrP (Ch)	YP_006375955	41.5
contig00158|39	0	1	UniRef100_H8LE69 Aspartate carbamoyltransferase n=205 Tax=Firmicutes RepID=H8LE69_ENTFU	pyrB (Ch)	YP_006375956	42.4
contig00158|38	0	1	UniRef100_J6YFW2 Dihydroorotase n=4 Tax=Enterococcus faecium RepID=J6YFW2_ENTFC	pyrC (Ch)	YP_006375957	42.8
contig00158|37	0	1	UniRef100_H8LE67 Carbamoyl-phosphate synthase small chain n=176 Tax=Firmicutes RepID=H8LE67_ENTFU	carA (Ch)	YP_006375958	40.7
contig00158|36	0	1	UniRef100_D4SK98 Carbamoyl-phosphate synthase large chain n=4 Tax=Enterococcus faecium RepID=D4SK98_ENTFC	carB (Ch)	YP_006375959	40.9
contig00158|35	0	1	UniRef100_L2IBE1 Dihydroorotate dehydrogenase B (NAD(+)), electron transfer subunit n=4 Tax=Enterococcus faecium RepID=L2IBE1_ENTFC	pyrK (Ch)	YP_006375960	40.7
contig00158|34	0	1	UniRef100_D4SKA0 Dihydroorotate dehydrogenase n=15 Tax=Enterococcus faecium RepID=D4SKA0_ENTFC	pyrD (Ch)	YP_006375961	43.9

Table S4 - Annotation of transferable region of *E. faecium* GEHPH2.1 using Best BLAST Hit and *E. faecium* DO as a reference strain (cont.)

E. Faecium GEHPH2.1	*E. faecium* HPH2	*E. faecium* GE1	Best BLAST Hit Annotation	*E. faecium* DO Annotation	*E. faecium* DO GenBank accession no.	GC content (%)
contig00158|33	0	1	UniRef100_L2NCL3 Orotidine 5'-phosphate decarboxylase n=10 Tax=Enterococcus faecium RepID=L2NCL3_ENTFC	pyrF (Ch)	YP_006375962	41.3
contig00158|32	0	1	UniRef100_H8LE62 Orotate phosphoribosyltransferase n=203 Tax=Firmicutes RepID=H8LE62_ENTFU	pyrE (Ch)	YP_006375963	39.2
contig00158|31	1	1	UniRef100_E4IAY5 Uncharacterized protein n=56 Tax=Enterococcus RepID=E4IAY5_ENTFC	HP (Ch) CDS 768	YP_006375964	31.4
contig00158|30	0	1	UniRef100_H8LE61 HD domain protein n=217 Tax=Enterococcus RepID=H8LE61_ENTFU	phosphohydrolase (Ch) CDS 769	YP_006375965	37.5
contig00158|29	0	1	UniRef100_D4SKA4 Carbonic anhydrase n=13 Tax=Enterococcus faecium RepID=D4SKA4_ENTFC	Carbonic anhydrase (Ch) CDS 770	YP_006375966	40.3
contig00158|28	0	1	UniRef100_L2IDJ2 LysR family transcriptional regulator n=11 Tax=Enterococcus faecium RepID=L2IDJ2_ENTFC	LysR transriptional regulator (Ch) CDS 771	YP_006375967	41.1
contig00158|27	0	1	UniRef100_J6L2A0 Fibronectin-binding protein A n=5 Tax=Enterococcus faecium RepID=J6L2A0_ENTFC	pavA (Ch)	YP_006375969	39.3
contig00158|26	0	1	UniRef100_L2IB53 Uncharacterized protein n=12 Tax=Enterococcus faecium RepID=L2IB53_ENTFC	HP (Ch) CDS 774	YP_006375971	37
contig00158|25	0	1	UniRef100_D0AF93 Uncharacterized protein n=42 Tax=Enterococcus faecium RepID=D0AF93_ENTFC	ABC transporter (Ch) CDS 775	YP_006375972	40.6
contig00158|24	1	1	UniRef100_H8LE54 Amino acid or sugar ABC transport system, permease protein n=168 Tax=Firmicutes RepID=H8LE54_ENTFU	HP (Ch) CDS 776	YP_006375973	41.6
contig00158|23	0	1	UniRef100_D4RGT0 ABC transporter, ATP-binding protein n=13 Tax=Enterococcus faecium RepID=D4RGT0_ENTFC	ABC transporter (Ch) CDS 777	YP_006375974	39.7
contig00158|22	0	1	UniRef100_D4SKB1 Shikimate dehydrogenase n=11 Tax=Enterococcus faecium RepID=D4SKB1_ENTFC	aroE (Ch)	YP_006375975	40.4
contig00158|21	0	0	UniRef100_H8LE51 Transcriptional regulator, PSR protein n=132 Tax=Enterococcus RepID=H8LE51_ENTFU	msrR (Ch)	YP_006375976	40.7
contig00158|19	0	1	UniRef100_D0AF98 Uncharacterized protein n=62 Tax=Enterococcus faecium RepID=D0AF98_ENTFC	Glyxalase (Ch) CDS 779 (smaller)	YP_006375978	41.1
contig00158|18	0	1	UniRef100_L2IBC7 DNA topoisomerase n=12 Tax=Enterococcus faecium RepID=L2IBC7_ENTFC	topA2 (Ch)	YP_006375979	38.2
contig00158|17	0	1	UniRef100_D0AFA1 Uncharacterized protein n=86 Tax=Enterococcus faecium RepID=D0AFA1_ENTFC	HP (Ch) CDS 780	YP_006375980	35.3
contig00158|16	0	1	UniRef100_L2I7G9 Addiction module antidote n=18 Tax=Enterococcus faecium RepID=L2I7G9_ENTFC	AbrB transcriptor regulator (Ch) CDS 781	YP_006375981	32.9
contig00158|15	0	1	UniRef100_D4RFQ1 Death-on-curing family protein n=11 Tax=Enterococcus faecium RepID=D4RFQ1_ENTFC	Death-on-curing family protein (Ch) CDS 782	YP_006375982	35.3
contig00158|13	1	0	UniRef100_H8LE45 L-lactate dehydrogenase n=204 Tax=Firmicutes RepID=H8LE45_ENTFU	dehydrogenase (Ch) CDS 783	YP_006375983	38.3
				ComG protein 5	YP_006375984	28.7
contig00158|12	1	0	UniRef100_C9B5N1 Helix-turn-helix domain-containing protein n=6 Tax=Enterococcus faecium RepID=C9B5N1_ENTFC	araC transcriprional regulator (Ch) CDS 785	YP_006375985	33.7
contig00158|11	1	0	UniRef100_E4I5F7 Uncharacterized protein n=43 Tax=Enterococcus faecium RepID=E4I5F7_ENTFC	HP (Ch) CDS 786	YP_006375986	35.4
contig00158|10	1	0	UniRef100_I3U1W7 MFS family major facilitator transporter n=13 Tax=Enterococcus faecium RepID=I3U1W7_ENTFC	MFS transporter (Ch) CDS 787	YP_006375987	38.8
contig00158|9	1	0	UniRef100_D4QWX6 Alpha-L-rhamnosidase n=21 Tax=Enterococcus faecium RepID=D4QWX6_ENTFC	Alpha-L-rhamnosidase (Ch) CDS 788	YP_006375988	37.1
contig00158|8	1	0	UniRef100_H8LE39 PTS system, ascorbate-specific IIC component n=125 Tax=Firmicutes RepID=H8LE39_ENTFU	PTS system (Ch) CDS 789	YP_006375989	40.4
contig00158|7	1	0	UniRef100_H8LE38 PTS system, ascorbate-specific IIB component n=189 Tax=Firmicutes RepID=H8LE38_ENTFU	PTS system (Ch) CDS 790	YP_006375990	36.1
contig00158|6	1	0	UniRef100_E4IQW8 Phosphoenolpyruvate-dependent sugar phosphotransferase system, EIIA 2 n=36 Tax=Enterococcus faecium RepID=E4IQW8_ENTFC	BglG antiterminator (Ch) CDS 791	YP_006375991	38.3
contig00158|5	1	0	UniRef100_H8LE36 Uncharacterized protein n=201 Tax=Firmicutes RepID=H8LE36_ENTFU	HP (Ch) CDS 793	YP_006375993	35.3
contig00158|4	1	0	UniRef100_H8LE35 Oxidoreductase family, NAD-binding Rossmann fold protein n=132 Tax=Firmicutes RepID=H8LE35_ENTFU	dehydrogenase (Ch) CDS 794	YP_006375994	35.4
contig00158|3	1	0	UniRef100_E4I5E9 Uncharacterized protein n=46 Tax=Firmicutes RepID=E4I5E9_ENTFC	HP (Ch) CDS 795	YP_006375995	29.8
contig00158|2	1	0	UniRef100_J7B871 Transcriptional regulator, RpiR family n=31 Tax=Enterococcus RepID=J7B871_ENTFC	rpiR (Ch)	YP_006375996	37
contig00158|1	1	0	UniRef100_H8LE33 Peptidase C60 n=134 Tax=Firmicutes RepID=H8LE33_ENTFU	srtC1 (Ch)	YP_006375997	39.5
contig00118|1	0	0	UniRef100_L2HBV4 Uncharacterized protein n=4 Tax=Enterococcus faecium RepID=L2HBV4_ENTFC	Δpilus (Ch) CDS 797	YP_006376000	38.4
contig00118|2	1	0	UniRef100_J6YPJ5 HTH domain protein n=1 Tax=Enterococcus faecium 503 RepID=J6YPJ5_ENTFC	ebpR (Ch)	YP_006376001	34.1
contig00118|3	1	0	UniRef100_H8LE28 TetR/AcrR family transcriptional regulator n=142 Tax=Firmicutes RepID=H8LE28_ENTFU	regulator (Ch) CDS 798	YP_006376002	36.7
contig00118|4	1	0	UniRef100_H8LE27 ABC transporter, ATP-binding protein n=195 Tax=Firmicutes RepID=H8LE27_ENTFU	ABC transporter (Ch) CDS 799	YP_006376003	36.4
contig00118|5	1	0	UniRef100_H8LE26 Efflux ABC transporter, permease protein n=128 Tax=Firmicutes RepID=H8LE26_ENTFU	ABC transporter (Ch) CDS 800	YP_006376004	38.6
contig00118|6	1	0	UniRef100_C9C101 Transcriptional regulator n=23 Tax=Enterococcus faecium RepID=C9C101_ENTFC	ciaR (Ch)	YP_006376005	34.7
contig00118|7	1	0	UniRef100_C9B5L3 Histidine kinase n=110 Tax=Enterococcus RepID=C9B5L3_ENTFC	ciaH (Ch)	YP_006376006	36.4
contig00118|8	1	0	UniRef100_D4QWZ2 Conserved protein n=51 Tax=Enterococcus RepID=D4QWZ2_ENTFC	HP (Ch) CDS 801	YP_006376007	39.2
contig00118|9	1	0	UniRef100_H8LE21 Site-specific tyrosine recombinase XerC-family n=134 Tax=Firmicutes RepID=H8LE21_ENTFU	integrase (Ch) CDS 802	YP_006376008	35.1
contig00118|10	1	0	UniRef100_C9C0Z7 AMP-dependent synthetase and ligase n=26 Tax=Enterococcus faecium RepID=C9C0Z7_ENTFC	ligase (Ch) CDS 803	YP_006376009	38.9
contig00118|11	1	0	UniRef100_E4I947 Uncharacterized protein n=55 Tax=Firmicutes RepID=E4I947_ENTFC	HP (Ch) CDS 804	YP_006376010	24.8
contig00118|12	1	0	UniRef100_H8LE19 Phosphomethylpyrimidine kinase n=196 Tax=Firmicutes RepID=H8LE19_ENTFU	thiD2 (Ch)	YP_006376011	37.9
contig00118|13	1	0	UniRef100_H8LE18 Uncharacterized protein n=199 Tax=Firmicutes RepID=H8LE18_ENTFU	HP (Ch) CDS 805	YP_006376012	42.4
contig00118|14	1	0	UniRef100_H8LE17 UPF0340 protein EFAU004_00917 n=145 Tax=Enterococcus RepID=H8LE17_ENTFU	ywlG (Ch)	YP_006376013	42.5
contig00118|15	1	0	UniRef100_H8LE16 Alpha amylase, catalytic domain protein n=88 Tax=Enterococcus RepID=H8LE16_ENTFU	malA (Ch)	YP_006376014	38
contig00118|16	1	0	UniRef100_H8LE15 ATP-dependent protease ATP-binding subunit ClpX n=64 Tax=Enterococcus faecium RepID=H8LE15_ENTFU	HP (Ch) CDS 806	YP_006376015	40
contig00118|17	1	0	UniRef100_H8LE14 Uncharacterized protein n=65 Tax=Enterococcus faecium RepID=H8LE14_ENTFU	HP (Ch) CDS 807	YP_006376016	37.1
contig00058|1	1	0	UniRef100_H8LE12 EDD domain protein, DegV family n=133 Tax=Firmicutes RepID=H8LE12_ENTFU	DegV family (ch) CDS 808	YP_006376017	37.8
contig00058|2	1	0	UniRef100_H8LE11 Polysaccharide biosynthesis family protein n=143 Tax=Firmicutes RepID=H8LE11_ENTFU	MATE superfamily (Ch) CDS 809	YP_006376018	38.8
contig00058|3	1	0	UniRef100_H8LE10 Branched-chain amino acid transport protein n=201 Tax=Firmicutes RepID=H8LE10_ENTFU	azlD (Ch)	YP_006376019	40.3
contig00058|4	1	0	UniRef100_C9BJ08 Branched-chain amino acid transporter n=164 Tax=Firmicutes RepID=C9BJ08_ENTFC	azlC (Ch)	YP_006376020	40.4
contig00058|5	1	0	UniRef100_H8LE08 Cation diffusion facilitator family transporter n=205 Tax=Firmicutes RepID=H8LE08_ENTFU	HP (Ch) CDS 810	YP_006376021	39.9
contig00058|6	1	0	UniRef100_H8LE07 Uncharacterized protein n=99 Tax=Enterococcus RepID=H8LE07_ENTFU	HP (Ch) CDS 811	YP_006376022	35.6

Table S4 - Annotation of transferable region of *E. faecium* GEHPH2.1 using Best BLAST Hit and *E. faecium* DO as a reference strain (cont.)

E. Faecium GEHPH2.1	*E. faecium* HPH2	*E. faecium* GE1	Best BLAST Hit Annotation	*E. faecium* DO Annotation	*E. faecium* DO GenBank accession no.	GC content (%)
contig00058|7	1	0	UniRef100_H8LE06 Uncharacterized protein n=198 Tax=Firmicutes RepID=H8LE06_ENTFU	MP (Ch) CDS 812	YP_006376023	37.2
contig00058|8	1	0	UniRef100_H8LE05 Uncharacterized protein n=181 Tax=Firmicutes RepID=H8LE05_ENTFU	HP (Ch) CDS 813	YP_006376024	37
contig00058|9	1	0	UniRef100_H8LE04 Uncharacterized protein n=136 Tax=Firmicutes RepID=H8LE04_ENTFU	HP (Ch) CDS 815	YP_006376026	38.6
contig00058|10	1	0	UniRef100_H8LE03 Uncharacterized protein n=209 Tax=Firmicutes RepID=H8LE03_ENTFU	HP (Ch) CDS 816	YP_006376027	35.4
contig00058|11	1	0	UniRef100_H8LE02 ATP-dependent Zn protease n=153 Tax=Enterococcus RepID=H8LE02_ENTFU	Zn protease (Ch) CDS 817	YP_006376028	38.8
contig00058|12	1	0	UniRef100_H8LE01 Na dependent nucleoside transporter, NupC family n=152 Tax=Firmicutes RepID=H8LE01_ENTFU	nupC (Ch)	YP_006376029	37.4
contig00058|13	0	0	UniRef100_H8LE00 Guanylate kinase n=151 Tax=Firmicutes RepID=H8LE00_ENTFU	kdpA (Ch)	YP_006376030	37.5
contig00058|14	1	0	UniRef100_C9BT11 Acetyltransferase n=96 Tax=Enterococcus RepID=C9BT11_ENTFC	acetyltrabsferase (Ch) CDS 818	YP_006376031	38.2
contig00058|15	1	0	UniRef100_J6YHG4 Uncharacterized protein n=51 Tax=Enterococcus faecium RepID=J6YHG4_ENTFC	HP (Ch) CDS 819	YP_006376032	39.1
contig00058|16	1	0	UniRef100_H8LDZ7 Uncharacterized protein n=79 Tax=Enterococcus RepID=H8LDZ7_ENTFU	HP (Ch) CDS 820	YP_006376033	39.1
contig00058|17	1	0	UniRef100_H8LDZ6 Amino acid ABC transporter, amino acid-binding/permease n=153 Tax=Enterococcus RepID=H8LDZ6_ENTFU	ABC transporter (Ch) CDS 821	YP_006376034	39.7
contig00058|18	1	0	UniRef100_J6L6Z4 ABC transporter, substrate-binding protein, family 3 n=64 Tax=Enterococcus faecium RepID=J6L6Z4_ENTFC	ABC transporter (Ch) CDS 822	YP_006376035	35.4
contig00058|19	1	0	UniRef100_S4EW44 Amidase n=1 Tax=Enterococcus faecium SD1C-2 RepID=S4EW44_ENTFC	amidase (Ch) CDS 823	YP_006376036	42.4
contig00058|20	1	0	UniRef100_E4I7Z7 Dihydroorotate oxidase n=45 Tax=Enterococcus faecium RepID=E4I7Z7_ENTFC	pyrD2 (Ch)	YP_006376037	39.1
contig00058|21	1	0	UniRef100_S4EMF4 Lysis protein n=1 Tax=Enterococcus faecium SD1C-2 RepID=S4EMF4_ENTFC	lipoprotein (Ch) CDS 824	YP_006376038	37.4
contig00058|22	1	0	UniRef100_H8LDZ1 Cyclopropane-fatty-acyl-phospholipid synthase n=97 Tax=Enterococcus RepID=H8LDZ1_ENTFU	cfa (Ch)	YP_006376039	36.6
contig00058|23	1	0	UniRef100_H8LDZ0 Integral membrane protein n=138 Tax=Firmicutes RepID=H8LDZ0_ENTFU	partial MP (Ch) CDS 825	YP_006376041	37.6
contig00058|24	1	0	UniRef100_H8LDY9 ABC transporter, permease protein n=179 Tax=Firmicutes RepID=H8LDY9_ENTFU	malD (Ch)	YP_006376042	38.6
contig00058|25	1	0	UniRef100_H8LDY8 ABC transporter, permease protein n=138 Tax=Firmicutes RepID=H8LDY8_ENTFU	malC (Ch)	YP_006376043	38.2
contig00058|26	1	0	UniRef100_J7D0P8 Maltodextrin-binding protein MdxE n=41 Tax=Enterococcus faecium RepID=J7D0P8_ENTFC	malX (Ch)	YP_006376044	39.4
contig00058|27	1	0	UniRef100_H8LDY6 Alpha amylase, catalytic domain protein n=87 Tax=Enterococcus RepID=H8LDY6_ENTFU	nplT (Ch)	YP_006376045	37.5
contig00058|28	1	0	UniRef100_C9C9M8 Integral membrane protein n=42 Tax=Enterococcus faecium RepID=C9C9M8_ENTFC	HP (Ch) CDS 826	YP_006376046	36.5
contig00058|29	1	0	UniRef100_H8LDY4 MerR family transcriptional regulator n=95 Tax=Enterococcus RepID=H8LDY4_ENTFU	merR (Ch) CDS 828	YP_006376048	35.6
contig00058|30	1	0	UniRef100_H8LDY3 Peroxiredoxin, Ohr family protein n=175 Tax=Firmicutes RepID=H8LDY3_ENTFU	osmC (Ch)	YP_006376049	39.7
contig00058|31	1	0	UniRef100_H8LDY2 DNA-binding transcriptional repressor MarR n=85 Tax=Enterococcus RepID=H8LDY2_ENTFU	marR2 (Ch)	YP_006376050	38.1
contig00058|32	1	0	UniRef100_U2NB72 Membrane protein n=31 Tax=Enterococcus faecium RepID=U2NB72_ENTFC	MP (Ch) CDS 829	YP_006376051	34.8
contig00081|72	0	0	UniRef100_C9BT30 Cell wall surface adhesion protein n=10 Tax=Enterococcus faecium RepID=C9BT30_ENTFC	Δfms22 (Ch)	YP_006376052	40.8
contig00081|71	1	0	UniRef100_H8LDX9 M protein trans-acting positive regulator n=104 Tax=Enterococcus RepID=H8LDX9_ENTFU	regulator (Ch) CDS 830	YP_006376053	31.9
contig00081|70	1	0	UniRef100_H8LDX8 Mannonate dehydratase n=98 Tax=Enterococcus RepID=H8LDX8_ENTFU	uxuA (Ch)	YP_006376054	39.6
contig00081|69	1	0	UniRef100_H8LDX7 Mannitol dehydrogenase protein n=80 Tax=Enterococcus faecium RepID=H8LDX7_ENTFU	uxuB (Ch)	YP_006376055	38.3
contig00081|68	1	0	UniRef100_H8LDX6 Transcriptional regulator, GntR family n=187 Tax=Firmicutes RepID=H8LDX6_ENTFU	kdgR (Ch)	YP_006376056	39.5
contig00081|67	1	0	UniRef100_H8LDX5 Alkyl hydroperoxide reductase, C subunit n=188 Tax=Firmicutes RepID=H8LDX5_ENTFU	ahpC (Ch)	YP_006376057	38.5
contig00081|66	1	0	UniRef100_I3U238 Peroxiredoxin subunit F n=86 Tax=Firmicutes RepID=I3U238_ENTFC	ahpF (Ch)	YP_006376058	39
contig00081|65	1	0	UniRef100_H8LDX3 Uncharacterized protein n=193 Tax=Firmicutes RepID=H8LDX3_ENTFU	HP (Ch) CDS 831	YP_006376059	37.6
contig00081|64	1	0	UniRef100_H8LDX2 Uncharacterized protein n=133 Tax=Firmicutes RepID=H8LDX2_ENTFU	HP (Ch) CDS 832	YP_006376060	35.8
contig00081|63	1	0	UniRef100_J6YHE4 Na+/H+ antiporter n=68 Tax=Enterococcus faecium RepID=J6YHE4_ENTFC	Na+/H+ transporter (Ch) CDS 833	YP_006376061	36.3
contig00081|62	1	0	UniRef100_M1RTD4 Penicillin-binding protein 5 n=1 Tax=Enterococcus faecium RepID=M1RTD4_ENTFC	pbp5 (Ch)	YP_006376062	39.8
contig00081|61	1	0	UniRef100_C9BT41 Cell envelope transcriptional attenuator n=34 Tax=Enterococcus faecium RepID=C9BT41_ENTFC	psr (Ch)	YP_006376064	38.1
contig00081|60	1	0	UniRef100_H8LDW5 Cell cycle protein, FtsW/RodA/SpoVE family n=76 Tax=Enterococcus faecium RepID=H8LDW5_ENTFU	ftsW3 (Ch)	YP_006376065	38.3
contig00081|59	1	0	UniRef100_H8LDW4 Uncharacterized protein n=194 Tax=Firmicutes RepID=H8LDW4_ENTFU	HP (Ch) CDS 837	YP_006376068	31.9
contig00081|58	1	0	UniRef100_H8LDW3 Flavin reductase-like domain protein n=131 Tax=Firmicutes RepID=H8LDW3_ENTFU	reductase (Ch) CDS 838	YP_006376069	37.6
contig00081|57	1	0	UniRef100_D4RGK8 Putative N-acetylmannosamine-6-phosphate 2-epimerase n=23 Tax=Enterococcus faecium RepID=D4RGK8_ENTFC	nanE (Ch)	YP_006376070	40.5
contig00081|56	1	0	UniRef100_H8LDW1 ASCH domain protein n=141 Tax=Firmicutes RepID=H8LDW1_ENTFU	HP (Ch) CDS 839	YP_006376071	35.3
contig00081|55	1	0	UniRef100_D4RGK6 1-deoxy-d-xylulose-5-phosphate synthase n=12 Tax=Enterococcus faecium RepID=D4RGK6_ENTFC	dxs (Ch)	YP_006376072	38.8
contig00081|54	1	0	UniRef100_J6PSU4 Uncharacterized protein n=18 Tax=Enterococcus faecium RepID=J6PSU4_ENTFC	HP (Ch) CDS 840	YP_006376073	32.4
contig00081|53	1	0	UniRef100_H8LDV8 Glutaminase n=144 Tax=Firmicutes RepID=H8LDV8_ENTFU	glsA (Ch)	YP_006376076	40.2
contig00081|52	1	0	UniRef100_H8LDV7 Amino acid permease family protein n=110 Tax=Enterococcus RepID=H8LDV7_ENTFU	gadC2 (Ch)	YP_006376077	39.6
contig00081|51	1	0	UniRef100_H8LDV6 Ammonium transporter family protein n=144 Tax=Firmicutes RepID=H8LDV6_ENTFU	amt (Ch)	YP_006376078	42.1
contig00081|50	1	0	UniRef100_C2HB96 Transglycosylase associated protein n=226 Tax=Firmicutes RepID=C2HB96_ENTFC	glsB1 (Ch)	YP_006376080	39.9
contig00081|49	1	0	UniRef100_H8L7N1 Alkaline shock protein 23 family protein n=202 Tax=Firmicutes RepID=H8L7N1_ENTFU	gls20 (Ch)	YP_006376081	34.8
contig00081|48	1	0	UniRef100_H8L7N2 Small integral membrane protein n=132 Tax=Firmicutes RepID=H8L7N2_ENTFU	HP (Ch) CDS 844	YP_006376082	34.9
contig00081|47	1	0	UniRef100_H8L7N3 Uncharacterized protein n=134 Tax=Firmicutes RepID=H8L7N3_ENTFU	HP (Ch) CDS 845	YP_006376083	38.4
contig00081|46	1	0	UniRef100_H8L7N5 Uncharacterized protein n=149 Tax=Firmicutes RepID=H8L7N5_ENTFU	HP (Ch) CDS 847	YP_006376085	34
contig00081|45	1	0	UniRef100_H8L7N6 Helix-turn-helix domain-containing protein n=195 Tax=Firmicutes RepID=H8L7N6_ENTFU	regulator (Ch) CDS 848	YP_006376086	34.6
contig00081|44	1	0	UniRef100_H8L7N8 Transglycosylase associated protein n=219 Tax=Firmicutes RepID=H8L7N8_ENTFU	glsB (Ch)	YP_006376088	41.2

Table S4 - Annotation of transferable region of *E. faecium* GEHPH2.1 using Best BLAST Hit and *E. faecium* DO as a reference strain (cont.)

E. Faecium GEHPH2.1	*E. faecium* HPH2	*E. faecium* GE1	Best BLAST Hit Annotation	*E. faecium* DO Annotation	*E. faecium* DO GenBank accession no.	GC content (%)
contig00081|43	1	0	UniRef100_L2K6B2 General stress protein n=1 Tax=Enterococcus faecium EnGen0016 RepID=L2K6B2_ENTFC	gls33 (Ch)	YP_006376089	38.2
contig00081|42	1	0	UniRef100_H8L7P0 Uncharacterized protein n=214 Tax=Firmicutes RepID=H8L7P0_ENTFU	HP (Ch) CDS 850	YP_006376090	38.1
contig00081|41	1	0	UniRef100_H8L7P1 Uncharacterized protein n=193 Tax=Firmicutes RepID=H8L7P1_ENTFU	HP (Ch) CDS 851	YP_006376091	37.4
contig00081|40	1	0	UniRef100_H8L7P2 Oxidoreductase, short chain dehydrogenase/reductase family protein n=102 Tax=Enterococcus faecium RepID=H8L7P2_ENTFU	SDR dehydrogenase (Ch) CDS 852	YP_006376092	41.9
contig00081|39	1	0	UniRef100_H8L7P3 Mg2+ cation transporter n=78 Tax=Enterococcus RepID=H8L7P3_ENTFU	corA3 (Ch)	YP_006376093	36.3
contig00081|38	1	0	UniRef100_H8L7P4 Uncharacterized protein n=134 Tax=Firmicutes RepID=H8L7P4_ENTFU	HP (Ch) CDS 853	YP_006376094	34.2
contig00081|37	1	0	UniRef100_H8L7P5 Glycosyl transferase family 8 n=127 Tax=Firmicutes RepID=H8L7P5_ENTFU	glycosyl transferase (Ch) CDS 854	YP_006376095	36.5
contig00081|36	1	0	UniRef100_J6EIZ9 Putative general stress protein A n=1 Tax=Enterococcus faecium ERV1 RepID=J6EIZ9_ENTFC	glycosyl transferase (Ch) CDS 855	YP_006376096	35.3
contig00081|35	1	0	UniRef100_H8L7P7 HAD superfamily hydrolase n=105 Tax=Enterococcus RepID=H8L7P7_ENTFU	Hydrolase (Ch) CDS 856	YP_006376097	37.8
contig00081|34	1	0	UniRef100_J7D0M2 Isochorismatase family protein n=1 Tax=Enterococcus faecium 504 RepID=J7D0M2_ENTFC	isochorismatase (Ch) CDS 857	YP_006376098	36
contig00081|33	1	0	UniRef100_Q3Y347 Uncharacterized protein n=53 Tax=Enterococcus faecium RepID=Q3Y347_ENTFC	HP (Ch) CDS 858	YP_006376099	32.1
contig00081|32	1	0	UniRef100_H8L7Q0 Uncharacterized protein n=143 Tax=Firmicutes RepID=H8L7Q0_ENTFU	HP (Ch) CDS 859	YP_006376100	33.3
contig00081|31	1	0	UniRef100_H8L7Q1 Toxin-antitoxin system, antitoxin component, Xre domain protein n=139 Tax=Enterococcus RepID=H8L7Q1_ENTFU	regulator (Ch) CDS 860	YP_006376101	36.2
contig00081|30	1	0	UniRef100_H8L7Q2 Pyrimidine dimer DNA glycosylase n=159 Tax=Firmicutes RepID=H8L7Q2_ENTFU	HP (Ch) CDS 861	YP_006376102	38.8
contig00081|29	1	0	UniRef100_E4ICY1 Uncharacterized protein n=48 Tax=Enterococcus faecium RepID=E4ICY1_ENTFC	Anti-toxin (Ch) CDS 862	YP_006376103	35.5
contig00081|28	1	0	UniRef100_C9BT75 Predicted protein n=49 Tax=Enterococcus faecium RepID=C9BT75_ENTFC	HP (ch) CDS 863	YP_006376104	24.4
contig00081|27	1	0	UniRef100_H8L7Q5 Major cold shock protein CspA n=214 Tax=Firmicutes RepID=H8L7Q5_ENTFU	cspA2 (Ch)	YP_006376105	39.8
contig00081|26	1	0	UniRef100_C2HBD0 Uncharacterized protein n=19 Tax=Enterococcus RepID=C2HBD0_ENTFC	HP (Ch) CDS 864	YP_006376106	32.7
contig00081|25	1	0	UniRef100_H8L8P0 HAD superfamily hydrolase n=134 Tax=Firmicutes RepID=H8L8P0_ENTFU	Hydrolase (Ch) CDS 866	YP_006376108	34.6
contig00081|24	1	0	UniRef100_S4EG01 Mannonate dehydratase n=1 Tax=Enterococcus faecium SD1C-2 RepID=S4EG01_ENTFC	uxuA2 (Ch)	YP_006376109	40.7
contig00081|23	1	0	UniRef100_H8L8P2 PTS system, mannose/fructose/sorbose-specific IIA component n=134 Tax=Firmicutes RepID=H8L8P2_ENTFU	PTS system (Ch) CDS 867	YP_006376110	41.2
contig00081|22	1	0	UniRef100_H8L8P3 Uncharacterized protein n=55 Tax=Enterococcus faecium RepID=H8L8P3_ENTFU	HP (Ch) CDS 868	YP_006376111	37.4
contig00081|21	1	0	UniRef100_H8L8P4 Uncharacterized protein n=107 Tax=Firmicutes RepID=H8L8P4_ENTFU	HP (Ch) CDS 869	YP_006376112	35.1
contig00081|20	1	0	UniRef100_H8L8P5 PTS system, mannose/fructose/sorbose-specific IIB component n=136 Tax=Firmicutes RepID=H8L8P5_ENTFU	PTS system (Ch) CDS 870	YP_006376113	33.1
contig00081|19	1	0	UniRef100_H8L8P6 PTS system, mannose/fructose/sorbose-specific IID component n=100 Tax=Enterococcus RepID=H8L8P6_ENTFU	manZ (Ch)	YP_006376114	40.3
contig00081|18	1	0	UniRef100_H8L8P7 PTS system, mannose/fructose/sorbose-specific IIC component n=129 Tax=Firmicutes RepID=H8L8P7_ENTFU	manY (Ch)	YP_006376115	41.2
contig00081|17	1	0	UniRef100_C9BT86 Iron-containing alcohol dehydrogenase n=3 Tax=Enterococcus faecium RepID=C9BT86_ENTFC	alcohol dehydrogenase (Ch) CDS 871	YP_006376116	38.3
contig00081|16	1	0	UniRef100_H8L8P9 Iron-containing alcohol dehydrogenase n=99 Tax=Firmicutes RepID=H8L8P9_ENTFU	alcohol dehydrogenase (Ch) CDS 872	YP_006376117	35.8
contig00081|15	1	0	UniRef100_E4ICW6 4-phosphoerythronate dehydrogenase n=46 Tax=Enterococcus faecium RepID=E4ICW6_ENTFC	serA2 (Ch)	YP_006376118	38.8
contig00081|14	1	0	UniRef100_J6S6N0 Phosphogluconate dehydrogenase n=2 Tax=Enterococcus faecium RepID=J6S6N0_ENTFC	gnd2 (Ch)	YP_006376119	37
contig00081|13	1	0	UniRef100_H8L8Q2 Phosphosugar isomerase transcriptional regulator n=137 Tax=Firmicutes RepID=H8L8Q2_ENTFU	gntR (Ch)	YP_006376120	35.2
contig00081|12	1	0	UniRef100_H8L8Q4 Esterase n=100 Tax=Firmicutes RepID=H8L8Q4_ENTFU	lipA (Ch)	YP_006376122	35.1
contig00081|11	1	0	UniRef100_C9C0P4 Alpha/beta hydrolase n=43 Tax=Enterococcus faecium RepID=C9C0P4_ENTFC	fms7 (Ch)	YP_006376123	38.1
contig00081|10	1	0	UniRef100_H8L8Q6 Isochorismatase family hydrolase n=106 Tax=Firmicutes RepID=H8L8Q6_ENTFU	Hydrolase (Ch) CDS 874	YP_006376124	36.3
contig00081|9	1	0	UniRef100_H8L8Q7 Transcriptional regulator Spx n=97 Tax=Firmicutes RepID=H8L8Q7_ENTFU	arsC3 (Ch)	YP_006376125	34.7
contig00081|8	1	0	UniRef100_H8L8Q8 Uncharacterized protein n=97 Tax=Firmicutes RepID=H8L8Q8_ENTFU	HP (Ch) CDS 875	YP_006376126	33.4
contig00081|7	1	0	UniRef100_H8L8Q9 Uncharacterized protein n=103 Tax=Enterococcus faecium RepID=H8L8Q9_ENTFU	HP (Ch) CDS 876	YP_006376127	29.7
contig00081|6	1	0	UniRef100_E4ICV6 Uncharacterized protein n=46 Tax=Firmicutes RepID=E4ICV6_ENTFC	HP (Ch) CDS 877	YP_006376128	35
contig00081|5	0	0	UniRef100_H8L8R0 LPXTG-motif protein cell wall anchor domain protein n=35 Tax=Firmicutes RepID=H8L8R0_ENTFU	fms2 (Ch)	YP_006376129	39.8
contig00081|4	1	0	UniRef100_H8L8R1 CAAX amino terminal protease family protein n=137 Tax=Firmicutes RepID=H8L8R1_ENTFU	abi (Ch)	YP_006376130	38.1
contig00081|3	1	0	UniRef100_C9BT99 Foldase protein PrsA n=105 Tax=Firmicutes RepID=C9BT99_ENTFC	prsA2 (Ch)	YP_006376131	30.9
contig00081|2	1	0	UniRef100_H8L8R3 Fructose-6-phosphate aldolase n=107 Tax=Firmicutes RepID=H8L8R3_ENTFU	tal (Ch)	YP_006376132	37.1
contig00081|1	0	0	UniRef100_H8L8R4 PTS system, glucitol/sorbitol-specific IIA component n=108 Tax=Firmicutes RepID=H8L8R4_ENTFU	gutA (Ch)	YP_006376133	33.8
contig00008|9	1	0	UniRef100_C9BPG5 PTS system, sorbitol-specific IIBC component n=48 Tax=Firmicutes RepID=C9BPG5_ENTFC	gutN - PTS system - EIIBC-GUT_N y C superfamily (Ch) CDS 878	YP_006376135	39.5
contig00008|8	1	0	UniRef100_H8L8R7 PTS system, glucitol/sorbitol-specific IIC component n=109 Tax=Firmicutes RepID=H8L8R7_ENTFU	PTS system - EIT-GUT superfamily (Ch) CDS 879	YP_006376136	40.4
contig00008|7	1	0	UniRef100_H8L8R8 Glucitol operon activator protein n=95 Tax=Firmicutes RepID=H8L8R8_ENTFU	srlR (Ch) - DeoR family transcriptional regulator (GutM)	YP_006376137	38
contig00008|6	1	0	UniRef100_H8L8R9 PRD domain protein n=74 Tax=Enterococcus faecium RepID=H8L8R9_ENTFU	sorbitol transcriptional regulator CDS 880	YP_006376138	33.3
contig00008|5	1	0	UniRef100_H8L8S0 Oxidoreductase, short chain dehydrogenase/reductase family protein n=109 Tax=Firmicutes RepID=H8L8S0_ENTFU	sorD (Ch)	YP_006376139	38.8
contig00008|4	1	0	UniRef100_H8L8S1 Uncharacterized protein n=170 Tax=Enterococcus RepID=H8L8S1_ENTFU	HP DUF378 superfamily (Ch) CDS 881	YP_006376140	35.2
contig00008|3	1	0	UniRef100_H8L8S2 Uncharacterized protein n=100 Tax=Enterococcus faecium RepID=H8L8S2_ENTFU	HP (Ch) CDS 882	YP_006376141	37.4
contig00008|2	1	0	UniRef100_H8L8S3 Alpha crystallin family heat shock protein n=97 Tax=Enterococcus faecium RepID=H8L8S3_ENTFU	heat shock protein Hsp20 (Ch) CDS 883	YP_006376142	35.5
contig00008|1	1	0	UniRef100_Q3XX58 Uncharacterized protein n=5 Tax=Enterococcus faecium RepID=Q3XX58_ENTFC	HP (Ch) CDS 885	YP_006376144	32.3
contig00010|1	1	0	UniRef100_H8L8S8 Site-specific tyrosine recombinase XerC-family n=90 Tax=Firmicutes RepID=H8L8S8_ENTFU	integrase (Ch) CDS 889	YP_006376148	36.7
contig00010|2	1	0	UniRef100_S4EAC6 Elongation factor 4 n=4 Tax=Enterococcus faecium RepID=S4EAC6_ENTFC	lepA GTP-binding (Ch)	YP_006376149	42.3
contig00010|3	1	0	UniRef100_H8L8T0 Acetyltransferase, GNAT family n=109 Tax=Firmicutes RepID=H8L8T0_ENTFU	acetyltranferase (Ch) CDS 890	YP_006376150	38.7
contig00010|4	1	0	UniRef100_H8L8T1 Nucleoside 2-deoxyribosyltransferase n=111 Tax=Firmicutes RepID=H8L8T1_ENTFU	nucleoside deoxyribosyltransferase (Ch) CDS 891	YP_006376151	38.8

Table S4 - Annotation of transferable region of *E. faecium* GEHPH2.1 using Best BLAST Hit and *E. faecium* DO as a reference strain (cont.)

E. Faecium GEHPH2.1	*E. faecium* HPH2	*E. faecium* GE1	Best BLAST Hit Annotation	*E. faecium* DO Annotation	*E. faecium* DO GenBank accession no.	GC content (%)
contig00010|5	1	0	UniRef100_E4IB57 Uncharacterized protein n=42 Tax=Enterococcus faecium RepID=E4IB57_ENTFC	HP (Ch) CDS 892	YP_006376153	38.1
contig00010|6	1	0	UniRef100_D0AFE3 Chaperone ClpB n=70 Tax=Firmicutes RepID=D0AFE3_ENTFC	ClpB (Ch)	YP_006376154	40
contig00010|7	1	0	UniRef100_R2NH85 Integral membrane protein n=1 Tax=Enterococcus faecium EnGen0191 RepID=R2NH85_ENTFC	MP (Ch) CDS 894	YP_006376155	38.1
contig00010|8	1	0	UniRef100_H8L8T5 Membrane protein n=112 Tax=Enterococcus faecium RepID=H8L8T5_ENTFU	HP (Ch) CDS 895	YP_006376156	36.3
contig00010|9	1	0	UniRef100_H8L8T6 Diacylglycerol kinase catalytic subunit n=102 Tax=Firmicutes RepID=H8L8T6_ENTFU	diacylglycerol kinase (Ch) CDS 896	YP_006376157	39.3
contig00010|10	1	0	UniRef100_H8L8T7 Peptidase T n=165 Tax=Firmicutes RepID=H8L8T7_ENTFU	pepT (Zinc-peptidase)	YP_006376158	38.9
contig00010|11	1	0	UniRef100_H8L8T8 Uncharacterized protein n=175 Tax=Firmicutes RepID=H8L8T8_ENTFU	HP (Ch) CDS 897	YP_006376159	40.8
contig00010|12	1	0	UniRef100_H8L8T9 SAM-dependent methyltransferase family protein n=102 Tax=Firmicutes RepID=H8L8T9_ENTFU	methyltransferase (Ch) CDS 898	YP_006376160	40.9
contig00010|13	1	0	UniRef100_H8L7Q4 ISL3 family transposase n=69 Tax=Enterococcus faecium RepID=H8L7Q4_ENTFU	ISEfa11 (ISL3 family)		38.4
contig00010|14	1	0	UniRef100_H8L8U0 Uncharacterized protein n=104 Tax=Firmicutes RepID=H8L8U0_ENTFU	Lipoprotein 9 (Ch) CDS 899	YP_006376161	39.9
contig00010|15	1	0	UniRef100_H8L8U1 Primosomal protein DnaI n=205 Tax=Firmicutes RepID=H8L8U1_ENTFU	dnaI (Ch)	YP_006376162	40.7
contig00010|16	1	0	UniRef100_H8L8U2 Replication initiation and membrane attachment protein n=105 Tax=Firmicutes RepID=H8L8U2_ENTFU	dnaB2 (Ch)	YP_006376163	39.2
contig00010|17	1	0	UniRef100_H8L8U3 Transcriptional repressor NrdR n=215 Tax=Firmicutes RepID=H8L8U3_ENTFU	nrdR (Ch)	YP_006376164	38.6
contig00010|18	1	0	UniRef100_H8L8U4 Uncharacterized protein n=99 Tax=Enterococcus RepID=H8L8U4_ENTFU	erpQ (Ch)	YP_006376165	39.9
contig00010|19	1	0	UniRef100_D0AFF5 Dephospho-CoA kinase n=73 Tax=Enterococcus RepID=D0AFF5_ENTFC	coaE (Ch)	YP_006376166	41.1
contig00010|20	1	0	UniRef100_H8L8U6 Formamidopyrimidine-DNA glycosylase n=103 Tax=Firmicutes RepID=H8L8U6_ENTFU	mutM (Ch)	YP_006376167	42.5
contig00010|21	1	0	UniRef100_C9BPJ4 DNA polymerase n=46 Tax=Firmicutes RepID=C9BPJ4_ENTFC	polA (Ch)	YP_006376168	39.8
contig00010|22	1	0	UniRef100_H8L8U8 Inhibitor of apoptosis-promoting Bax1 n=127 Tax=Firmicutes RepID=H8L8U8_ENTFU	MP (Ch) CDS 900	YP_006376169	39.9
contig00010|23	1	0	UniRef100_H8L8U9 MaoC like domain protein n=158 Tax=Firmicutes RepID=H8L8U9_ENTFU	dehydratase (Ch) CDS 901	YP_006376170	38.5
contig00010|24	1	0	UniRef100_H8L8V0 UDP-N-acetylmuramate--L-alanine ligase n=129 Tax=Enterococcus RepID=H8L8V0_ENTFU	murC (Ch)	YP_006376171	38.3
contig00010|25	1	0	UniRef100_H8L8V1 Uncharacterized protein n=126 Tax=Firmicutes RepID=H8L8V1_ENTFU	HP - YdcF-like superfamily (Ch) CDS 902	YP_006376172	39
contig00010|26	1	0	UniRef100_H8L8V2 Protein SprT-like n=150 Tax=Firmicutes RepID=H8L8V2_ENTFU	Zinc-metalloprotease (Ch) CDS 903	YP_006376173	39.3
contig00010|27	1	0	UniRef100_H8L8V3 RNA binding protein S1 n=119 Tax=Firmicutes RepID=H8L8V3_ENTFU	tex (Ch)	YP_006376174	41.3
contig00010|28	1	0	UniRef100_J6EWM7 Ser/Thr phosphatase family protein n=1 Tax=Enterococcus faecium ERV1 RepID=J6EWM7_ENTFC	pphA (Ch)	YP_006376175	40.1
contig00010|29	1	0	UniRef100_H8L8V5 UPF0176 protein EFAU004_01558 n=126 Tax=Firmicutes RepID=H8L8V5_ENTFU	rhoD (Ch) CDS 904	YP_006376176	41.6
contig00010|30	0	0	UniRef100_H8L8V7 Glyoxalase family protein n=143 Tax=Firmicutes RepID=H8L8V7_ENTFU	gloA (Ch)	YP_006376177	38.3
contig00064|32	1	0	UniRef100_S4EXV9 Putative ATP synthase F0, A subunit n=1 Tax=Enterococcus faecium SD1C-2 RepID=S4EXV9_ENTFC	Na+ symporter (Ch) CDS 905	YP_006376178	40.7
contig00064|31	1	0	UniRef100_H8L8V9 Uncharacterized protein n=227 Tax=Firmicutes RepID=H8L8V9_ENTFU	HP (Ch) CDS 906	YP_006376179	38.2
contig00064|30	1	0	UniRef100_H8L8W0 Phosphoglycolate phosphatase n=116 Tax=Firmicutes RepID=H8L8W0_ENTFU	HAD hydrolase (Ch) CDS 907	YP_006376180	38.5
contig00064|29	1	0	UniRef100_S4E0B6 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase n=2 Tax=Enterococcus faecium RepID=S4E0B6_ENTFC	gpmA3 (Ch)	YP_006376181	39.3
contig00064|28	1	0	UniRef100_H8L8W2 Uncharacterized protein n=126 Tax=Enterococcus RepID=H8L8W2_ENTFU	HP (Ch) CDS 908	YP_006376182	35.6
contig00064|27	1	0	UniRef100_L2RPN8 Uncharacterized protein n=3 Tax=Enterococcus faecium RepID=L2RPN8_ENTFC	regulator (Ch) CDS 909	YP_006376183	36
contig00064|25	1	0	UniRef100_H8L8W4 Uncharacterized protein n=131 Tax=Firmicutes RepID=H8L8W4_ENTFU	HP (Ch) CDS 910	YP_006376184	37.3
contig00064|24	1	0	UniRef100_H8L8W5 Uncharacterized protein n=94 Tax=Firmicutes RepID=H8L8W5_ENTFU	HP (Ch) CDS 911	YP_006376185	40.4
contig00064|23	1	0	UniRef100_H8L8W6 Uncharacterized protein n=133 Tax=Firmicutes RepID=H8L8W6_ENTFU	HP (Ch) CDS 912	YP_006376186	42.7
contig00064|22	1	0	UniRef100_H8L8W7 ABC transporter, permease protein n=123 Tax=Firmicutes RepID=H8L8W7_ENTFU	lacG2 (Ch)	YP_006376187	38
contig00064|21	1	0	UniRef100_H8L8W8 ABC transporter, permease protein n=128 Tax=Firmicutes RepID=H8L8W8_ENTFU	lacF2 (Ch)	YP_006376188	36.7
contig00064|20	1	0	UniRef100_U2NFT9 ABC transporter substrate-binding protein n=1 Tax=Enterococcus faecium CRL1879 RepID=U2NFT9_ENTFC	ABC transporter (Ch) CDS 913	YP_006376189	41.7
contig00064|19	1	0	UniRef100_H8L8X0 Response regulator receiver domain protein n=90 Tax=Firmicutes RepID=H8L8X0_ENTFU	regulator (Ch) CDS 914	YP_006376190	38.2
contig00064|18	1	0	UniRef100_C9BY79 Response regulator receiver n=1 Tax=Enterococcus faecium 1,231,408 RepID=C9BY79_ENTFC	araC-like (Ch) CDS 915	YP_006376191	35.8
contig00064|17	1	0	UniRef100_H8L8X2 Sensor histidine kinase n=120 Tax=Firmicutes RepID=H8L8X2_ENTFU	Kinase (Ch) CDS 916	YP_006376192	37.8
contig00064|16	0	0	UniRef100_R2NHB3 Prephenate dehydratase n=1 Tax=Enterococcus faecium EnGen0191 RepID=R2NHB3_ENTFC	pheA	YP_006376193	43.2
contig00064|15	0	1	UniRef100_D4RAC9 Shikimate kinase n=15 Tax=Enterococcus faecium RepID=D4RAC9_ENTFC	aroK	YP_006376194	38.6
contig00064|14	0	1	UniRef100_D4SM47 3-phosphoshikimate 1-carboxyvinyltransferase n=43 Tax=Enterococcus faecium RepID=D4SM47_ENTFC	aroA	YP_006376195	45.5
contig00064|13	0	1	UniRef100_L2IDW0 Prephenate dehydrogenase n=34 Tax=Enterococcus faecium RepID=L2IDW0_ENTFC	tyrA	YP_006376196	40.7
contig00064|12	0	1	UniRef100_D4SM45 Chorismate synthase n=38 Tax=Enterococcus faecium RepID=D4SM45_ENTFC	aroC	YP_006376197	45
contig00064|11	0	1	UniRef100_D4SM44 3-dehydroquinate synthase n=19 Tax=Enterococcus faecium RepID=D4SM44_ENTFC	aroB	YP_006376198	42.3
contig00064|10	0	1	UniRef100_C2HBL1 3-deoxy-7-phosphoheptulonate synthase n=31 Tax=Enterococcus faecium RepID=C2HBL1_ENTFC	aroF	YP_006376199	42.7
contig00064|9	0	1	UniRef100_D4RAD5 Beta galactosidase small chain n=25 Tax=Enterococcus faecium RepID=D4RAD5_ENTFC	lacZ	YP_006376201	41.1
contig00064|8	0	1	UniRef100_J6YIB9 Glycosyl hydrolase family 2, TIM barrel domain protein n=15 Tax=Enterococcus faecium RepID=J6YIB9_ENTFC	lacL	YP_006376202	39.1
contig00064|7	0	0	UniRef100_S4EL11 Alpha-galactosidase n=1 Tax=Enterococcus faecium OC2A-1 RepID=S4EL11_ENTFC	galA2	YP_006376203	40.2
contig00064|6	0	1	UniRef100_D4RAD8 GalR n=15 Tax=Enterococcus faecium RepID=D4RAD8_ENTFC	galR/purR	YP_006376204/05	41.8
contig00064|4	0	1	UniRef100_D4RAD9 Alpha/beta hydrolase domain protein n=26 Tax=Enterococcus faecium RepID=D4RAD9_ENTFC	peptidase (Ch) CDS 918	YP_006376206	39.3
contig00064|3	0	1	UniRef100_D4SM37 Methionine synthase, vitamin-B12 independent n=12 Tax=Enterococcus faecium RepID=D4SM37_ENTFC	absent in DO		41.9

Table S4 - Annotation of transferable region of *E. faecium* GEHPH2.1 using Best BLAST Hit and *E. faecium* DO as a reference strain (cont.)

E. faecium HPH2- wild type strain; *E. faecium* GE1- recipient strain; *E. faecium* TCHPH2.2-transconjugant. Blue box with "1"- Sequence of the transferred *pbp5* genetic plataform present in both the transconjugant (TCHPH2.2) and in wild type strain (HPH2) but not in the recipient strain (GE1). White box with "0"- Sequence of the transconjugant (TCHPH2.2) absent either in wild type (HPH2) and/or recepient strains (GE1). White box with "1"- Sequence of the transconjugant (TCHPH2.2) also present in the recipient strain (GE1) but absent in the wild type (HPH2), thus not being not part of the transfered region.

Table S5. Epidemiological data of all *E. faecium* strains used in this study bioinformatic analysis.

Strain	Strain type analyzed	Clade	PBP5 alleles	Ampicillin Susceptibility	ST	BAPS	Source	Country	Date	*pbp* 5 platform	GeneBank accession number
E980	WT	A0	C30	S	94	1.2	Community surveillance	Netherlands	1998	IX	ABQA01000020.1
E1972	WT	A0	C30	NA	94	1.2	Clinical isolate	Germany	2000	IX	AHXR01000007.1
E1861	WT	A0	C31	S	289	1.2	Hospital surveillance	Spain	2001	IX	AHXP01000002.1
E1007	WT	B	C47	S	61	1.2	Community surveillance	Netherlands	1998	I	AHWP01000007.1
E2039	WT	B	C47	NA	296	1.2	Clinical isolate	Germany	2000	I	AHXS01000006.1
D356	WT	B	C47	NA	361	1.2	NA	NA	NA	I	ATIT01000093.1
Com15	WT	B	C48	S (P)	583	1.2	Community surveillance	USA	2006	I	ACBD01000014.1
E1604	WT	B	C66	S	75	1.2	Cheese	Norway	1956	I-like[l]	AHXD01000013.1
E1613	WT	B	C33	S	77	1.3	Fish burger	Norway	1964	I	AHXE01000011.1
TX1337RF	WT	B	C34	NA	172	1.3	Human GI tract	NA	NA	I	AMAA01000092.1
Com12	WT	B	C21	S (P)	107	1.5	Community surveillance	USA	2006	I	ACBC01000012.1
TX1330	WT	B	C21	S	107	1.5	Human GI tract	NA	NA	I	ACHL01000102.1, ACHL01000101.1
PC4.1	WT	B	C21	NA	720	1.5	Community surveillance	Australia	NA	I	ADMM0100002.1
E1590	WT	B	C36	S	163	1.5	Community surveillance	Ireland	2001	I	AHXC01000004.1
E3083	WT	B	C47	S	327	1.5	Clinical isolate	Netherlands	2000	I	AHXZ01000002.1
E3548	WT	B	C49	S	328	1.5	Clinical isolate	Netherlands	2004	I	AHYB01000002.1
E1133	WT	A1R	C11	R	117	2.1a	Hospital surveillance	USA	2001	VIII-like[k]	AHWR01000016.1
E4453	WT	A1R	C11	R	192	2.1a	Dog	Netherlands	2008	I	AEDZ01000364.1
U0317	WT	A1R	C15	R	78	2.1a	Clinical isolate	Netherlands	2005	I	ABSW01000111.1
E2560	WT	A1R	C15	R	78	2.1a	Clinical isolate	Netherlands	2006	I	AHYI01000006.1
E6045	WT	A1R	C15	NA	78	2.1a	Clinical isolate	Portugal	2010	IV	AHYL01000021.1
VRE108	WT	A1R	C15	NA	192	2.1a	Human	Denmark	2010	I	AIVC01000031.1
1,231,502	WT	A1R	C15	R	203	2.1a	Clinical isolate	USA	NA	I	GG688486.1, ACAX01000001.1
S447	WT	A1R	C15	R	203	2.1a	Clinical isolate	USA	2004	I	JH806868.1, AMAD01000018.1
R446	WT	A1R	C15	R	203	2.1a	Clinical isolate	USA	2004	I	JH808025.1, AMAJ01000023.1
ERV165	WT	A1R	C15	NA	203	2.1a	Non-clinical isolate	NA	NA	I	AMAV01000037.1
E422	WT	A1R	C15	R	203	2.1a	Clinical isolate	Ecuador	2006-2008	I-like[d]	ZP_1842609.1, AMAZ01000004.1
Aus0085	WT	A1R	C15	R	203	2.1a	Clinical isolate	Australia	2009	I	NC_021994.1
V689	WT	A1R	C15	R	412	2.1a	Clinical isolate	Venezuela	NA	I	AMAC01000037.1
R499	WT	A1R	C15	R	412	2.1a	Human	NA	NA	I	AMAF01000040.1
R496	WT	A1R	C15	R	412	2.1a	Human	NA	NA	I	AMAH01000068.1
ERV168	WT	A1R	C15	NA	412	2.1a	Non-clinical isolate	NA	NA	I	AMAU01000153.1
ERV161	WT	A1R	C15	NA	412	2.1a	Clinical isolate	NA	NA	I	AMAW01000027.1
P1140	WT	A1R	C15	R	412	2.1a	Clinical isolate	Peru	2006-2008	I	JH808980.1, AMAM01000021.1
VRE13	WT	A1R	C15	NA	412	2.1a	Human	Denmark	2010	I	AIVE01000014.1
515	WT	A1R	C15	R	549	2.1a	Human	NA	NA	I	AMBE01000026.1
E2297	WT	A1R	C16	NA	117	2.1a	Clinical isolate	USA	2001	XX	AHXV01000007.1
ERV1	WT	A1R	C24	NA	412	2.1a	Human airways	NA	NA	I	JH813696.1, AMAY01000126.1
E1321	WT	A1R	C27	R	78	2.1a	Clinical isolate	Italy	1999	II-like[d]	AHYF01000006.1
C621	WT	A1R	C38	NA	412	2.1a	Clinical isolate	Colombia	2006-2008	I	AMBB01000025.1
E0333	WT	A1R	C4	R	80	2.1a	Clinical isolate	Israel	1997	XIV-like[g]	AHWL01000001.1
HM1071	WT	A1R	C4	R (P)	117	2.1a	NA	NA	1994	XV	AITX01000027.1
E6012	WT	A1R	C41	NA	78	2.1a	Hospital surveillance	Latvia	2010	I-like[a]	AHYK01000012.1
E2369	WT	A1R	C45	R	78	2.1a	Clinical isolate	Hungary	2005	XIX	AHYH01000012.1
GESN71_1	TC	A1R	C68	R	393	2.1a	Piggery	Portugal	2006	I	This study, JN208885.1
E4389	WT	A1R	C7	R	78	2.1a	Dog	Denmark	2007	XIV	AHYJ01000006.1, AHYJ01000005.1
E1644	WT	A1R	C9	R	78	2.1a	Clinical isolate	Germany	2002	I	AHYG01000012.1
UAA724	WT	A1R	C11	NA	50	2.1b	NA	France	1993	I	AIUO01000031.1
C373	WT	A1R	C11	R	148	2.1b	Community surveillance	Portugal	2001	I	This study
E4452	WT	A1R	C11	R	266	2.1b	Dog	Netherlands	2008	I	AEOU01000130.1
E0680	WT	A1M	C22	S	151	2.1b	Pig	Germany	NA	VII	AHWN01000006.1
E0045	WT	A1M	C23	S	9	2.1b	Poultry	UK	1992	V-like[j]	AHWH01000023.1
E1185	WT	A1M	C23	S	26	2.1b	Clinical isolate	France	NA	V-like[j]	AHWS01000017.1
HM1072	WT	A1M	C23	R (P)	26	2.1b	NA	NA	1994	V	AITY01000037.1
9731349-1	WT	A1M	C23	NA	82	2.1b	Pig	Denmark	1997	V	AITA01000032.1
7330381-1	WT	A1M	C23	NA	133	2.1b	Pig	Denmark	2001	V	AISO01000021.1
9930238-2	WT	A1M	C23	NA	133	2.1b	Pig	Denmark	1999	V	AITJ01000019.1
841V03	WT	A1M	C23	S	147	2.1b	Pig	Denmark	2003	V	AISU01000016.1
E1575	WT	A1M	C23	S	158	2.1b	Poultry	Belgium	1995	V-like[j]	AHWZ01000016.1
9830565-4	WT	A1M	C23	NA	185	2.1b	Pig	Denmark	1998	V	AITG01000020.1
7330614-1	WT	A1M	C23	NA	185	2.1b	Pig	Denmark	2001	V	AISR01000013.1
HM1073	WT	A1M	C23	R (P)	771	2.1b	NA	NA	1994	V	AITZ01000019.1
E0688	WT	A1M	C23	S	5	2.1b	Pig	Spain	NA	V	AHWO01000001.1
E1552	WT	A1M	C23	S	5	2.1b	Hospital surveillance	Netherlands	2002	V	AHWW01000007.1
HF50104	WT	A1M	C23	S	5	2.1b	Pig	USA	NA	V	AITR01000020.1
HF50215	WT	A1M	C23	S	5	2.1b	Pig	USA	NA	V	AITW01000030.1
E0269	WT	A1S	C28	S	9	2.1b	Poultry	Netherlands	1996	X	AHWK01000016.1, AHWK0100001.1
E0164	WT	A1S	C28	S	26	2.1b	Poultry	Netherlands	1996	I	AHWJ01000010.1
E1630	WT	A1S	C32	S	69	2.1b	Community surveillance	Netherlands	1981	I	AHXK01000019.1
E3346	WT	A1S	C32	S	69	2.1b	Clinical isolate	Netherlands	2002	I	AHYA01000007.1
504	WT	A1M	C35	R	649	2.1b	Human	NA	NA	I	AMBM01000063.1
E1627	WT	A1M	C39	S	66	2.1b	Hospital surveillance	Netherlands	1979	I	AHXJ01000003.1
UAA910	WT	A1M	C55	NA	66	2.1b	NA	Switzerland	1996	V-like[j]	AIUS01000017.1
E4215	WT	A1M	C44	NA	310	2.1b	Poultry	Sweden	2004	VI	AHYE01000033.1
109_A1	WT	A1M	C55	NA	123	2.1b	Human	Denmark	1995-1998	V-like[j]	AISL01000023.1
UAA718	WT	A1M	C55	NA	636	2.1b	NA	France	1994	V	AIUI01000020.1
UAA719	WT	A1M	C55	NA	636	2.1b	NA	France	1994	V-like[j]	AIUJ01000018.1
UAA431	WT	A1M	C56	NA	636	2.1b	NA	France	1989	V-like[j]	AJDJ01000012.1
UAA715	WT	A1M	C56	NA	636	2.1b	NA	France	1994	V-like[j]	AIUG01000030.1
UAA714	WT	A1M	C56	NA	636	2.1b	NA	France	1994	V	AIUF01000015.1
E1634	WT	A1M	C57	S	66	2.1b	Non-clinical isolate	NA	1982	V-like[j]	AHXL01000003.1
F9730129-1	WT	A1M	C58	NA	245	2.1b	Poultry	Denmark	1997	XIII	AITN01000033.1
UAA722	WT	A1M	C60	NA	9	2.1b	NA	France	1994	XII	AIUM01000025.1
UAA723	WT	A1R	C72	NA	50	2.1b	NA	France	1993	II	AIUN01000017.1
GE28798_1	WT	A1R	C8	R	310	2.1b	Clinical isolate	Portugal	1999	III	This study
E2134	WT	A1R	C9	R	12	2.1b	Poultry	Netherlands	2004	I	AHXU01000020.1
E1293	WT	A1R	C9	R	50	2.1b	Clinical isolate	Italy	NA	I	AHWU01000013.1
UAA1023	WT	A1R	C9	NA	50	2.1b	NA	France	1996	I	AIZZ01000046.1
UAA1024	WT	A1R	C9	NA	50	2.1b	NA	France	1996	I	AJAA01000028.1
P1123	WT	A1R	C15	R	79	2.3a	Clinical isolate	Peru	2006-2008	I	JH810542.1, AMAP01000100.1
P1986	WT	A1R	C15	R	494	2.3a	Clinical isolate	Peru	2006-2008	I	JH808239.1, AMAK01000017.1
E1573	WT	A1R	C17	S	21	2.3a	Bison	Belgium	1994	I	AHWX01000001.1
GMD5E	WT	A1R	C17	NA	92	2.3a	Human GI tract	NA	NA	I-like[a]	AJRF02000007.1
D355	WT	A1R	C17	NA	92	2.3a	NA	NA	NA	I	ATIU01000010.1
UAA430	WT	A1R	C17	NA	640	2.3a	NA	France	1989	I	AJDI01000015.1
164306	WT	A1M	C2	R	190	2.3a	Clinical isolate	Portugal	1998	V	This study, JN208882.1
D353	WT	A1M	C22	NA	832	2.3a	NA	NA	NA	I	ATIW01000019.1

Table S5. Epidemiological data of all *E. faecium* strains used in this study bioinformatic analysis (cont.).

Strain	Strain type analyzed	Clade	PBP5 alleles	Ampicillin Susceptibility	ST	BAPS	Source	Country	Date	*pbp5* platform	GeneBank accession number
UAA210	WT	A1M	C23	NA	25	2.3a	NA	France	1986	V	AJBN01000025.1
UAA407	WT	A1M	C23	NA	25	2.3a	NA	France	1986	V	AJDG01000015.1
E1620	WT	A1M	C23	S	67	2.3a	Clinical isolate	Netherlands	1957	I	AHXF01000060.1
EnGen0308	WT	A1M	C23	NA	88	2.3a	NA	NA	NA	V	AJDM01000021.1
UAA1022	WT	A1M	C23	NA	88	2.3a	NA	Switzerland	1996	V-like[j]	AIZY01000017.1
E1626	WT	A1R	C24	R	92	2.3a	Clinical isolate	Netherlands	1965	I	AHXI01000005.1
E1679	WT	A1R	C27	R	114	2.3a	Clinical isolate	Brazil	1998	I-like[d]	ABSC01000284.1
E1904	WT	A1R	C27	R	210	2.3a	Clinical isolate	Netherlands	2001	I	AHXQ01000004.1
10/96A	WT	A1R	C27	NA	281	2.3a	Clinical isolate	Brazil	1996	I	AXOL01000027.1
E1050	WT	A1R	C43	S	92	2.3a	Community surveillance	Netherlands	1998	I	AHWQ01000005.1
VAN327	WT	A0	C54	S	417	2.3a	NA	NA	NA	I-like[a]	ASEC01000004.1
VAN335	WT	A0	C54	S	417	2.3a	Poultry	Denmark	2010	I-like[a]	AIUZ01000021.1
HM1074	WT	A1M	C59	R (P)	79	2.3a	NA	NA	1994	V	AIUA01000011.1
D344	WT	A1R	C71	R	25	2.3a	Clinical isolate	USA	NA	NA	AF362954.1
GMD1E	WT	A1R	C74	NA	92	2.3a	Human GI tract	NA	NA	I-like[e]	AJQX01000102.1
E1039	WT	A1M	C14	S	42	2.3b	Community surveillance	Netherlands	1998	I-like[c]	ACOS01000009.1
E0679	WT	A1M	C23	S	150	2.3b	Pig	Belgium	NA	V	AHWM01000004.1
H196	WT	A1M	C1	R	390	3.1	Clinical isolate	Portugal	2002	V	This study, KC479673.1
1,231,408	WT	A1R	C11	R	582	3.1	Clinical isolate	USA	2006	I-like[d]	GG688547.1, ACBB01000047.1
GEH323_3	TC	A1R	C12	R	280	3.1	Clinical isolate	Portugal	2002	I	This study, KC479674.1
E1071	WT	A1R	C13	S	32	3.1	Hospital surveillance	Netherlands	2000	I	ABQI01000087.1
P1139	WT	A1R	C15	NA	280	3.1	Clinical isolate	Peru	2006-2008	I	JH809204.1, AMAN01000049.1
513	WT	A1R	C15	R	736	3.1	Human	NA	NA	I	AMBG01000061.1
NY1-1	WT	A1R	C15	NA	736	3.1	Human GI tract	NA	NA	I	AJDR01000051.1
NY1-2	WT	A1R	C15	NA	736	3.1	Clinical isolate	NA	NA	I	AJDS01000052.1
NY1-3	WT	A1R	C15	NA	736	3.1	Human GI tract	NA	NA	I	AJDT01000045.1
NY1-4	WT	A1R	C15	NA	736	3.1	Clinical isolate	NA	NA	I	AJDU01000056.1
NY1-5	WT	A1R	C15	NA	736	3.1	Human GI tract	NA	NA	I	AJDV01000041.1
NY1-6	WT	A1R	C15	NA	736	3.1	Clinical isolate	NA	NA	I	AJDW01000039.1
NY3-1 (X2)	WT	A1R	C15	NA	736	3.1	Human GI tract	NA	NA	I	AJDX01000030.1
NY2-1 (X2)	WT	A1R	C15	NA	736	3.1	Human GI tract	NA	NA	I	ASWQ01000008.1
NY2-2 (X2)	WT	A1R	C15	NA	736	3.1	NA	NA	NA	I	ASWR01000005.1
NY2-3 (X2)	WT	A1R	C15	NA	736	3.1	Clinical isolate	NA	NA	I	ASWS01000005.1
R497	WT	A1R	C15	R	752	3.1	Human	NA	NA	I	AMAG01000075.1
E1623	WT	A1R	C17	S	22	3.1	Clinical isolate	Netherlands	1960	I	AHXH01000005.1
VAN342	WT	A1R	C17	S	22	3.1	Poultry	Denmark	2010	I	AIVA01000020.1
H17243	WT	A1R	C17	NA	22	3.1	Human	Denmark	1995	I	AITO01000025.1
D352	WT	A1R	C17	NA	32	3.1	NA	NA	NA	I	ATIX01000015.1
E1576	WT	A1R	C17	S	159	3.1	Oyester	South Africa	2001	I	AHXA01000008.1
GEHPH2_1	TC	A1R	C20	R	125	3.1	Clinical isolate	Portugal	2007	I	This study
E1636	WT	A1M	C23	R	106	3.1	Clinical isolate	Netherlands	1961	I-like[b]	ABRY01000062.1
503	WT	A1R	C25	R	280	3.1	Human	NA	NA	I	JH809465.1, AMBN01000040.1
H352	WT	A1R	C4	R	280	3.1	Clinical isolate	Portugal	2000	II	This study
E1622	WT	A1R	C42	S	104	3.1	Mouse	Netherlands	1959	I	AHXG01000003.1
VAN219	WT	A1M	C62	S	784	3.1	Poultry	Denmark	2010	I	AIUW01000023.1
VAN222	WT	A1M	C62	S	784	3.1	Poultry	Denmark	2010	I	AIUX01000013.1
VAN476	WT	A1M	C62	S	785	3.1	NA	NA	NA	I	ASEB01000017.1
P1190	WT	A1R	C7	R	125	3.1	Clinical isolate	Peru	2006-2008	XVII	JH808818.1, AMAL01000087.1
NRRL B-2354 (ATCC	WT	A1R	C73	S	860	3.1	Milk and dairy utensils	USA	1927	I	CP004063.1
E1578	WT	A1R	C17	S	160	3.2	Pig	Germany	2001	I	AHXB01000009.1
7330446-2	WT	A1M	C23	NA	6	3.2	Pig	Denmark	2001	V	AISP01000010.1
9730357-1	WT	A1M	C23	NA	6	3.2	Pig	Denmark	1997	V	AISZ01000017.1
9730219-1	WT	A1M	C23	NA	6	3.2	Pig	Denmark	1997	V	AISY01000007.1
9731352-4	WT	A1M	C23	NA	6	3.2	Pig	Denmark	1997	V	AITB01000017.1
9830512-2	WT	A1M	C23	NA	6	3.2	Pig	Denmark	1998	V	AITE01000025.1
HF50204	WT	A1M	C23	NA	6	3.2	Pig	USA	NA	V	AITV01000023.1
7330519-3	WT	A1M	C23	NA	6	3.2	Pig	Denmark	2001	V	AISQ01000013.1
7330884-2	WT	A1M	C23	NA	6	3.2	Pig	Denmark	2001	V-like[j]	AISS01000009.1
7430166-3	WT	A1M	C23	NA	6	3.2	Pig	Denmark	2002	V	AIST01000016.1
9830091-5	WT	A1M	C23	NA	6	3.2	Pig	Denmark	1998	V	AITC01000013.1
9931110-4	WT	A1M	C23	NA	6	3.2	Pig	Denmark	1999	V	AITK01000010.1
A17	WT	A1M	C23	NA	6	3.2	Pig	Denmark	1995	V-like[j]	AITL01000019.1
E8SV3	WT	A1M	C23	NA	6	3.2	Pig	Denmark	1995	V	AITM01000017.1
H17575	WT	A1M	C23	NA	6	3.2	Human	Denmark	1995	V	AITQ01000016.1
S658-3	WT	A1M	C23	NA	6	3.2	Pig	Denmark	2002	V	AIUD01000018.1
HF50105	WT	A1M	C23	S	6	3.2	Pig	USA	NA	V	AITS01000013.1
HF50106	WT	A1M	C23	S	6	3.2	Pig	USA	NA	V	AITT01000026.1
HF50203	WT	A1M	C23	S	6	3.2	Pig	USA	NA	V	AITU01000017.1
509	WT	A1R	C11	R	17	3.3a	Human	NA	NA	VIII-like[k]	AMBJ01000257.1
511	WT	A1R	C15	R	17	3.3a	Human	NA	NA	I	AMBH01000038.1
514	WT	A1R	C15	R	17	3.3a	Human	NA	NA	I	AMBF01000038.1
VRE110	WT	A1R	C15	NA	18	3.3a	Human	Denmark	2010	I	AIVD01000049.1
R494	WT	A1R	C15	R	664	3.3a	Human	NA	NA	I	AMAI01000028.1
1,231,410	WT	A1R	C16	R	17	3.3a	Clinical isolate	USA	NA	I-like[d]	GG962468.1, ACBA01000014.1
R501	WT	A1R	C16	R	17	3.3a	Human	NA	NA	I	AMAE01000246.1
ERV69	WT	A1R	C16	NA	17	3.3a	Human	NA	NA	I	AMAR01000209.1
ERV38	WT	A1R	C16	NA	17	3.3a	Human	NA	NA	I	AMAS01000051.1
DO[m]	WT	A1R	C17	R	18	3.3a	Clinical isolate	USA	1992	XI	NC_017960.1
C68	WT	A1R	C18	R	16	3.3a	Hospital surveillance	USA	1996	VIII	66703717.1, ACJQ01000082.1
GEE4_1	TC	A1R	C19	R	132	3.3a	Hospital sewage	Portugal	2011	II	This study, JN208889.1
UAA950	WT	A1R	C24	NA	16	3.3a	NA	USA	1996	I	AJDP01000022.1
UAA949	WT	A1R	C24	NA	16	3.3a	NA	USA	1996	XVI	AJDO01000031.1
UAA951	WT	A1R	C24	NA	16	3.3a	NA	USA	1996	XVI	AIZT01000035.1
E417	WT	A1R	C24	R	17	3.3a	Clinical isolate	Ecuador	2006-2008	I	JH813696.1, AMBA01000202.1
ERV26	WT	A1R	C26	NA	17	3.3a	Human airways	NA	NA	I	JH809881.1, AMAT01000050.1
E1392	WT	A1R	C27	R	64	3.3a	Hospital surveillance	UK	2000	I	AHWV01000002.1
ERV102	WT	A1R	C38	NA	17	3.3a	Human oral cavity	NA	NA	I	JH811939.1, AMAX01000171.1
E1162	WT	A1R	C4	R	17	3.3a	Clinical isolate	France	NA	NA	ABQJ01000017.1
TX0133a04	WT	A1R	C4	R	17	3.3a	Clinical isolate	USA	NA	II-like[g]	AEBC01000061.1
TX0133C	WT	A1R	C4	R	17	3.3a	Clinical isolate	USA	NA	II-like[g]	AEBG01000194.1
TX0082	WT	A1R	C4	R	17	3.3a	Clinical isolate	USA	NA	XIV-like[g]	AEBU01000140.1
TX0133B	WT	A1R	C4	R	17	3.3a	Clinical isolate	USA	NA	II-like[g]	AECI01000046.1
TX0133a01	WT	A1R	C4	R	17	3.3a	Clinical isolate	USA	2006	II-like[g]	AECJ01000122.1
UAA825	WT	A1R	C4	NA	17	3.3a	NA	France	1996	II-like[c]	AIUQ01000018.1
UAA725	WT	A1R	C4	NA	17	3.3a	NA	France	1994	II	AIUP01000033.1

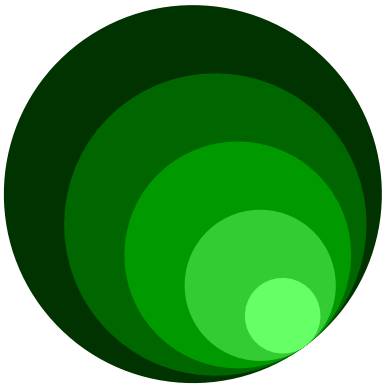
Table S5. Epidemiological data of all *E. faecium* strains used in this study bioinformatic analysis (cont.).

Strain	Strain type analyzed	Clade	PBP5 alleles	Ampicillin Susceptibility	ST	BAPS	Source	Country	Date	*pbp* 5 platform	GeneBank accession number
UAA911	WT	A1R	C4	NA	17	3.3a	NA	Switzerland	1996	II-like[f]	AIUT01000036.1
UAA909	WT	A1R	C4	NA	17	3.3a	NA	Switzerland	1996	II-like[h]	AIUR01000020.1, AIUR01000021.1
UAA1007	WT	A1R	C4	NA	17	3.3a	NA	NA	1996	II	AIZV01000020.1, AIZV01000021.1
UAA1484	WT	A1R	C4	NA	17	3.3a	NA	NA	1992	II	AJAF01000050.1
UAA720	WT	A1R	C4	NA	17	3.3a	NA	France	1993	II	AIUK01000047.1
UAA952	WT	A1R	C4	NA	17	3.3a	NA	USA	1996	II	AIZU01000018.1
GE70411_2	TC	A1R	C4	R	670	3.3a	Clinical isolate	Portugal	1997	II	This study, JN208888.1
HPH6	WT	A1R	C5	R	18	3.3a	Clinical isolate	Portugal	2007	II	This study, KC479675.1
529940	WT	A1R	C6	R	16	3.3a	Clinical isolate	Portugal	2000	II	This study, JN208883.1
TX0133A	WT	A1R	C64	R	17	3.3a	Clinical isolate	USA	2006	II-like[g]	AECH01000021.1
VRE84	WT	A1R	C69	NA	17	3.3a	Human	Denmark	2010	I	AIVF01000028.1
AUS0004	WT	A1R	C7	R	17	3.3a	Clinical isolate	Australia	1998	II	NC_017022.1
UAA1025	WT	A1R	C7	NA	17	3.3a	NA	France	1996	II-like[g]	AJAB01000030.1
UAA1019	WT	A1R	C7	NA	17	3.3a	NA	NA	1996	II	AIZX01000035.1
1,230,933	WT	A1R	C7	R	18	3.3a	Clinical isolate	USA	2005	NA	ACAS01000028.1
P1137	WT	A1R	C7	R	18	3.3a	Clinical isolate	Peru	2006-2008	XVIII	AMAO01000169.1
510	WT	A1R	C7	R	18	3.3a	Human	NA	NA	XIV-like[g]	AMBI01000132.1
ERV99	WT	A1R	C7	NA	18	3.3a	Clinical isolate	NA	NA	XIV-like[g]	AMAQ01000006.1
C1904	WT	A1R	C7	NA	18	3.3a	Clinical isolate	Colombia	2006-2008	XIV-like[g]	AMBD01000237.1
C497	WT	A1R	C7	R	18	3.3a	Clinical isolate	Colombia	2006-2008	XIV-like[g]	AMBC01000119.1
506	WT	A1R	C7	R	18	3.3a	Human	NA	NA	II-like[g]	JH813945.1, AMBK01000250.1
E1731	WT	A1R	C7	R	18	3.3a	Clinical isolate	Tanzania	NA	II	AHXO01000011.1
E2883	WT	A1R	C7	R	18	3.3a	Clinical isolate	Netherlands	2002	XIV-like[g]	AHXX01000007.1
UAA1433	WT	A1R	C7	NA	18	3.3a	NA	France	2000	II	AJAE01000027.1
VD79C1_5	TC	A1R	C7	R	18	3.3a	Community surveillance	Portugal	2001	II	This study
GEE49_1	TC	A1R	C8	R	132	3.3a	Hospital sewage	Portugal	2001	III	This study, JN208884.1
SN449	WT	A1R	C8	R	132	3.3a	Piggery	Portugal	2007	III	This study, JN208887.1
E197	WT	A1R	C8	R	368	3.3a	Hospital sewage	Portugal	2001	III	This study
UAA947	WT	A1R	C9	NA	18	3.3a	NA	USA	1996	I	AIZR01000020.1
UAA716	WT	A1M	C22	NA	87	3.3b	NA	France	1994	VII	AIUH01000039.1
E0120	WT	A0	C37	S	27	3.3b	Clinical isolate	Netherlands	1995	I	AHWI01000003.1
E1574	WT	A0	C37	S	27	3.3b	Dog	Belgium	1995	I	AHWY01000003.1
E2071	WT	A0	C37	S	27	3.3b	Poultry	Denmark	2001	I	AHXT01000013.1
E2966	WT	A1R	C17	NA	326	4	Clinical isolate	Netherlands	2005	I	AHXY01000021.1
E2620	WT	B	C29	S	331	4	Clinical isolate	Netherlands	2006	I	AHXW01000008.1
E1258	WT	A1S	C40	S	127	7.1	Clinical isolate	Spain	NA	I	AHWT01000005.1
D357	WT	A1S	C40	NA	127	7.1	NA	NA	NA	I	ATIS01000033.1
1,231,501	WT	B	C47	S	52	7.1	Clinical isolate	USA	2005	I	ACAY01000033.1
1,141,733	WT	B	C51	S	52	7.1	Clinical isolate	USA	2005	I	ACAZ01000019.1
UAA944	WT	A1R	C70	NA	20	7.1	NA	USA	1996	XXI	AIUU01000031.1
UAA945	WT	A1R	C70	NA	20	7.1	NA	USA	1996	XXI	AIUV01000032.1
VAN345	WT	A1R	C75	S	38	7.1	Poultry	Denmark	2010	I	AIVB01000020.1
VAN332	WT	A1R	C75	S	38	7.1	Poultry	Denmark	2010	I	AIUY01000015.1
SE97F1	WT	A1R	C10	R	NA	NA	Poultry	Portugal	2001	I	This study, KC479676.1
GMD4E	WT	A1R	C17	NA	New	NA	Human GI tract	NA	NA	I-like[a]	AJRD01000046.1
GMD2E	WT	A1R	C17	NA	New	NA	Human GI tract	NA	NA	I-like[a]	AJQZ01000046.1
GMD3E	WT	A1R	C17	NA	New	NA	Human GI tract	NA	NA	I-like[a]	AJRB03000042.1
H17494	WT	A1R	C17	NA	NA	NA	Human	Denmark	1995	I	AITP01000026.1
7230532-1	WT	A1M	C23	NA	New	NA	Pig	Denmark	2000	V	AISN01000018.1
KH36SYN	WT	A1M	C23	NA	NA	NA	Human	Netherlands	NA	V	AIUC01000009.1
EnGen0305	WT	A1M	C23	NA	New	NA	NA	NA	NA	V-like[i]	AJDL01000022.1
UAA721	WT	A1M	C23	NA	New	NA	NA	France	1994	V-like[j]	AIUL01000016.1
SN194	WT	A1R	C3	R	NA	NA	Piggery	Portugal	2006	V	This study, JN208886.1
BM4107	WT	B	C46	S	NA	NA	Clinical isolate	NA	NA	I	AF364092.1
D63r	WT	B	C50	S (P)	NA	NA	Clinical isolate	France	NA	NA	X84860.1
505	WT	A0	C52	R	New	NA	Human	NA	NA	I	AMBL01000029.1, AMBL01000072.1
UAA1280	WT	A0	C53	NA	New	NA	NA	France	1998	I	AJAD01000011.1
D366	WT	A1M	C61	S	NA	NA	NA	NA	NA	NA	X84859.1
9439	WT	A1R	C63	R	NA	NA	NA	NA	NA	NA	X92687.1
EFM-1	WT	A1M	C65	R (P)	NA	NA	NA	NA	NA	NA	X84861.1
H80721	WT	A1R	C67	R	NA	NA	NA	NA	NA	NA	X84862.1
BM4538	WT	A1R	C7	NA	NA	NA	Clinical isolate	France	2001	II	AXOJ01000014.1
NEF1	WT	A1R	C7	R	NA	NA	NA	France	2009	II-like[h]	AXOK01000016.1, AXOK01000017.1

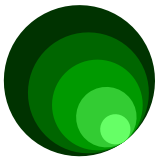
Abbreviations: ND- not determined; NA-not available; USA-United States of America; UK-United Kingdom; WT-wild type; R-resistant; S-susceptible; R(P)-Resistance to penicillin, ampicillin resistance phenotype unkown; S(P)- susceptible to penicillin. [a]extra stop codon in FtsW; [b]sequenced by NGS not possible to identify sequence before pbp5 gene; [c]extra stop codon in psr gene; [d]sequenced by NGS not possible to identify sequence before ftsW gene; [e]extra stop codon in ftsW gene;sequenced by NGS not possible to identify sequence after ion efflux gene; [f]sequenced by NGS not possible to identify sequence before HisPh; [g]sequenced by NGS not possible to identify sequence before ISEfm1; [h]sequenced by NGS not possible to identify sequence after ISEfm1 and before HisPh gene; [i]sequenced by NGS not possible to identify sequence before ISEf1; [j]different genetic background before ISEf1; [k]sequenced by NGS not possible to identify sequence after ISEnfa3; lextra stop codon in pbp5 gene; [m]*E. faecium* DO strain is classified as ampicillin resistance (MIC=16 mg/L) by Galloway-Peña *et al* [Antimicrob Agents Chemother. 2011, 55(7): 3272–3277]. The other *E. faecium* with amino acid combination of C17 were classified as susceptible by Willems *et al*. [mBio. 2012, 3(4): e00151-12]; [n]E1636 was classified as ampicillin resistant (CMI=25mg/L) though the *pbp5* sequence did not present mutations associated with ampicillin resistance the authors suggest that this low level resistance migh be related with other mechanism of resistance (van Schaik *et al* BMC genomics. 2010, 11:239).

Knowledge has to be improved, challenged, and increased constantly, or it vanishes.

Peter Drucker



Chapter 7



Fitness Costs of *vanA* Carrying Plasmids Influences the Biology of Vancomycin-Resistant *Enterococcus faecium*

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In preparation

ABSTRACT

Background: Vancomycin resistance *Enterococcus faecium* has increasingly been reported worldwide. The predominant genotype, Tn*1546*-*vanA*, is mostly located on RepA_N and Inc18 family plasmids. The aim of this study was to determine the fitness cost imposed by the acquisition of Tn*1546*-plasmids in different hosts.

Methods: Globally spread Tn*1546*-plasmids containing RepA_N (3 pRUM, 2 pLG1) and/or Inc18 (n=4) and/or pHTβ (n=1) replication modules and prototype plasmids RepA_N (pRUM) and Inc18 (pRE25, pIP501) were analyzed. Plasmid transferability and fitness cost were assessed using *E. faecium* (GE1RF, 64/3) and *Enterococcus faecalis* (JH2-2, FA202, UV202) strains. Growth curves were performed using Bioscreen C and Relative Growth Rates were calculated in presence/absence of vancomycin. Plasmid stability was analysed after 300 generations. Whole genome sequencing (Illumina MiSeq) of both non-evolved and evolved strains (GE1RF and 64/3, n=49) was performed. SNP calling was preformed using breseq software using non-evolved strains for comparison.

Results: All *E. faecium* plasmids were transferred into different *E. faecium* backgrounds but only plasmids carrying Inc18 family modules were transferred into *E. faecalis*. Most Tn*1546*-plasmids and prototype-plasmids reduced the host's fitness (-2%-18%). Fitness cost of Tn*1546* expression varies according with the transposon variant and background (9%-49%). Stability of Tn*1546*-plasmids was verified in all cases, sometimes with loss of phenotypic resistance and plasmid modules. Mutation analysis revealed point mutations and/or indels mostly in the chromosome of evolved strains associated with essential bacterial functions (e.g. DNA replication and repair, carbohydrate and amino acid metabolism).

Conclusions: The host-specificity of *E. faecium* Tn*1546*-plasmids might play an important role in the confinement of vancomycin resistance almost exclusively to this species. Even though Tn*1546*-plasmids seem to impose a significant burden to the host strain, their stability in the absence of selective pressure and high conjugation rates might explain their maintenance *E. faecium* populations, perpetuating the presence of VRE strains worldwide.

INTRODUCTION

Vancomycin Resistant Enterococci (VRE) have increasingly been reported worldwide since their first description in the late 1980s, and have become endemic in many geographical areas [1]. Resistance to vancomycin has been mostly associated with *Enterococcus faecium* while vancomycin resistant *Enterococcus faecalis* remains infrequent [2,3].

In *E. faecium* several genotypes have been associated with vancomycin resistance, being *vanA* among the most prevalent [1,2]. The *van*A gene cluster is part of Tn*1546,* a member of the Tn*3* family, usually associated with conjugative plasmids [1,4–6]. A number of Tn*1546* variants have been described but only those containing IS*1216V* and IS*Ef1* are widely distributed among human, animal, and environmental strains [7–9]. Tn*1546* is frequently associated with enterococcal plasmids belonging to Inc18 (pIP501 and pRE25 derivatives) and RepA_N (derivatives of narrow-host plasmids pRUM and pLG1) families [1,5,10–12]. The localization of Tn*1546* in broad host range plasmids, as those of the Inc18 family, is a potential threat for dissemination to other bacterial species, as *Staphylococcus aureus* [2,5,12].

Because of the frequent location of Tn*1546* in broad-host plasmids with high conjugative efficiency, the tight association of only few types of Tn*1546* with particular hospital associated BAPS groups (BAPS 2.1a, 3.3a1 and 3.3a2), and the infrequent transfer to other species was unexpected, and remains to be explained. A possible hypothesis is that various Tn*1546* variants could spread in their plasmid vehicles across bacterial populations, but only a limited number of hosts ensure their stable maintenance [1,12–14]. These hosts are probably those with a higher intrinsic fitness, and/or resistant to other antibiotics, and/or where the uptake of plasmids harboring particular Tn*1546* variants might impose a lower fitness cost, in the absence or during antibiotic exposure.

Only a few studies have evaluated the fitness cost of vancomycin resistance and/or Tn*1546* carrying plasmids, being frequently limited to a single recipient strain and/or involving insufficiently characterized plasmids [15–20]. However, when the fitness cost of vancomycin resistance (*vanA*) was evaluated in *S. aureus*, in the presence and absence of induction, it was shown that in the absence of the inducer (vancomycin) the fitness cost of Tn*1546* was negligible and probably due to basal induction of the vancomycin resistance operon. However, an important reduction of bacterial host fitness was observed when the vancomycin resistance operon was induced by vancomycin, probably due to the complex metabolic changes associated with the expression of the *vanA* operon [17]. A few studies performed in *E. faecium* regarding the fitness cost of Tn*1546* carrying plasmids showed that these plasmids reduce the fitness of the host. However, they appear to be stable, and the initial fitness cost seems to be rapidly reduced by compensatory changes in sequential evolution experiments [15,16].

The aim of this study was to determine the fitness cost imposed by the acquisition of worldwide disseminated enterococcal plasmids carrying different Tn*1546* variants in different hosts. The intrinsic fitness of different *E. faecium* host specific populations was also studied.

MATERIAL AND METHODS

Tn*1546-vanA* plasmids. *E. faecium* strains harbouring highly transferable narrow or broad host plasmids involved in the spread of Tn*1546-van*A worldwide were analysed (Figure 1). These strains were classified as *E. faecium* BAPS 3.3a1 (ST18, ST132), BAPS 3.3a2 (ST17, ST16), BAPS 2.1a (ST78) BAPS 2.1b (ST5) and BAPS 2.3a (ST25) [13,14]. The Tn*1546-vanA* plasmids selected for this study have been known for their apparent epidemicity and persistence in the hospital setting [21]. These plasmids contained sequences associated with Inc18 (rep_1 and rep_2), RepA_N (rep_{17} and rep_{20}) and pHTβ (rep_{22}) plasmid families. Prototype plasmids for Inc18 [pIP501 and pRE25 (GenBank NC_008445)] and RepA_N plasmid families [pRUM (GenBank NC_005000)] were also studied for comparative purposes [22–24].

Plasmid analysis and transferability. Plasmid content was detected by Pulsed Field Gel Electrophoresis (PFGE) of *S1* nuclease digested genomic DNA and characterization of replication initiator proteins (REP), relaxases (REL) and maintenance systems (partition and toxin-antitoxin systems) by PCR, hybridization and further sequencing [21,25–28].

Transferability of plasmids among enterococcal species was determined by filter mating experiments at 1:1 donor-recipient ratio and overnight incubation. Transconjugants were selected on Brain Heart Infusion (BHI) agar plates containing fusidic acid (20μg/mL), rifampicin (30μg/mL) and vancomycin (6μg/mL) or erythromycin (20μg/mL) after incubation at 37ºC (24h) [29,30]. Conjugation frequencies were calculated as the proportion of transconjugants *per* recipient CFUs. Transconjugants were confirmed by PCR detection of *vanA* and *ermB* genes [31,32]; and by PFGE of *Sma*I-digested genomic DNA (Takara Bio Inc., Shiga, Japan) [33]. Rifampicin and fusidic acid-resistant *E. faecium* GE1RF (ST515, BAPS 2.3b) and 64/3(ST21, BAPS 2.3a), and *E. faecalis* JH2-2, FA202 and UV202 (all belonging to ST8, BAPS 1), were used as recipient strains.

Growth kinetics. The growth kinetics of field *E. faecium* isolates (see supplementary table S1), laboratory receptor *E. faecium* strains GE1RF and 64/3 and *E. faecalis* strains JH2-2, and transconjugants harbouring Tn*1546-van*A carrying plasmids were performed in the incubator/spectrophotometer Bioscreen C (ThermoLab Systems, Vantaa, Finland) adapting the method described by Foucault *et al* (Foucault et al., 2009). Briefly, strains were grown overnight (18h) at 37ºC in BHI broth with vancomycin (1/50 of the MIC) and without vancomycin. Overnight cultures were diluted 1:1000 into fresh BHI broth, approximately 10^5 bacteria/mL, and 300μl of this bacterial suspension was transferred into a 100-well microplate. Optical density (OD) was measured at 600nm every 15 min for 20h. In order to guarantee culture optical homogeneity the plates were agitated for 10s before each OD measurement. For each strain, 5 biological replicates were essayed in duplicate in each experiment (10 readings *per* strain *per* experiment). Two independent experiments were performed for all strains analysed [17]. Conditions of induction or non-induction with vancomycin were maintained throughout the entire experiment. Variants of this procedure were used to analyze possible changes in the *E. faecium* growth dynamics at 42ºC.

Growth rates (μ) were determined in the interval estimated to be exponential phase using the GrowthRates 2.1 program [34]. The fitness cost of the plasmids and expression of Tn*1546-vanA* was determined by calculating the RGR_{PL} and RGR_{Tn} using the following formulas:

$$RGRPl = \frac{\mu(transconjugant\ without\ induction)}{\mu(isogenic\ plasmid\ free\ strain)}$$

$$RGRTn = \frac{\mu(transconjugant\ with\ induction)}{\mu(transconjugant\ without\ induction)}$$

Plasmid stability. Three independent colonies from each strain (receptors and transconjugants, see supplementary Figure S1) were inoculated into 5mL of BHI broth and incubated at 37ºC overnight (22-24h). Subsequently, cultures were diluted 1:1000 into fresh BHI broth and incubated at 37ºC overnight (22-24h). This procedure was repeated up to 300 generations. Bacterial cultures were plated at 0, 100, 200 and 300 generations on BHI agar plates and subsequently 100 colonies of each plate were randomly picked and re-streaked into BHI agar plates and BHI agar plates supplemented with the appropriate antibiotic (6μg/mL of vancomycin for strains containing pH182, pH311 and pBM4165, or 20μg/mL of erythromycin for the strains containing pIP501, pRE25 and pRUM) [22–24]. Plasmid loss frequency was initially calculated by determining the ratio of susceptible colonies to the total number of colonies. Antibiotic susceptibility (stability/loss) was further confirmed phenotypically by disc diffusion method, and genotypically by PCR detection of *vanA* and *ermB* genes [31,32]. The presence/absence of plasmid was confirmed by PFGE-*S1* nuclease digested genomic DNA as well as by confirming the presence of all plasmid modules previously observed in each of the plasmids studied as described above (Table 1).

Analysis of evolved strains. Whole genome sequencing (WGS, Illumina MiSeq) of 49 transconjugants, 24 *E. faecium* GE1 (7 non-evolved and 17 evolved strains) and 25 *E. faecium* 64/3 (7 non-evolved and 18 evolved strains), obtained in the plasmid stability experiment was performed. Sequencing was carried out with an Ilumina MiSeq platform to obtain 100-200 bp paired-end reads (supplementary Figure S1). DNA extraction (Promega Wizard Genomic DNA Purification kit) was done according manufacturer instructions. For non-evolved strains, reads were revised and sequencing errors were corrected with Lighter software [35], the best k-mer length was estimated with KmerGenie [36], and the final assembly was performed with Spades [37]. SNPs analysis of evolved strains was performed against non-evolved strains using Breseq v0.26.1 pipeline (http://barricklab.org/twiki/bin/view/Lab/ToolsBacterial GenomeResequencing)[38]. Protein functions were assigned according Uniprot and KEGG databases. Plasmid analysis of non-evolved strains was performed by PLACNET in order to determine both plasmid content in these strains and to identify the location of mutations in evolved strains [39].

Clonal relatedness and genome size. *E. faecium* field strains used in this study and their epidemiological characteristics are listed in Table S1. Clonal relatedness between *E. faecium* stains was established by PFGE using *Sma*I-digested genomic DNA (Takara Bio Inc., Shiga, Japan), multilocus sequence typing (MLST) and Bayesian analysis of population structure (BAPS) as previously described

[13,14,33,40]. Genome size was established by PFGE using *Asc*I-digested genomic DNA (New England Biolabs, Ipswich, MA, USA). The following electrophoresis conditions were used: 10 to 40s pulses for 26h at 14ºC and 6V/cm^2. OG1 was used a DNA weight marker [41,42].

Statistical analysis. Statistics were calculated using R analytical Studio [43], ANOVA and Tukey's honest significance test (for multiple all in all comparison). Chi-Square test was used when comparing two populations. Values of $p<0.01$ were considered statistically significant.

RESULTS

Fitness cost of Tn*1546-vanA* carrying plasmids in laboratory *E. faecium* and *E. faecalis* strains.

Both Tn*1546-van*A carrying plasmids and prototype plasmids of the Inc18 and RepA_N plasmid families analysed in this study conferred different fitness cost on distinct clonal backgrounds (Figure 1 and 2).

With the exception of pE1644 (rep_{20}+rel_6, fitness gain of 2.0%, not statistically significant), all tested *E. faecium* Tn*1546-vanA* carrying plasmids conferred a fitness cost to *E. faecium* GE1RF (n=7, ranging from 4.4% to 9.2%) and also in *E. faecium* 64/3 (n=5), although to a lesser extent (0.3% and 7.7%). The two *E. faecium* plasmids that transferred to *E. faecalis* JH2-2 (pBM4165 and pH305) showed a high fitness cost in this strain (17.5% and 39.4%, respectively).

Among plasmids of the Inc18 family, pIP816 (rep_1+rep_2+rel_6+$TA_{Axe\text{-}Txe}$) exhibited the highest fitness cost values in both GE1RF (9.2%) and 64/3 (7.7%). Conversely, pIP501 and pRE25 did not impose a significant cost to the *E. faecium* strains studied, only pRE25 showed a significant fitness cost in *E. faecalis* JH2-2 (13.9%). Other mosaic plasmids containing plasmid modules of the Inc18 family (pE1651, rep_1; pBM4165, rep_1+rep_2; pH311, rep_1+rep_2+rel_6+$TA_{\varepsilon\zeta}$ and pH305, rel_6+$TA_{\varepsilon\zeta}$), reduced the fitness of *E. faecium* GE1RF [6.8%, 6.9%, 4.4% (not statistically significant) and 4.6%, respectively]. Aside from pIP816, the only plasmid that showed a significant fitness cost in *E. faecium* 64/3 was also an Inc18+RepA_N plasmid (pBM4165, 5.0%) (Figure 1 and 2).

The RepA_N-pRUM family plasmid studied (pH182, rep_{17}+rel_6) also conferred a significant fitness cost to *E. faecium* GE1RF (5.9%) as did the pRUM prototype in both *E. faecium* backgrounds (GE1RF - 18.4%, and 64/3 – 4.2%).

Fitness cost of Tn*1546-vanA* expression in isogenic *E. faecium* and *E. faecalis* backgrounds.

The fitness cost of Tn*1546-vanA* expression varies according with the transposon variant being expressed and with the clonal and plasmid backgrounds in which they are located (Figures 1 and 2).

The expression of Tn*1546* variant A when located in pBM4165 (Inc18+RepA_N) conferred a fitness cost of 9.4%, in *E. faecium* GE1RF, 13.6% in *E. faecalis* JH2-2 and no cost in *E. faecium* 64/3.

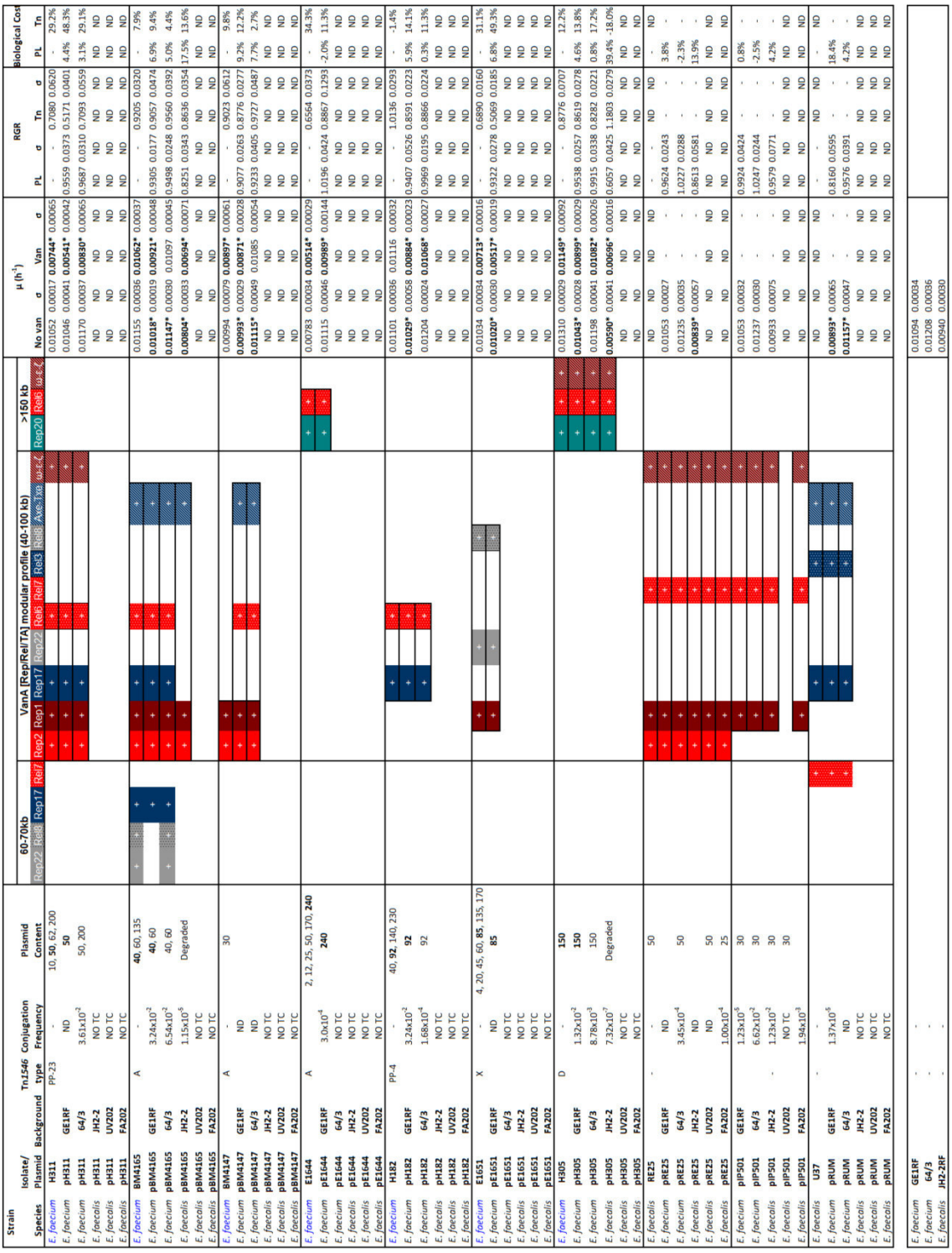


Figure 1. Tn*1546-vanA* and prototype plasmids used in this study.

Rep1 – $rep_{1/pIP501}$; Rep2 – $rep_{2/pRE25}$; Rep17 – $rep_{17/pRUM}$; Rep22 – $rep_{22/pHTβ}$; Rel3 – $rel_{3/pRUM}$; Rel6 – $rel_{6/pEF1}$; Rel7 – $rel_{7/pIP501}$; Rel8 – $rel_{8/pHTβ}$; ωεζ – Toxin-Antitoxin system of plasmid pSM19035; Axe-Txe – Toxin-Antitoxin system of plasmid pRUM. *statistically significant. Plasmid in bold - plasmid carrying Tn*1546-vanA*. **Abbreviations:** ND – Not Determined; NO TC – No transconjugant was obtained; No Van – No vancomycin induction; Van – Vancomycin induction; RGR – Relative Growth Rate; PL – Plasmid; Tn – Transposon Tn*1546-vanA*.

The Tn*1546* variants PP-4 (containing an IS*Ef1* insertion in the *vanX-vanY* intergenic region), when expressed in pH182 (RepA_N), and variant D, in plasmid pH305 (Inc18+RepA_N), conferred a similar fitness cost in *E. faecium* GE1RF and *E. faecium* 64/3 (14.1% *vs* 11.3% and 13.8% *vs* 17.2%, respectively).

Interestingly, in the case of *E. faecalis* JH2-2 the expression of Tn*1546* variant D improves the fitness of the background by 18.0% (Figures 1 and 2).

Other transposon variants exhibiting indels and duplications as Tn*1546* variant PP-23, in pH311 (Inc18+RepA_N) or Tn*1546* variant X, in pE1651 (Inc18+pHTβ) imposed a high fitness cost in GE1RF (48.3% *vs* 49.3%) and 64/3 strains (29.1%, only analysed for pH311) (Figures 1 and 2).

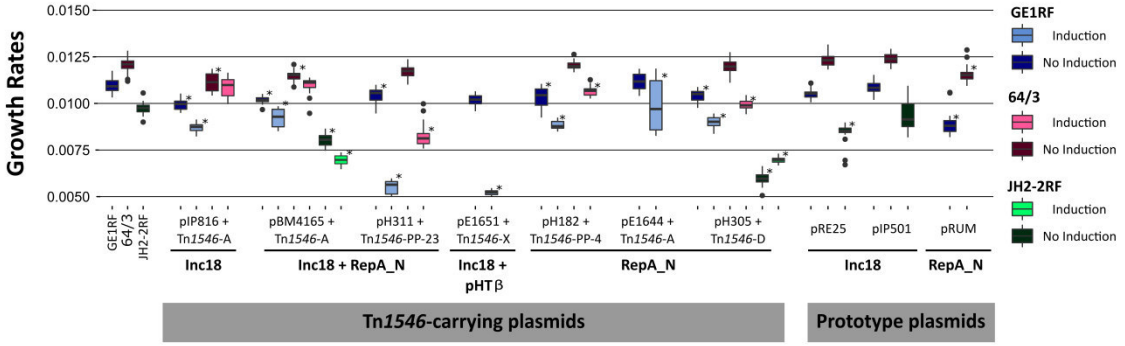


Figure 2. Box and whiskers plot representing growth rates of *E. faecium* Tn*1546-vanA* carrying plasmids with and without vancomycin induction and prototype plasmids (pRE25, pIP501 and pRUM).

Plasmid and Tn*1546-vanA* expression was tested in different backgrounds (*E. faecium* GE1RF and 64/3 and *E. faecalis* JH2-2) when plasmids conjugate into those backgrounds. Plasmids are classified according with RIP plasmid family and Tn*1546* type. *statistically significant.

Plasmid Host-Range.

Plasmid transferability rates (conjugation frequencies) varied among plasmids and, for the same plasmid, between the recipients strains used. Most of *E. faecium* plasmids transferred at high frequencies (10^{-2} to 10^{-4}) into all *E. faecium* receptors used. Two plasmids (pH305 and pBM4165) transferred to both *E. faecalis* and *E. faecium* at different frequencies (10^{-7} and 10^{-5}, respectively) (Figure 1).

While the Inc18 family prototype plasmids pRE25 and pIP501 were transferred from *E. faecalis* to most of the *E. faecalis* and *E. faecium* recipients used (10^{-2} to 10^{-5}); the original pRUM plasmid from *E. faecium* was only able to be transferred into *E. faecium* recipients (≈10^{-5}) (Figure 1).

In host stability experiments.

E. faecium Tn*1546*-carrying plasmids were highly stable up to 300 generations in both *E. faecium* GE1RF and *E. faecium* 64/3, even though the presence of these plasmids was not always associated with a resistance phenotype. In general all plasmids were more stable in *E. faecium* 64/3 than in *E. faecium* GE1RF (Figure 3 and S1).

For the plasmid pH311, the few *E. faecium* GE1RF and *E. faecium* 64/3 isolates for which vancomycin resistant phenotype reversion occurred (1.0-3.0%) contained a plasmid carrying the same backbone, and the *vanA* gene (3/5). Characterization of Tn*1546* showed changes in its structure that might be related with the absence of vancomycin resistance. For strains harbouring pH182, the phenotype reversion also

occurred sporadically (1.0-4.0%) but in this case, it was accompanied by the loss of Tn*1546* and changes in plasmid size in most cases, although without the loss of the plasmid and plasmid modules. Conversely, the reversion to a vancomycin susceptible phenotype was frequent (up to 100%) in strains containing plasmid pBM4165 and occurred more rapidly in *E. faecium* GE1RF (100% at 200 generations) than in *E. faecium* 64/3 (up to 92.0% at 300 generations). The reversion of phenotype was accompanied by the loss of Tn*1546* without the loss of the plasmid or plasmid modules (Figure 3 and S1).

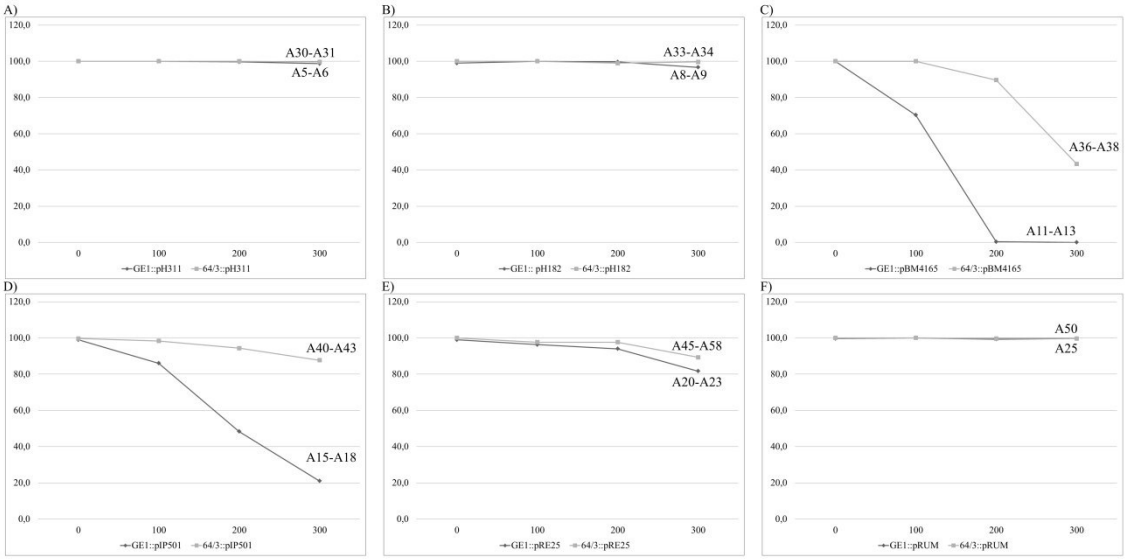


Figure 3. Antibiotic resistant phenotype loss of Tn*1546-vanA* carrying plasmids in *E. faecium* GE1RF and *E. faecium* 64/3.

Antibiotic resistance does not always correspond with plasmid loss. A) Antibiotic resistance phenotype loss for pH311 (A5 - GE1RF::pH311$_{300}$::*vanA*; A6 - GE1RF::pH311$_{300}$::Δ*vanA*; A30 - 64/3::pH311$_{300}$::*vanA*; A31 - 64/3::pH311$_{300}$::Δ*vanA*); B) Antibiotic resistance phenotype loss for pH182 (A8 - GE1RF::pH182$_{300}$::*vanA*; A9 - GE1RF::pH182$_{300}$::Δ*vanA*; A33 - 64/3::pH182$_{300}$::*vanA*; A34 - 64/3::pH182$_{300}$::Δ*vanA*); C) Antibiotic resistance phenotype loss for pBM4165 (A11 - GE1RF::pBM4165$_{100}$::*vanA*; A12 - GE1RF::pBM4165$_{100}$::Δ*vanA*; A13 - GE1RF::pBM4165$_{300}$::Δ*vanA*; A36 - 64/3::pBM4165$_{100}$::*vanA*; A37 - 64/3::pBM4165$_{300}$::*vanA*; A38 - 64/3::pBM4165$_{300}$::Δ*vanA*); D) Antibiotic resistance phenotype loss for pIP501 (A15 - GE1RF::pIP501$_{100}$::*ermB*; A16 - GE1RF::pIP501$_{100}$::Δ*ermB*; A17 - GE1RF::pIP501$_{300}$::*ermB*; A18 - GE1RF::pIP501$_{300}$::Δ*ermB*; A40 - 64/3::pIP501$_{100}$::*ermB*; A41 - 64/3::pIP501$_{100}$::Δ*ermB*; A42 - 64/3::pIP501$_{300}$::*ermB*; A43 - 64/3::pIP501$_{300}$::Δ*ermB*); E) Antibiotic resistance phenotype loss for pRE25 (A20 - GE1RF::pRE25$_{100}$::*ermB*; A21 - GE1RF::pRE25$_{100}$::Δ*ermB*; A22 -. GE1RF::pRE25$_{300}$::*ermB*; A23 - GE1RF::pRE25$_{300}$::Δ*ermB*; A45 - 64/3::pRE25$_{100}$::*ermB*; A46 - 64/3::pRE25$_{100}$::Δ*ermB*; A47 - 64/3::pRE25$_{300}$::*ermB*; A48 - 64/3::pRE25$_{300}$::Δ*ermB*); F) Antibiotic resistance phenotype loss for pRUM (A25 - GE1RF::pRUM$_{300}$::*ermB*; A50 - 64/3::pRUM$_{300}$::*ermB*).

The behaviour of prototype plasmids pRUM and pRE25 was similar to that of Tn*1546*-carrying plasmids pH311 and pH182, respectively. Strains harbouring pRUM rarely lost the erythromycin resistant phenotype (up to 1.0%) in both *E. faecium* GE1RF and *E. faecium* 64/3, and also plasmid size and backbone integrity were maintained in most cases. Prototype plasmid pRE25 was found to be less stable in *E. faecium* than pRUM (plasmid loss of 7.0-27.0% in *E. faecium* GE1RF and 0-17.0% in *E. faecium* 64/3) and, in some instances, the loss of erythromycin resistance was accompanied by plasmid loss but in other cases the plasmid was present with different size due to then loss of one or more plasmid modules that constitute the plasmid backbone. Contrarily, the prototype plasmid pIP501 had low stability in *E. faecium* backgrounds, being easily lost, particularly in *E. faecium* GE1RF (68.0-96.0% *vs* 8.0-23.0% in *E. faecium* 64/3). The reversion to an erythromycin susceptible phenotype frequently coincided with plasmid loss (Figure 3 and S1).

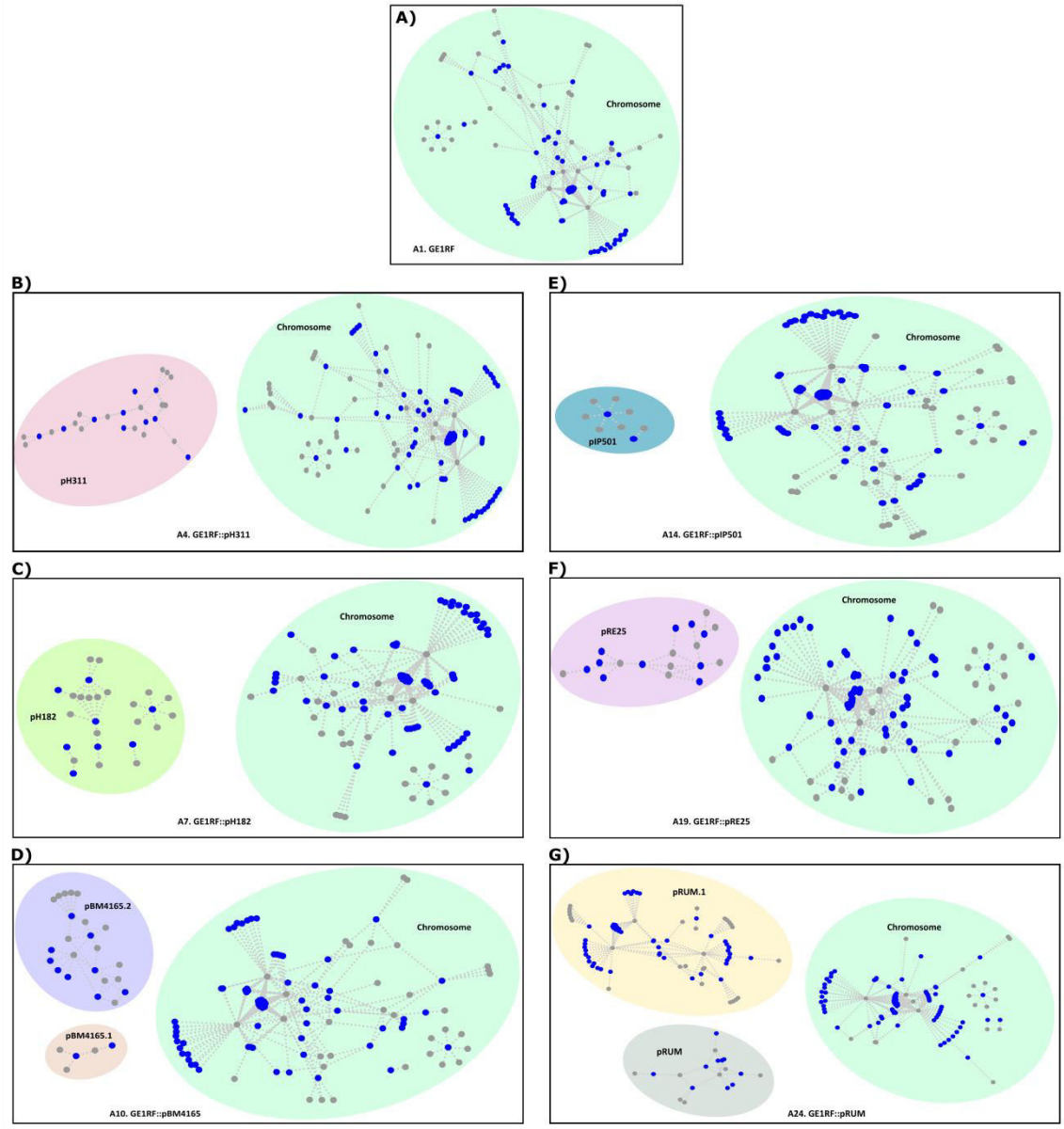


Figure 4. PLACNET analysis of non-evolved strains in *E. faecium* GE1RF.

A) PLACNET of GE1RF; B) PLACNET analysis of GE1RF::pH311; C) PLACNET analysis of GE1RF::pH182; D) PLACNET analysis of GE1RF::pBM4165; E) PLACNET analysis of GE1RF::pIP501; F) PLACNET analysis of GE1RF::pRE25; G) PLACNET analysis of GE1RF::pRUM.

Whole genome analysis of evolved strains.

PLACNET analysis of non-evolved strains helped to identify the possible plasmids present in these strains (Figures 4 and 5). All evolved strains sequenced showed point mutations, deletions, duplications and possibly recombination regions when they were compared with their corresponding parental strains. These alterations in the genomes were found both in the chromosome and plasmids. In general more mutations were detected in *E. faecium* 64/3 than in *E. faecium* GE1RF (Figures 6 and 7).

The most frequent functions affected in the chromosome of *E. faecium* GE1RF and *E. faecium* 64/3 were: DNA replication and repair (6 *E. faecium* GE1RF and 17 *E. faecium* 64/3), transposition and genome plasticity (7 *E. faecium* GE1RF and 7 *E. faecium* 64/3), environmental information processing (3

E. faecium GE1RF and 10 *E. faecium* 64/3), tissue adherence (5 *E. faecium* GE1RF and 5 *E. faecium* 64/3), transcription regulators (11 *E. faecium* 64/3), inorganic ion transport and metabolism (1 *E. faecium* GE1RF and 8 *E. faecium* 64/3), cellular and cell wall enzymes (2 *E. faecium* GE1RF and 3 *E. faecium* 64/3), and genetic information processing (1 *E. faecium* GE1RF and 3 *E. faecium* 64/3).

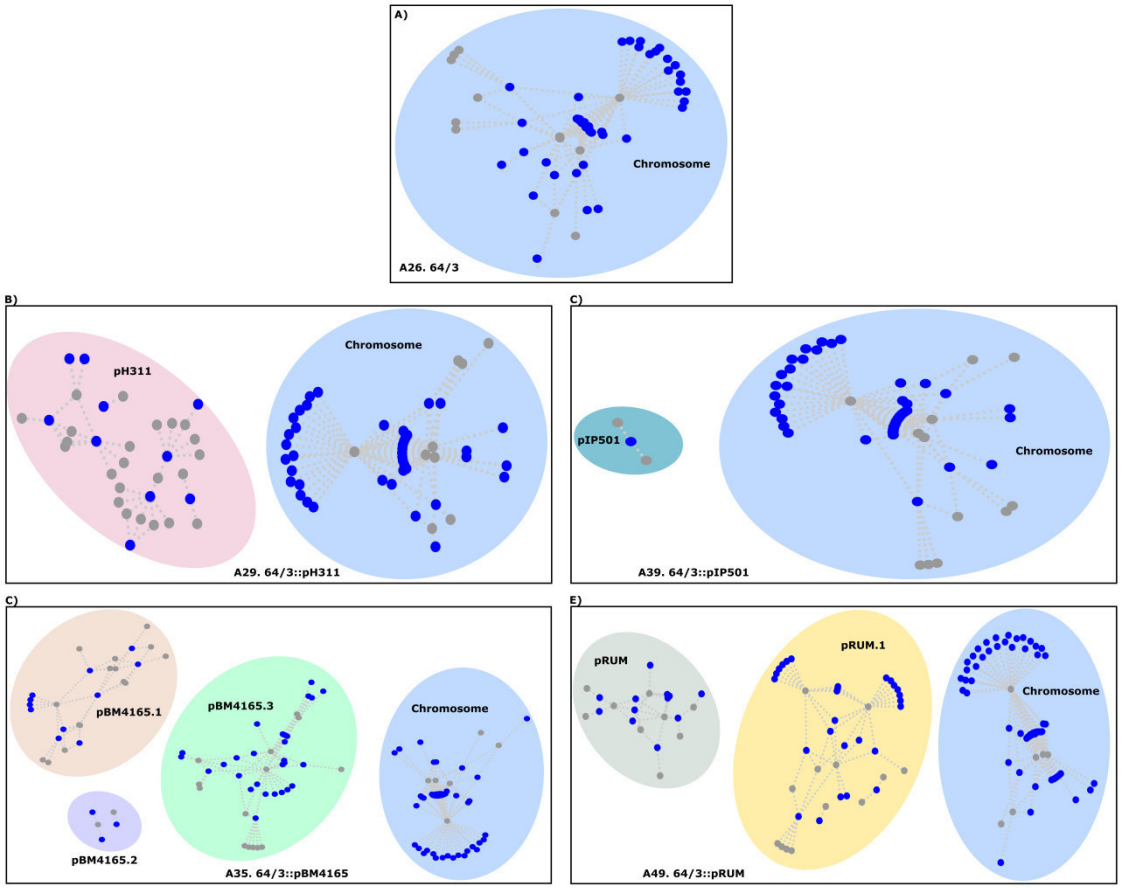
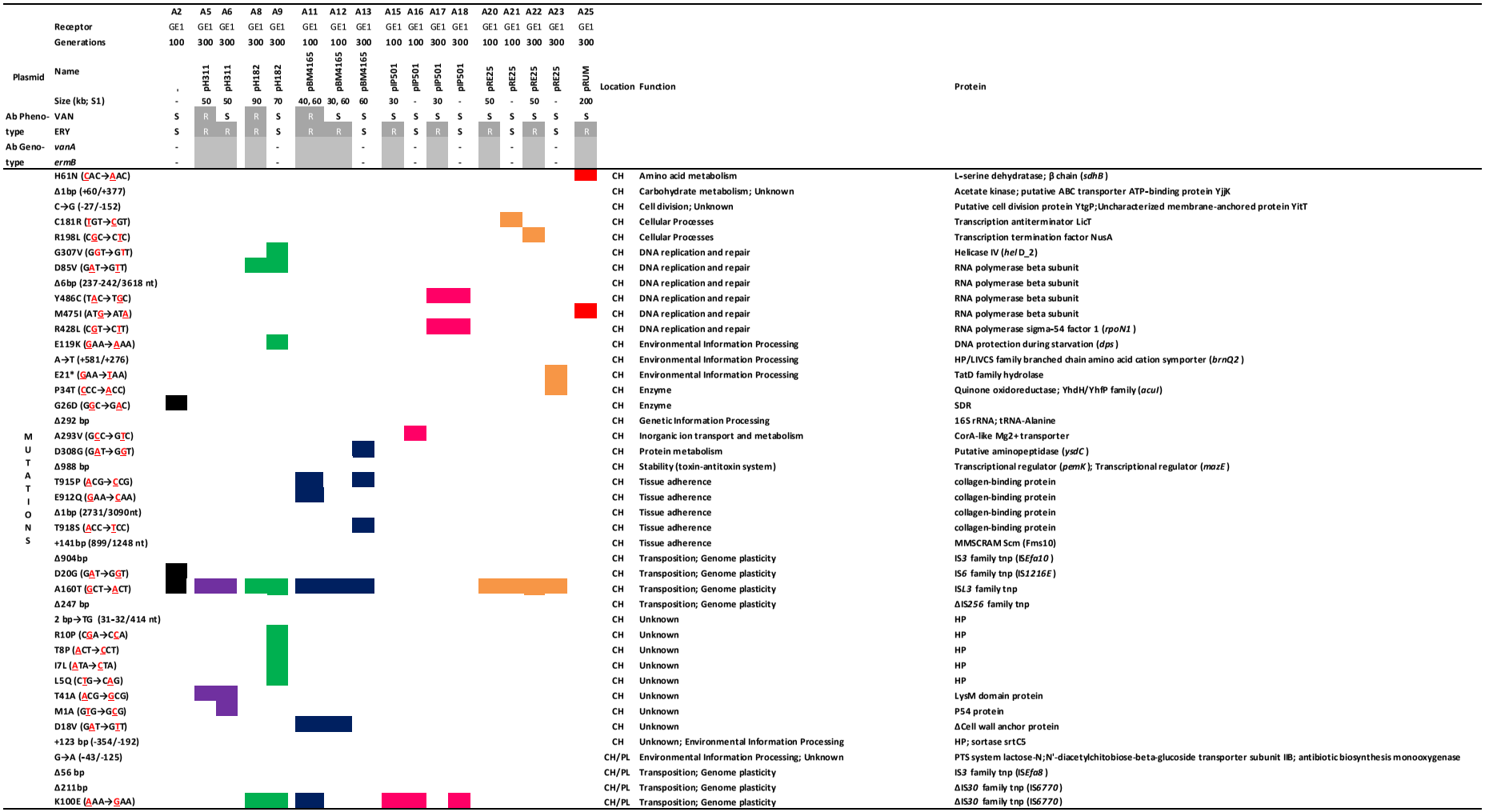


Figure 5. PLACNET analysis of non-evolved strains in *E. faecium* 64/3.

A) PLACNET of 64/3; B) PLACNET analysis of 64/3::pH311; C) PLACNET analysis of 64/3::pBM4165; D) PLACNET analysis of 64/3::pIP501; E) PLACNET analysis of 64/3::pRUM.

Plasmid changes included point mutations in REP of the Inc18 family (for plasmids pH311, pBM4165 and pIP501 in *E. faecium* GE1RF not in *E. faecium* 64/3); deletions of plasmid modules (REP, REL and TA systems) and antibiotic resistance determinants. The last ones were found in both *E. faecium* GE1RF and *E. faecium* 64/3 justifying loss of plasmid modules and phenotypical resistance to vancomycin and/or erythromycin.

In *E. faecium* GE1RF, an A160T mutation within an IS*L3*-like element, a 292bp deletion in the intergenic region encoding for 16S rRNA and tRNA, and a 211bp deletion within IS*6770* were observed for most strains sequenced. Aside from the deletion in IS*6770*, a point mutation (K100E) was also found in some strain pairs (Figure 6).



		A2	A5	A6	A8	A9	A11	A12	A13	A15	A16	A17	A18	A20	A21	A22	A23	A25			
	Receptor	GE1	GE1	GE1	GE1	GE1	GE1	GE1	GE1	GE1	GE1	GE1	GE1	GE1	GE1	GE1	GE1	GE1			
	Generations	100	300	300	300	300	100	100	300	100	100	300	300	100	100	300	300	300			
Plasmid	Name	-	pH311	pH311	pH182	pH182	pBM4165	pBM4165	pBM4165	pIP501	pIP501	pIP501	pIP501	pRE25	pRE25	pRE25	pRE25	pRUM			
	Size (kb; S1)	-	50	50	90	70	40, 60	30, 60	60	30	-	30	-	50	-	50	-	200			
Ab Pheno-	VAN	S	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S			
type	ERY	S	R	R	R	S	R	R	S	R	S	R	S	R	S	R	S	R			
Ab Geno-	*vanA*	-				-			-		-		-		-		-				
type	*ermB*	-				-			-		-		-		-		-				
	H61N (CAC→AAC)																		CH	Amino acid metabolism	L-serine dehydratase; β chain (*sdhB*)
	Δ1bp (+60/+377)																		CH	Carbohydrate metabolism; Unknown	Acetate kinase; putative ABC transporter ATP-binding protein YjjK
	C→G (-27/-152)																		CH	Cell division; Unknown	Putative cell division protein YtgP;Uncharacterized membrane-anchored protein YitT
	C181R (TGT→CGT)																		CH	Cellular Processes	Transcription antiterminator LicT
	R198L (CGC→CTC)																		CH	Cellular Processes	Transcription termination factor NusA
	G307V (GGT→GTT)																		CH	DNA replication and repair	Helicase IV (*helD_2*)
	D85V (GAT→GTT)																		CH	DNA replication and repair	RNA polymerase beta subunit
	Δ6bp (237-242/3618 nt)																		CH	DNA replication and repair	RNA polymerase beta subunit
	Y486C (TAC→TGC)																		CH	DNA replication and repair	RNA polymerase beta subunit
	M475I (ATG→ATA)																		CH	DNA replication and repair	RNA polymerase beta subunit
	R428L (CGT→CTT)																		CH	DNA replication and repair	RNA polymerase sigma-54 factor 1 (*rpoN1*)
	E119K (GAA→AAA)																		CH	Environmental Information Processing	DNA protection during starvation (*dps*)
	A→T (+581/+276)																		CH	Environmental Information Processing	HP/LIVCS family branched chain amino acid cation symporter (*brnQ2*)
	E21* (GAA→TAA)																		CH	Environmental Information Processing	TatD family hydrolase
	P34T (CCC→ACC)																		CH	Enzyme	Quinone oxidoreductase; YhdH/YhfP family (*acuI*)
	G26D (GGC→GAC)																		CH	Enzyme	SDR
	Δ292 bp																		CH	Genetic Information Processing	16S rRNA; tRNA-Alanine
MUTATIONS	A293V (GCC→GTC)																		CH	Inorganic ion transport and metabolism	CorA-like Mg2+transporter
	D308G (GAT→GGT)																		CH	Protein metabolism	Putative aminopeptidase (*ysdC*)
	Δ988 bp																		CH	Stability (toxin-antitoxin system)	Transcriptional regulator (*pemK*); Transcriptional regulator (*mazE*)
	T915P (ACG→CCG)																		CH	Tissue adherence	collagen-binding protein
	E912Q (GAA→CAA)																		CH	Tissue adherence	collagen-binding protein
	Δ1bp (2731/3090nt)																		CH	Tissue adherence	collagen-binding protein
	T918S (ACC→TCC)																		CH	Tissue adherence	collagen-binding protein
	+141bp (899/1248 nt)																		CH	Tissue adherence	MMSCRAM Scm (Fms10)
	Δ904bp																		CH	Transposition; Genome plasticity	IS*3* family tnp (IS*Efa10*)
	D20G (GAT→GGT)																		CH	Transposition; Genome plasticity	IS*6* family tnp (IS*1216E*)
	A160T (GCT→ACT)																		CH	Transposition; Genome plasticity	IS*L3* family tnp
	Δ247 bp																		CH	Transposition; Genome plasticity	Δ*IS256* family tnp
	2 bp→TG (31-32/414 nt)																		CH	Unknown	HP
	R10P (CGA→CCA)																		CH	Unknown	HP
	T8P (ACT→CCT)																		CH	Unknown	HP
	I7L (ATA→CTA)																		CH	Unknown	HP
	L5Q (CTG→CAG)																		CH	Unknown	HP
	T41A (ACG→GCG)																		CH	Unknown	LysM domain protein
	M1A (GTG→GCG)																		CH	Unknown	P54 protein
	D18V (GAT→GTT)																		CH	Unknown	ΔCell wall anchor protein
	+123 bp (-354/-192)																		CH	Unknown; Environmental Information Processing	HP; sortase srtC5
	G→A (-43/-125)																		CH/PL	Environmental Information Processing; Unknown	PTS system lactose-N;N'-diacetylchitobiose-beta-glucoside transporter subunit IIB; antibiotic biosynthesis monooxygenase
	Δ56 bp																		CH/PL	Transposition; Genome plasticity	IS*3* family tnp (IS*Efa8*)
	Δ211bp																		CH/PL	Transposition; Genome plasticity	Δ*IS30* family tnp (*IS6770*)
	K100E (AAA→GAA)																		CH/PL	Transposition; Genome plasticity	Δ*IS30* family tnp (*IS6770*)

Figure 6. Table of mutations in evolved strains in *E. faecium* GE1RF background.

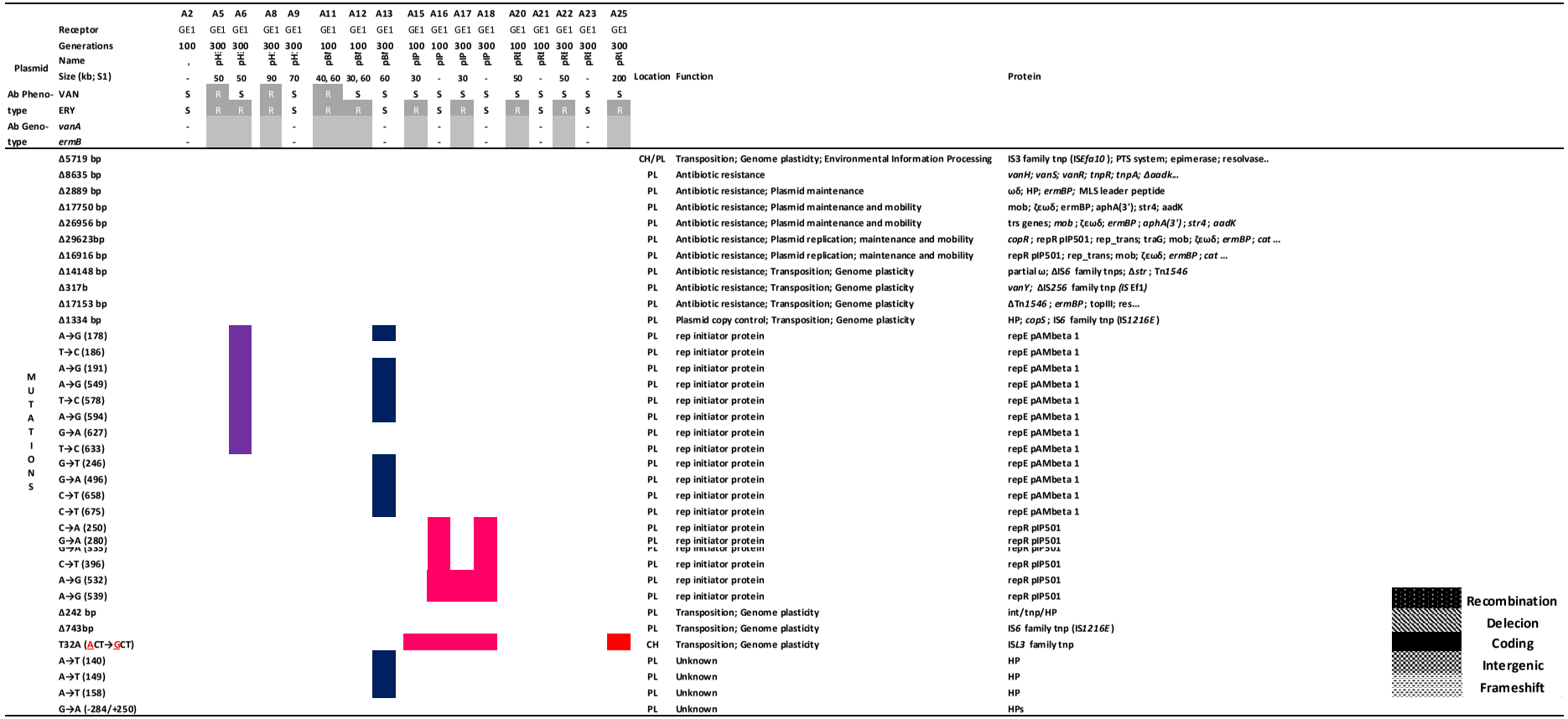


Figure 6. Table of mutations in evolved strains in *E. faecium* GE1RF background.

Each color represents a pair of evolved strains. Uniprot and KEGG databases were used to assess protein functions of mutate proteins (due to the size of the figure it will be provided only in digital format).

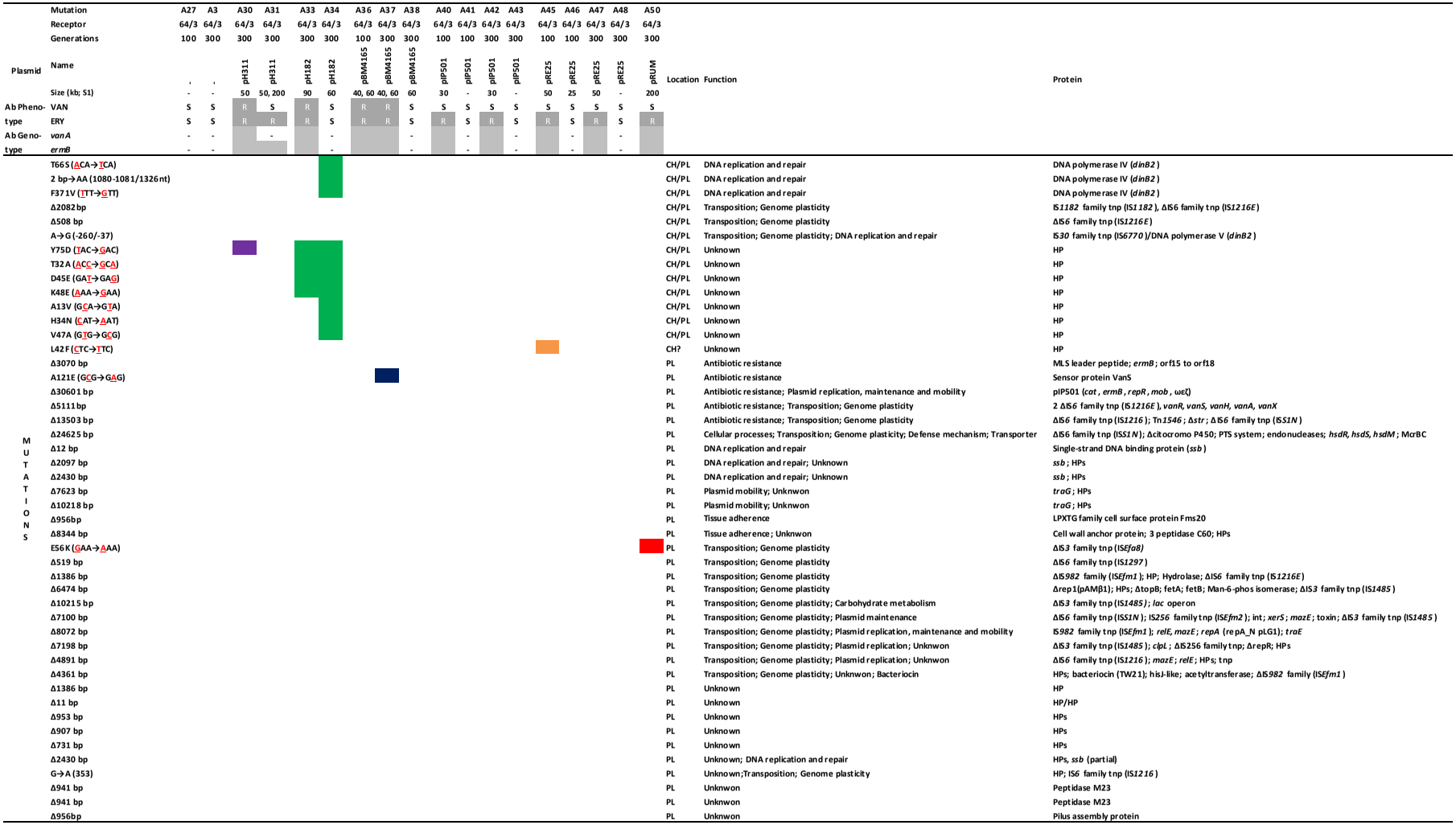
	Mutation	A27	A3	A30	A31	A33	A34	A36	A37	A38	A40	A41	A42	A43	A45	A46	A47	A48	A50	Location	Function	Protein
	Receptor	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3			
	Generations	100	300	300	300	300	300	100	300	300	100	100	300	300	100	100	300	300	300			
Plasmid	Name	-	-	pH311	pH311	pH182	pH182	pBM4165	pBM4165	pBM4165	pIP501	pIP501	pIP501	pIP501	pRE25	pRE25	pRE25	pRE25	pRUM			
	Size (kb; S1)	-	-	50	50, 200	90	60	40, 60	40, 60	60	30	-	30	-	50	25	50	-	200			
Ab Pheno-	VAN	S	S	R	S	R	S	R	R	S	S	S	S	S	S	S	S	S	S			
type	ERY	S	S	R	R	R	S	R	R	S	R	S	R	S	R	S	R	S	R			
Ab Geno-	*vanA*	-	-		-		-			-		-		-		-		-				
type	*ermB*	-	-				-			-		-		-		-		-				
MUTATIONS	A→T (-207/-254)																			CH	Amino acid metabolism	Arginine deiminase (*arcA*)/Crp family transcriptional regulator
	Q410P (CAA→CCA)																			CH	Carbohydrate metabolism	Sucrose-6-phosphate hydrolase (*scrB*)
	I85V (ATT→GTT)																			CH	Carbohydrate metabolism	Sucrose-6-phosphate hydrolase (*scrB*)
	T→C (-39/-122)																			CH	Carbohydrate metabolism; Transcriptional regulator	N-acylglucosamine-6-phosphate 2-epimerase (*nanE*)/phosphosugar-binding transcriptional (*rpiR*)
	T→C (-51/-110)																			CH	Carbohydrate metabolism; Transcriptional regulator	N-acylglucosamine-6-phosphate 2-epimerase (*nanE*)/phosphosugar-binding transcriptional (*rpiR*)
	A→G (-90/-71)																			CH	Carbohydrate metabolism; Transcriptional regulator	N-acylglucosamine-6-phosphate 2-epimerase (*nanE*)/phosphosugar-binding transcriptional (*rpiR*)
	C→T (+33/+99)																			CH	Carbohydrates metabolism; Biosynthesis of secondary metabolites	Aldose-1-epimerase (*galM4*)/glycerol-3-phosphate acyltransferase PlsY (*plsY*)
	Q18R (CAA→CGA)																			CH	Defence mechanism	Restriction endonudease subunit S
	E135G (GAA→GGA)																			CH	DNA replication and repair	DNA polymerase IV (*dinB1*)
	S30N (AGT→AAT)																			CH	DNA replication and repair	DNA polymerase IV (*dinB2*)
	R29N (AGG→AAT)																			CH	DNA replication and repair	DNA polymerase IV (*dinB2*)
	L334S (TTG→TCG)																			CH	DNA replication and repair	DNA-directed RNA polymerase sigma subunit SigL
	Q396* (CAA→TAA)																			CH	DNA replication and repair	RNA polymerase sigma-54 factor 1 (*rpoN1*)
	M1K (ATG→AAG)																			CH	DNA replication and repair	RNA polymerase sigma-54 factor 1 (*rpoN1*)
	D405E (GAC→GAG)																			CH	DNA replication and repair	RNA polymerase sigma-54 factor 1 (*rpoN1*)
	Q12* (CAG→TAG)																			CH	DNA replication and repair	RNA polymerase sigma-54 factor 1 (*rpoN1*)
	T53K (ACG→AAG)																			CH	Environmental Information Processing	Mannose permease IIC component (PTS system; *manY*)
	W140* (TGG→TGA)																			CH	Environmental Information Processing	Mannose permease PTS family mannose/fructose/sorbose porter component IIC (*manY*)
	9 bp x2																			CH	Environmental Information Processing	Mannose permease PTS family mannose/fructose/sorbose porter component IIC (*manY*)
	+A (-293/-413)																			CH	Environmental Information Processing	Metal-dependent phosphohydrolase (*ybeZ*)/phosphate starvation-inducible protein PhoH
	G8S (GGT→AGT)																			CH	Environmental Information Processing	Oligopeptide ABC superfamily ATP binding cassette transporter (*dppE*)
	Δ5bp (153-157/915 nt)																			CH	Environmental Information Processing	PTS family mannose/fructose/sorbose porter component IID (*maz1*)
	K99R (AAA→AGA)																			CH	Environmental Information Processing	PTS system beta-glucoside-specific EIIBCA component (*bglF*)
	*95L (TAG→TTG)																			CH	Environmental Information Processing	PTS system fructose IIA component
	Δ15bp																			CH	Environmental Information Processing	PTS system trehalose-specific EIIBC component (*treB*)
	Q484P (CAA→CCA)																			CH	Environmental Information Processing	PTS system trehalose-specific EIIBC component (*treB*)
	2 bp→AG (860-861/1020 nt)																			CH	Cell Wall Enzymes	N-acetylmuramoyl-L-alanine amidase
	A→G (214)																			CH	Cell Wall Enzymes	N-acetylmuramoyl-L-alanine amidase (partial)
	G→A (-477/+699)																			CH	Enzymes	SGNH subfamily hydrolase/asparaginyl-tRNA synthase
	Δ292bp																			CH	Genetic Information Processing	16S rRNA; tRNA-Alanine
	G→A (-1/+16)																			CH	Genetic Information Processing	tRNA-Asp/tRNA-Met
	A→G (195/1558nt)																			CH	Genetic Information Processing	Δ16S ribosomal RNA
	E289* (GAA→TAA)																			CH	Inorganic ion transport and metabolism	CorA-like Mg2+ transporter protein (*corA2*)
	L312* (TTA→TAA)																			CH	Inorganic ion transport and metabolism	CorA-like Mg2+ transporter protein (*corA2*)
	Q183K (CAA→AAA)																			CH	Inorganic ion transport and metabolism	CorA-like Mg2+ transporter protein (*corA2*)
	D181Y (GAT→TAT)																			CH	Inorganic ion transport and metabolism	CorA-like Mg2+ transporter protein (*corA2*)
	A123E (GCA→GAA)																			CH	Inorganic ion transport and metabolism	CorA-like Mg2+ transporter protein (*corA2*)
	Δ1bp (281/2835 nt)																			CH	Inorganic ion transport and metabolism	CorA-like Mg2+ transporter protein (*corA2*)
	Q12* (CAG→TAG)																			CH	Inorganic ion transport and metabolism	CorA-like Mg2+ transporter protein (*corA2*)
	S185R (AGC→AGA)																			CH	Inorganic ion transport and metabolism	CorA-like Mg2+ transporter protein (*corA2*)
	E334* (GAA→TAA)																			CH	Lipid metabolism	Alpha-monoglucosyl-diacylglycerol synthase (*mgs*)
	R219W (CGG→TGG)																			CH	Lipid metabolism	Lipoteichoic acid synthase LtaS Type IIc (*ltaS1*)
	2bp→CG (2494-5/3402 nt)																			CH	Tissue adherence	Collagen-binding protein
	2 bp→CG (2594-5/3123nt)																			CH	Tissue adherence	Collagen-binding protein
	I865T (ATA→ACA)																			CH	Tissue adherence	Collagen-binding protein
	Δ374bp																			CH	Tissue adherence	LPXTG-motif cell wall anchor domain protein

Figure 7. Table of mutations in evolved strains in *E. faecium* 64/3 background.

	Mutation	A27	A3	A30	A31	A33	A34	A36	A37	A38	A40	A41	A42	A43	A45	A46	A47	A48	A50			
	Receptor	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3			
	Generations	100	300	300	300	300	300	100	300	300	100	100	300	300	100	100	300	300	300			
Plasmid	Name	-	-	pH311	pH311	pH182	pH182	pBM4165	pBM4165	pBM4165	pIP501	pIP501	pIP501	pIP501	pRE25	pRE25	pRE25	pRE25	pRUM			
	Size (kb; S1)	-	-	50	50, 200	90	60	40, 60	40, 60	60	30	-	30	-	50	25	50	-	200			
Ab Pheno-type	VAN	S	S	R	S	R	S	R	R	S	S	S	S	S	S	S	S	S	S			
	ERY	S	S	R	R	R	S	R	R	S	R	S	R	S	R	S	R	S	R			
Ab Geno-type	*vanA*	-	-		-		-			-		-		-		-		-				
	ermB	-	-				-			-		-		-		-		-				
	Δ39bp																			CH	Tissue adherence	Pilus subunit protein EbpCfm (*fms9*)
	Δ374 bp																			CH	Tissue adherence	Surface protein; Collagen-binding protein
	Y51H (TAC→CAC)																			CH	Transcription regulator	ArpU family phage transcriptional regulator
	Δ404bp																			CH	Transcription regulator	ArpU family phage transcriptional regulator
	A→G (263)																			CH	Transcription regulator	ArpU family phage transcriptional regulator
	A→C (294)																			CH	Transcription regulator	ArpU family phage transcriptional regulator
	A→G (189)																			CH	Transcription regulator	ArpU family phage transcriptional regulator
	34bp x 2																			CH	Transcription regulator	DeoR family transcriptional regulator (*srlR*)
	+A (149/1026nt)																			CH	Transcription regulator	HTH-type transcriptional repressor (*purR*)
	A513T (GCT→ACT)																			CH	Transcription regulator	Transcriptor regulator LevR family
	E20K (GAA→AAA)																			CH	Transcription regulator	Transcriptor regulator ompR family
	Δ1bp (177/714 nt)																			CH	Transcription regulator	Trehalose operon transcrip repressor (*treR*)
	+T (178/714nt)																			CH	Transcription regulator	Trehalose operon transcrip repressor (*treR*)
	C→T (+124/-118)																			CH	Transcription regulator; DNA repair	phosphosugar-binding transcriptional (*rpiR*)/resolvase family site-specific recombinase
	C→T (-14/-83)																			CH	Transcription regulator; Tansporter	Trehalose operon transcrip repressor (*treR*)/PTS family trehalose porter component IIABC (*treB*)
	Δ3bp (493-495/1785NT)																			CH	Transporters	ABC-type multidrug transport system, ATPase and permease component
	A358T (GCT→ACT)																			CH	Transporters	Putative ABC transporter ATP-binding protein YheS (*yheS4*)
	I194T (ATA→ACA)																			CH	Transposition; Genome plasticity	IS*1182* family tnp (IS*Efa12*)
	C→A (-197/-157)																			CH	Transposition; Genome plasticity	IS*200*-like family tnp/IS*3* family tnp (IS*Efa10*)
	A→G (-245/-109)																			CH	Transposition; Genome plasticity	IS*200*-like family tnp/IS*3* family tnp (IS*Efa10*)
	T→C (-299/-55)																			CH	Transposition; Genome plasticity	IS*200*-like family tnp/IS*3* family tnp (IS*Efa10*)
	A→G (-329/-55)																			CH	Transposition; Genome plasticity	IS*200*-like family tnp/IS*3* family tnp (IS*Efa10*)
	T→C (-343/-11)																			CH	Transposition; Genome plasticity	IS*200*-like family tnp/IS*3* family tnp (IS*Efa10*)
	A→C (349)																			CH	Transposition; Genome plasticity	IS*3* family tnp (IS*Efa10*)
	H64R (CAT→CGT)																			CH	Transposition; Genome plasticity	IS*3* family tnp (IS*Efa10*)
	K103E (AAA→GAA)																			CH	Transposition; Genome plasticity	IS*3* family tnp (IS*Efa10*)
	M89T (ATG→ACG)																			CH	Transposition; Genome plasticity	IS*30* family tnp (IS*6770*)
	A104G (GCT→GGT)																			CH	Transposition; Genome plasticity	IS*30* family tnp (IS*6770*)
	S92A (TCC→GCC)																			CH	Transposition; Genome plasticity	IS*6* family tnp (IS*1216V*)
	T→G (-19/+129)																			CH	Transposition; Genome plasticity; Unknown	IS*982* family tnp (IS*Efm1*)/HP
	K212Q (AAG→CAG)																			CH	Unknown	HP
	I146T (ATA→ACA)																			CH	Unknown	HP
	Δ1bp (992/1218 nt)																			CH	Unknown	HP
	G338V (GGT→GTT)																			CH	Unknown	HP
	C→A (-34/+99)																			CH	Unknown	HP/Organic hydroperoxide resistance protein (*ohrB*)
	15 bp x2																			CH	Unknown	HP/Organic hydroperoxide resistance protein (*ohrB*)
	T99A (ACT→GCT)																			CH	Unknown	LysM domain protein
	G→A (-127/-85)																			CH	Unknown	TerC family integral membrane protein/signal peptidase I (*spsB*)
	A375 (GCC→TCC)																			CH	Unknown	Uncharacterized membrane protein YfcA
	A→G (-381/+174)																			CH	Unknown; Defense mechanism	HP/restriction endonuclease subunit S
	T→C (-415/+140)																			CH	Unknown; Defense mechanism	HP/restriction endonuclease subunit S
	S44A (TCT→GCG)																			CH/PL	DNA replication and repair	DNA polymerase IV (*dinB2*)
	2 bp→CT (173-174/1326nt)																			CH/PL	DNA replication and repair	DNA polymerase IV (*dinB2*)
	E390D (GAA→GAC)																			CH/PL	DNA replication and repair	DNA polymerase IV (*dinB2*)
	2 bp→AC (1176-1177/1326nt)																			CH/PL	DNA replication and repair	DNA polymerase IV (*dinB2*)
	3 bp→AAA (184-186/1326nt)																			CH/PL	DNA replication and repair	DNA polymerase IV (*dinB2*)
	E58A (GAA→GCA)																			CH/PL	DNA replication and repair	DNA polymerase IV (*dinB2*)

MUTATIONS

Figure 7. Table of mutations in evolved strains in *E. faecium* 64/3 background.



	Mutation	A27	A3	A30	A31	A33	A34	A36	A37	A38	A40	A41	A42	A43	A45	A46	A47	A48	A50			
	Receptor	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3	64/3			
	Generations	100	300	300	300	300	300	100	300	300	100	100	300	300	100	100	300	300	300			
Plasmid	Name	-	-	pIH311	pIH311	pIH182	pIH182	pBM4165	pBM4165	pBM4165	pIP501	pIP501	pIP501	pIP501	pRE25	pRE25	pRE25	pRE25	pRUM	Location	Function	Protein
	Size (kb; S1)	-	-	50	50, 200	90	60	40, 60	40, 60	60	30	-	30	-	50	25	50	-	200			
Ab Pheno-type	VAN	S	S	R	S	R	S	R	R	S	S	S	S	S	S	S	S	S	S			
	ERY	S	S	R	R	R	S	R	R	S	R	S	R	S	R	S	R	S	R			
Ab Geno-type	*vanA*	-	-		-		-			-		-		-		-		-				
	ermB	-	-				-			-		-		-		-		-				
MUTATIONS	T66S (ACA→TCA)																			CH/PL	DNA replication and repair	DNA polymerase IV (*dinB2*)
	2 bp→AA (1080-1081/1326 nt)																			CH/PL	DNA replication and repair	DNA polymerase IV (*dinB2*)
	F371V (TTT→GTT)																			CH/PL	DNA replication and repair	DNA polymerase IV (*dinB2*)
	Δ2082 bp																			CH/PL	Transposition; Genome plasticity	IS*1182* family tnp (IS*1182*), ΔIS6 family tnp (IS*1216E*)
	Δ508 bp																			CH/PL	Transposition; Genome plasticity	Δ*IS6* family tnp (IS*1216E*)
	A→G (-260/-37)																			CH/PL	Transposition; Genome plasticity; DNA replication and repair	IS*30* family tnp (IS*6770*)/DNA polymerase V (*dinB2*)
	Y75D (TAC→GAC)																			CH/PL	Unknown	HP
	T32A (ACC→GCA)																			CH/PL	Unknown	HP
	D45E (GAT→GAG)																			CH/PL	Unknown	HP
	K48E (AAA→GAA)																			CH/PL	Unknown	HP
	A13V (GCA→GTA)																			CH/PL	Unknown	HP
	H34N (CAT→AAT)																			CH/PL	Unknown	HP
	V47A (GTG→GCG)																			CH/PL	Unknown	HP
	L42F (CTC→TTC)																			CH?	Unknown	HP
	Δ3070 bp																			PL	Antibiotic resistance	MLS leader peptide; *ermB*; orf15 to orf18
	A121E (GCG→GAG)																			PL	Antibiotic resistance	Sensor protein VanS
	Δ30601 bp																			PL	Antibiotic resistance; Plasmid replication, maintenance and mobility	pIP501 (*cat*, *ermB*, *repR*, *mob*, ωε)
	Δ5111 bp																			PL	Antibiotic resistance; Transposition; Genome plasticity	2 Δ*IS6* family tnp (IS*1216E*), *vanR, vanS, vanH, vanA, vanX*
	Δ13503 bp																			PL	Antibiotic resistance; Transposition; Genome plasticity	Δ*IS6* family tnp (IS*1216*); Tn*1546*; Δ*str*; Δ*IS6* family tnp (IS*S1N*)
	Δ24625 bp																			PL	Cellular processes; Transposition; Genome plasticity; Defense mechanism; Transporter	Δ*IS6* family tnp (IS*S1N*); Δcitocromo P450; PTS system; endonucleases; *hsdR, hsdS, hsdM*; McrBC
	Δ12 bp																			PL	DNA replication and repair	Single-strand DNA binding protein (*ssb*)
	Δ2097 bp																			PL	DNA replication and repair; Unknown	*ssb*; HPs
	Δ2430 bp																			PL	DNA replication and repair; Unknown	*ssb*; HPs
	Δ7623 bp																			PL	Plasmid mobility; Unknwon	*traG*; HPs
	Δ10218 bp																			PL	Plasmid mobility; Unknwon	*traG*; HPs
	Δ956bp																			PL	Tissue adherence	LPXTG family cell surface protein Fms20
	Δ8344 bp																			PL	Tissue adherence; Unknwon	Cell wall anchor protein; 3 peptidase C60; HPs
	E56K (GAA→AAA)																			PL	Transposition; Genome plasticity	Δ*IS3* family tnp (IS*Efa8*)
	Δ519 bp																			PL	Transposition; Genome plasticity	Δ*IS6* family tnp (IS*1297*)
	Δ1386 bp																			PL	Transposition; Genome plasticity	Δ*IS982* family (IS*Efm1*); HP; Hydrolase; Δ*IS6* family tnp (IS*1216E*)
	Δ6474 bp																			PL	Transposition; Genome plasticity	Δrep1(pAMβ1); HPs; ΔtopB; fetA; fetB; Man-6-phos isomerase; Δ*IS3* family tnp (IS*1485*)
	Δ10215 bp																			PL	Transposition; Genome plasticity; Carbohydrate metabolism	Δ*IS3* family tnp (IS*1485*); *lac* operon
	Δ7100 bp																			PL	Transposition; Genome plasticity; Plasmid maintenance	Δ*IS6* family tnp (IS*S1N*); IS*256* family tnp (IS*Efm2*); int; *xerS*; *mazE*; toxin; Δ*IS3* family tnp (IS*1485*)
	Δ8072 bp																			PL	Transposition; Genome plasticity; Plasmid replication, maintenance and mobility	IS*982* family tnp (IS*Efm1*); *relE, mazE*; *repA* (repA_N pLG1); *traE*
	Δ7198 bp																			PL	Transposition; Genome plasticity; Plasmid replication; Unknwon	Δ*IS3* family tnp (IS*1485*); *clpL*; ΔIS256 family tnp; ΔrepR; HPs
	Δ4891 bp																			PL	Transposition; Genome plasticity; Plasmid replication; Unknwon	Δ*IS6* family tnp (IS*1216*); *mazE*; *relE*; HPs; tnp
	Δ4361 bp																			PL	Transposition; Genome plasticity; Unknwon; Bacteriocin	HPs; bacteriocin (TW21); hisJ-like; acetyltransferase; Δ*IS982* family (IS*Efm1*)
	Δ1386 bp																			PL	Unknown	HP
	Δ11 bp																			PL	Unknown	HP/HP
	Δ953 bp																			PL	Unknown	HPs
	Δ907 bp																			PL	Unknown	HPs
	Δ731 bp																			PL	Unknown	HPs
	Δ2430 bp																			PL	Unknown; DNA replication and repair	HPs, *ssb* (partial)
	G→A (353)																			PL	Unknown;Transposition; Genome plasticity	HP; IS6 family tnp (IS*1216*)
	Δ941 bp																			PL	Unknwon	Peptidase M23
	Δ941 bp																			PL	Unknwon	Peptidase M23
	Δ956bp																			PL	Unknwon	Pilus assembly protein

Figure 7. Table of mutations in evolved strains in *E. faecium* 64/3 background.

Each color represents a pair of evolved strains. Uniprot and KEGG databases were used to assess protein functions of mutate proteins (due to the size of the figure it will be provided only in digital format).

Among *E. faecium* 64/3, a frameshift mutation in N-acetylmuramoyl-L-alanine amidase (involved in peptidoglycan metabolism) and a point mutation in ArpU family transcriptional regulator were detected in some strains pairs (Figure 7). For some strain pairs, specific genome changes were found in evolved strains of *E. faecium* GE1RF and *E. faecium* 64/3 strains.

For pairs carrying pH182 plasmid in *E. faecium* GE1RF (A8-A9), we detected a mutation in the beta subunit of RNA polymerase (D85V) and in *E. faecium* 64/3 (A33-A34), a 34bp duplication in a transcription regulator of the DeoR family; two point mutations (S44A and E399D) in the DNA polymerase IV (*dinB2*); and five point mutations in several hypothetical proteins.

For the pair carrying plasmid pBM4165 in *E. faecium* 64/3 (A36-A38), the following changes were observed: a mutation (*95L) in the stop codon of a fructose PTS system that could have restored protein function, a mutation in the intergenic region of tRNA-Asp/tRNA-Met, a 374bp deletion in a protein with LPXTG-motif possibly associated with tissue adherence as well as several deletions of plasmid modules were found in this pair of evolved strains.

For strains carrying pIP501 in *E. faecium* GE1RF (A15-A18), a T32A mutation in an IS*30* family transposase was found. In the 300 generations evolved strains (A17-A18) two point mutations in DNA replication and repair associated proteins, RNA polymerase β-subunit (Y486C) and *rpoN1* (R428L) were also found. Several mutations on the *repR* gene of pIP501 were found in the evolved erythromycin susceptible strains (A16 and A18). In case of strains carrying pIP501 in *E. faecium* 64/3 (A40-A43), a 2bp frameshift mutation in a collagen binding protein (cell wall adherence) and an intergenic mutation between TerC family integral membrane protein and signal peptidase I (*spsB*) were found. A point mutation (D181Y) in a CorA-like Mg^{2+} transporter protein (inorganic ion transport and metabolism) was found strains susceptible to erythromycin (A41 and A43).

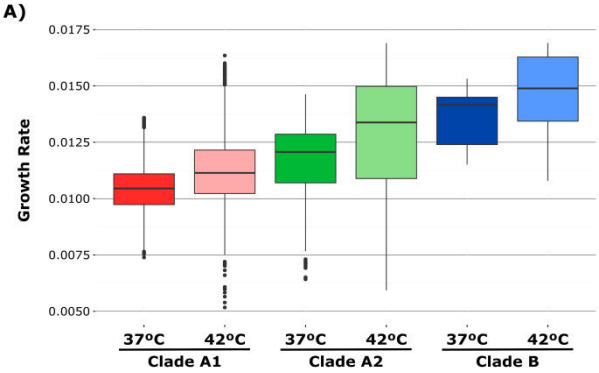
In *E. faecium* GE1RF strains carrying pRE25 (A20-A23), an intergenic mutation between a hypothetical protein and a LIVCS family branched chain amino acid cation symporter (*brnQ2*) was found. Several deletions were also found in the erythromycin susceptible strains (A21 and A23). For strains carrying pRE25 in *E. faecium* 64/3 (A45-A48) several point mutations were found: in an oligopeptide ABC superfamily ATP binding cassette transporter (*dppE*; G8S); in a putative ABC transporter ATP-binding protein YheS (*yheS4*; A358T); and in an IS*1216V* belonging to the IS*6* family (S92A). Also a 1bp deletion was found in a CorA-like Mg^{2+} transporter protein in erythromycin susceptible strains (A46 and A48).

E. faecium PFGE-estimation of genome sizes.

Genome sizes were highly variable, ranging from 2.05Mb [1st vancomycin resistant *E. faecium* (VREfm), ST25 (BAPS 2.3a)] and 3.08Mb, [1st linezolid resistant *E. faecium* isolated ST203 (BAPS 2.1a)] with an average genome size of 2.51±0.28Mb (Supplementary Table S1). Animal associated Clade A2 hade the smaller average genome (2.32±0.23Mb) and hospital associated Clade A1 had the largest genomes (2.66±0.23Mb) (Figure 8). No significant differences in genome size were observed between VSEfm and

VREfm strains (2.50±0.25Mb and 2.52±0.33Mb, respectively) (Figure 9). However, significant (p<0.0001) differences in average genome size were found when analysing VREfm and VSEfm for ampicillin resistance with ASEfm having a smaller genome size compared to AREfm (Figure 9).

A)

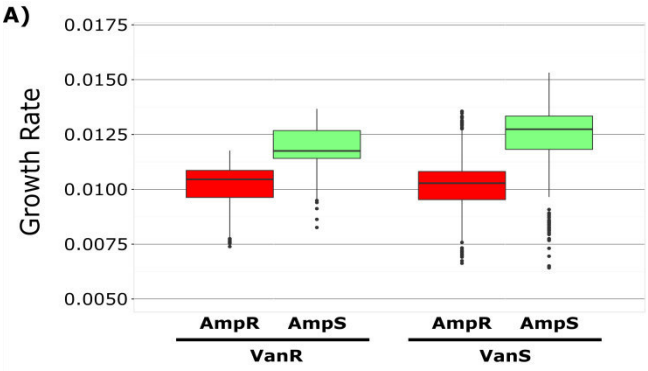


B)

		GR T=37ºC	SD (±)	GR T=42ºC	SD (±)	Genome size (Mbp)	SD (±)
Clade B	BAPS 1	0.01362	0.00116	0.01538	0.00295	2.55	0.157
Clade A1	BAPS 2.1a	0.01034	0.00152	0.01133	0.00196	2.70	0.232
	BAPS 3.3a1	0.01132	0.00121	0.01294	0.00182	2.62	0.244
	BAPS 3.3a2	0.00976	0.00099	0.01001	0.00146	2.64	0.173
	Total	**0.01049**	**0.00142**	**0.01142**	**0.00212**	**2.66**	**0.226**
Clade A2	BAPS 2.1b	0.01192	0.00195	0.01274	0.00307	2.29	0.163
	BAPS 2.3a	0.01212	0.00116	0.01437	0.00143	2.16	0.094
	BAPS 2.3b	0.01100	0.00030	0.01181	0.00032	2.38	0.000
	BAPS 3.1	0.01100	0.00179	0.01239	0.00252	ND	ND
	BAPS 3.2	0.01157	0.00125	0.01258	0.00125	2.41	0.285
	BAPS 3.3b	0.01102	0.00145	0.01101	0.00152	2.42	0.107
	BAPS 7	0.01073	0.00225	0.01070	0.00387	2.40	0.296
	Total	**0.01156**	**0.00171**	**0.01261**	**0.00295**	**2.32**	**0.231**

Figure 8. Growth Rate analysis of *E. faecium* clades at 37ºC and 42ºC. A) Box and whiskers plot of the different *E. faecium* clades (A1, A2 and B) at 37ºC and 42ºC. B) Summary table of Growth Rates and Genome size by clade and BAPS group.

A)



B)

		GR T=37ºC	SD (±)	GR T=42ºC	SD (±)	Genome size (Mbp)	SD (±)
VanS	AmpS	0.01241	0.00162	0.01241	0.00162	2.40	0.214
	AmpR	0.01025	0.00145	0.01025	0.00145	2.63	0.221
VanR	AmpS	0.01180	0.00107	0.01203	0.00064	2.28	0.314
	AmpR	0.01010	0.00110	0.01019	0.00099	2.64	0.260

Figure 9. Growth Rates of *E. faecium* wild type strains according with vancomycin and ampicillin resistance. A) Box and whiskers plot representing growth rates of E. faecium wild type strains according with vancomycin and ampicillin resistance; B) Summary table of growth rates and Genome size by vancomycin and ampicillin resistance.

Fitness of *E. faecium* representative wild-type clones at 37ºC and 42ºC.

E. faecium presented a significantly higher (p<0.01) average growth rate at 42ºC (0.01236±0.00284) than at 37ºC (0.01127±0.00178). A significant difference (p<0.0001) in growth rates was observed between isolates of the three *E. faecium* genomic clades, with Clade B having the best average growth rate at 37ºC and 42 ºC (Figure 8). BAPS 1 (equivalent to Clade B) had the significantly higher (p<0.0001) average growth rate compared to all other BAPS groups. At 37ºC average growth rate within Clade A2 varied greatly, even though, statistically significant differences were only observed between some BAPS groups (BAPS 3.1: BAPS 2.1b and BAPS2.3a; BAPS 7: BAPS 2.1b and BAPS 2.3a; BAPS3.3b – BAPS2.3a). Within

Clade A1 the differences in average GR between the three BAPS groups were statistically significant ($p<0.0001$) (Figure 8). VSEfm strains were found to have a significantly higher ($p<0.0001$) GR than VREfm strains. Moreover, the analysis of ampicillin resistance within VSEfm and VREfm showed that AREfm have a significant ($p<0.0001$) lower GR than those ASEfm (Figure 9).

DISCUSSION

The plasmid modules present in *E. faecium* Tn*1546-vanA* carrying plasmids seem to influence plasmid host range, as has also been demonstrated in previous works where *E. faecium* plasmids transfer into *E. faecalis* only when modules of broad host range Inc18 (rep_1) family plasmids are present [30].

All the plasmids analysed in this work conferred variable fitness cost depending on the bacterial recipient *E. faecium* and *E. faecalis* hosts. The results seem to demonstrate that some *E. faecium* clades or lineages might be more adapted to the presence of mobile genetic elements than others and, probably, that some plasmids are also better adapted to some clades or species than others [44].

Induction of the expression of Tn*1546-vanA* showed a high fitness cost, variable in depending on the Tn*1546* variant, and the bacterial host in which it was being expressed, in line with what was demonstrated for vancomycin resistant *S. aureus* strains. In *S. aureus* the fitness cost was attributed to the metabolic changes that the complex vancomycin resistance mechanism induces in the cell, leading to the elimination of chromosomal encoded peptidoglycan precursors (D-Ala-D-Ala) which are replaced by Tn*1546* encoded precursors (D-Ala-D-Lac) [6,17]. The differences in fitness observed between different Tn*1546* variants might be due to the efficiency of expression of enzymes encoded in Tn*1546*, as variant A (the first described) presented a lower fitness cost compared with the others variants studied, suggesting that the presence of ISs and other genetic material within Tn*1546* might hamper its' function.

Interestingly, and in spite of their fitness cost, most Tn*1546-vanA* carrying plasmids were stably maintained after 300 generations in the absence of selective pressure. Some of these plasmids carry known, highly effective, TA systems, which might account for their stability even when the vancomycin resistance genotype/phenotype was lost. Reversion of vancomycin resistant phenotype was seldom observed similarly to what has been described previously [45]. However, in the case of plasmid pBM4165 phenotype reversion was frequent and mostly due to the deletion of the part of Tn*1546*. It would be interesting to further study these strains in order to understand this phenomenon as it might carry some possible ideas for new therapeutic innovations in the treatment of VRE.

Even though, Inc18 plasmid family prototype, pIP501, had a low fitness cost for the host, it was easily lost in the 300 generations sequential evolution experiment. We may hypothesise that either TA system present in our copy of this plasmid was non-functional or that at the beginning of the experiment there was a mixed population, relatively frequent in Enterococci, of host cells (Host::pIP501 and Host::ΔpIP501) and that the difference in fitness between the populations was enough for the host cells without pIP501 to outgrow those with pIP501 [46].

The majority of the changes observed in evolved strains were in the chromosome. This observation is in consonance with other studies that show compensatory mutations associated with host-plasmid adaptation are located in the host's chromosome [46,47]. The changes observed in the plasmids were mostly related with deletion of plasmid modules although some mutations were found, as in the case of pIP501, that might be related with plasmid replication within the cell. The higher number of mutations in Tn*1546-vanA* carrying plasmids host compared to the plasmid-free evolved host suggests that the majority of them might be related with the presence of the plasmid.

The majority of mutations found in evolved strains were related with DNA replication and repair mechanisms indicating that increased mutation rates might be relevant for a rapid host-plasmid adaptation [48,49]. Alterations in the global transcription regulation (leading to higher transcription rates in non-evolved strains and back to normal levels in evolved strains) have also been described for streptomycin resistant mutants of *Salmonella Typhimurium* and in a *Pseudomonas aeruginosa* carrying a mercury resistance plasmid as a consequence of resistance adaptation [45,49,50]. Changes found in carbohydrate and amino acid metabolism, several PST systems and inorganic ion transport systems might be related with the metabolic burden imposed by the plasmid leading to o higher nutrient uptake and processing [49]. Curiously, even though the same functions were affected in *E. faecium* GE1RF and *E. faecium* 64/3 the mutations for the strains of the different background carrying the same plasmids here different indicating that even when confronted with the same plasmid, the hosts might have alternative evolutionary pathways [45]. Further research is needed in order to understand the role these mutations might play in host-plasmid adaptation.

The differences in genome size observed between clades A1, A2 and B were consistent with previous works describing that the clade A1 had a larger genome followed by clade B and clade A2 [51,52]. There are not clear relations between small genome size and higher replication rate [53]. Our observations were consistent with the reduction in fitness due to the presence of a higher number of acquired antibiotic resistance and virulence genes in clade A1. These differences might also account, up to some extent, for the differences found in fitness between clades, with more susceptible clade B having a better fitness than multi-drug resistant clade A1 [16–18,54–56]. The differences in fitness among clades were maintained at 42ºC, temperature at which *E. faecium* seems to had a significantly better fitness, probably related of the ubiquitous lifestyle of this species [51,57]. Interestingly, no significant differences in fitness were found between VSE and VRE *E. faecium* in this study, possibly due to the tight regulation of Tn*1546* by the two component system *vanS-vanR*, and also by the fact that Tn*1546* carrying plasmids seem to be highly adapted to certain *E. faecium* clonal backgrounds [17,18].

In summary, the stringent *E. faecium* host-specificity of Tn*1546-vanA* carrying plasmids might explain, up to some extent, the confinement of vancomycin resistance almost exclusively to this species. Even though the majority of Tn*1546-vanA* carrying plasmids studied seem to impose a significant burden to the host strain, their stability in the absence of selective pressure as well as their high conjugation rates

between *E. faecium* strains might explain their maintenance and conjugative spread among *E. faecium* populations, perpetuating the presence of VRE strains worldwide.

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REFERENCES

1. Freitas AR, Tedim AP, Francia MV et al. *Multi-Level Population Genetic Analysis of vanA and vanB Enterococcus faecium Causing Nosocomial Outbreaks in 27 Countries (1986-2012).* J Antimicrob Chemother 2016; *in print*.

2. Werner G, Coque TM, Hammerum AM et al. *Emergence and spread of vancomycin resistance among enterococci in Europe.* Euro Surveill 2008; 13: pii: 19046.

3. Kuch A, Willems RJL, Werner G et al. *Insight into antimicrobial susceptibility and population structure of contemporary human Enterococcus faecalis isolates from Europe*. J Antimicrob Chemother 2012; 67: 551–8.

4. Arthur M, Molinas C, Depardieu F et al. *Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in Enterococcus faecium BM4147.* J Bacteriol 1993; 175: 117–27.

5. Hegstad K, Mikalsen T, Coque TM et al. *Mobile genetic elements and their contribution to the emergence of antimicrobial resistant Enterococcus faecalis and Enterococcus faecium.* Clin Microbiol Infect 2010; 16: 541–54.

6. Courvalin P. *Vancomycin resistance in gram-positive cocci.* Clin Infect Dis 2006; 42 Suppl 1: S25–34.

7. Woodford N, Adebiyi AM, Palepou MF et al. *Diversity of VanA glycopeptide resistance elements in enterococci from humans and nonhuman sources*. Antimicrob Agents Chemother 1998; 42: 502–8.

8. Novais C, Freitas AR, Sousa JC et al. *Diversity of Tn1546 and its role in the dissemination of vancomycin-resistant enterococci in Portugal.* Antimicrob Agents Chemother 2008; 52: 1001–8.

9. López M, Sáenz Y, Alvarez-Martínez MJ et al. *Tn1546 structures and multilocus sequence typing of vanA-containing enterococci of animal, human and food origin*. J Antimicrob Chemother 2010; 65: 1570–5.

10. Clewell DB, Weaver KE, Coque TM et al. *Extrachromosomal and Mobile Elements in Enterococci: Transmission, Maintenance, and Epidemiology. In*: Gilmore MS, Clewell DB, Ike Y, Shankar N, eds. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Boston: Massachusetts Eye and Ear Infirmary, 2014.

11. Dahl KH, Lundblad EW, Rokenes TP et al. *Genetic linkage of the vanB2 gene cluster to Tn5382 in vancomycin-resistant enterococci and characterization of two novel insertion sequences*. Microbiology 2000; 146: 1469–79.

12. Lanza VF, Tedim AP, Martínez JL et al. The Plasmidome of Firmicutes: *Impact on the Emergence and the Spread of Resistance to Antimicrobials*. Microbiol Spectr 2015; 3: PLAS – 0039–2014.

13. Tedim AP, Ruiz-Garbajosa P, Corander J et al. *Population Biology of Intestinal Enterococcus Isolates from Hospitalized and Nonhospitalized Individuals in Different Age Groups*. Appl Environ Microbiol 2015; 81: 1820–31.

14. Willems RJL, Top J, van Schaik W et al. *Restricted gene flow among hospital subpopulations of Enterococcus faecium*. MBio 2012; 3: e00151–12.

15. Johnsen PJ, Simonsen GS, Olsvik O et al. *Stability, persistence, and evolution of plasmid-encoded VanA glycopeptide resistance in enterococci in the absence of antibiotic selection in vitro and in gnotobiotic mice*. Microb Drug Resist 2002; 8: 161–70.

16. Johnsen PJ, Townsend JP, Bøhn T et al. *Retrospective evidence for a biological cost of vancomycin resistance determinants in the absence of glycopeptide selective pressures*. J Antimicrob Chemother 2011; 66: 608–10.

17. Foucault M-L, Courvalin P, Grillot-Courvalin C. *Fitness cost of VanA-type vancomycin resistance in methicillin-resistant Staphylococcus aureus*. Antimicrob Agents Chemother 2009; 53: 2354–9.

18. Starikova I, Al-Haroni M, Werner G et al. *Fitness costs of various mobile genetic elements in Enterococcus faecium and Enterococcus faecalis.* J Antimicrob Chemother 2013; 68: 2755–65.

19. Foucault M-L, Depardieu F, Courvalin P et al. *Inducible expression eliminates the fitness cost of vancomycin resistance in enterococci.* Proc Natl Acad Sci U S A 2010; 107: 16964–9.

20. Ramadhan AA, Hegedus E. *Survivability of vancomycin resistant enterococci and fitness cost of vancomycin resistance acquisition*. J Clin Pathol 2005; 58: 744–6.

21. Freitas AR, Novais C, Tedim AP et al. *Microevolutionary Events Involving Narrow Host Plasmid Influences Local Fixation of Vancomycin-Resistance in Enterococcus*. PLoS One 2013: e60589.

22. Thompson JK, Collins MA. *Completed sequence of plasmid pIP501 and origin of spontaneous deletion derivatives*. Plasmid 2003; 50: 28–35.

23. Schwarz F V, Perreten V, Teuber M. *Sequence of the 50-kb conjugative multiresistance plasmid pRE25 from Enterococcus faecalis RE25*. Plasmid 2001; 46: 170–87.

24. Grady R, Hayes F. *Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multi-drug-resistant, clinical isolate of Enterococcus faecium*. Mol Microbiol 2003; 47: 1419–32.

25. Garcillán-Barcia MP, Francia MV, de la Cruz F. *The diversity of conjugative relaxases and its application in plasmid classification.* FEMS Microbiol Rev 2009; 33: 657–87.

26. Goicoechea P, Romo M, Coque TM et al. *Identification of enterococcal plasmids by multiplex-PCR-based relaxase typing* (Abstract number: P1669). *In*: 18th European Congress of Clinical Microbiology and Infectious Diseases. Barcelona, Spain, 2008.

27. Barton BM, Harding GP, Zuccarelli AJ. *A general method for detecting and sizing large plasmids.* Anal Biochem 1995; 226: 235–40.

28. Jensen LB, Garcia-Migura L, Valenzuela AJS et al. *A classification system for plasmids from enterococci and other Gram-positive bacteria*. J Microbiol Methods 2010; 80: 25–43.

29. Heaton MP, Handwerger S. *Conjugative mobilization of a vancomycin resistance plasmid by a putative Enterococcus faecium sex pheromone response plasmid*. Microb Drug Resist 1995; 1: 177–83.

30. Werner G, Freitas AR, Coque TM et al. *Host range of enterococcal vanA plasmids among Gram-positive intestinal bacteria*. J Antimicrob Chemother 2011; 66: 273–82.

31. Depardieu F, Perichon B, Courvalin P. *Detection of the van alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR.* J Clin Microbiol 2004; 42: 5857–60.

32. Sutcliffe J, Grebe T, Tait-Kamradt A et al. *Detection of erythromycin-resistant determinants by PCR.* Antimicrob Agents Chemother 1996; 40: 2562–6.

33. Coque TM, Willems RJL, Fortún J et al. *Population structure of Enterococcus faecium causing bacteremia in a Spanish university hospital: setting the scene for a future increase in vancomycin resistance?* Antimicrob Agents Chemother 2005; 49: 2693–700.

34. Hall BG, Acar H, Nandipati A et al. *Growth rates made easy.* Mol Biol Evol 2014; 31: 232–8.

35. Song L, Florea L, Langmead B. *Lighter: fast and memory-efficient sequencing error correction without counting.* Genome Biol 2014; 15: 509.

36. Chikhi R, Medvedev P. *Informed and automated k-mer size selection for genome assembly.* Bioinformatics 2014; 30: 31–7.

37. Bankevich A, Nurk S, Antipov D et al. *SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing.* J Comput Biol 2012; 19: 455–77.

38. Deatherage DE, Barrick JE. *Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq.* Methods Mol Biol 2014; 1151: 165–88.

39. Lanza VF, de Toro M, Garcillán-Barcia MP et al. *Plasmid flux in Escherichia coli ST131 sublineages, analyzed by plasmid constellation network (PLACNET), a new method for plasmid reconstruction from whole genome sequences*. PLoS Genet 2014; 10: e1004766.

40. Homan WL, Tribe D, Poznanski S et al. *Multilocus sequence typing scheme for Enterococcus faecium*. J Clin Microbiol 2002; 40: 1963–71.

41. Liu SL, Hessel A, Sanderson KE. *Genomic mapping with I-Ceu I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in Salmonella spp., Escherichia coli, and other bacteria.* Proc Natl Acad Sci U S A 1993; 90: 6874–8.

42. Murray BE, Singh K V, Ross RP et al. *Generation of restriction map of Enterococcus faecalis OG1 and investigation of growth requirements and regions encoding biosynthetic function*. J Bacteriol 1993; 175: 5216–23.

43. RStudio Team. *RStudio: Integrated Development Environment for R. 2015*. Available at: http://www.rstudio.com/.

44. Laverde Gomez JA, van Schaik W, Freitas AR et al. *A multiresistance megaplasmid pLG1 bearing a hylEfm genomic island in hospital Enterococcus faecium isolates.* Int J Med Microbiol 2011; 301: 165–75.

45. Andersson DI, Hughes D. *Antibiotic resistance and its cost: is it possible to reverse resistance?* Nat Rev Microbiol 2010; 8: 260–71.

46. San Millan A, Peña-Miller R, Toll-Riera M et al. *Positive selection and compensatory adaptation interact to stabilize non-transmissible plasmids*. Nat Commun 2014; 5: 5208.

47. Bouma JE, Lenski RE. *Evolution of a bacteria/plasmid association.* Nature 1988; 335: 351–2

48. Björkholm B, Sjölund M, Falk PG et al. *Mutation frequency and biological cost of antibiotic resistance in Helicobacter pylori.* Proc Natl Acad Sci U S A 2001; 98: 14607–12.

49. Harrison E, Guymer D, Spiers AJ et al. *Parallel compensatory evolution stabilizes plasmids across the parasitism-mutualism continuum.* Curr Biol 2015; 25: 2034–9.

50. Maisnier-Patin S, Berg OG, Liljas L et al. *Compensatory adaptation to the deleterious effect of antibiotic resistance in Salmonella typhimurium.* Mol Microbiol 2002; 46: 355–66.

51. Lebreton F, van Schaik W, McGuire AM et al. *Emergence of epidemic multi-drug-resistant Enterococcus faecium from animal and commensal strains.* MBio 2013; 4: e00534-13.

52. Kim EB, Jin G-D, Lee J-Y et al. *Genomic Features and Niche-Adaptation of Enterococcus faecium Strains from Korean Soybean-Fermented Foods.* PLoS One 2016; 11: e0153279.

53. Mira A, Ochman H, Moran NA. *Deletional bias and the evolution of bacterial genomes.* Trends Genet 2001; 17: 589–96.

54. Andersson DI, Levin BR. *The biological cost of antibiotic resistance.* Curr Opin Microbiol 1999; 2: 489–93.

55. Björkman J, Andersson DI. *The cost of antibiotic resistance from a bacterial perspective.* Drug Resist Updat 2000; 3: 237–45.

56. Johnsen PJ, Townsend JP, Bøhn T et al. *Factors affecting the reversal of antimicrobial-drug resistance.* Lancet Infect Dis 2009; 9: 357–64.

57. Lebreton F, Willems RJL, Gilmore MS. *Enterococcus Diversity, Origins in Nature, and Gut Colonization*. *In*: Gilmore MS, Clewell DB, Ike Y, Shankar N, eds. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection.* Boston: Massachusetts Eye and Ear Infirmary: Massachusetts Eye and Ear Infirmary, 2014.

Table S1. Epidemiological, virulence and mobilome characteristics of wild type strains used in this study. In plasmid content the plasmid in bold is the one carrying Tn*1546* transposon.

Name	Clonal Relation				Origin	Type	Country	Date	AbR		Tn*1546*	VF	Bacteriocins									Plasmid content	rep14a	rel1	rep18c	rep18	rep18c	rel2	rep6	rep17	rep20	rel3	rel5	TA1	rep1	rep2	rep21	rel6	rel7	TA2	rep22	rel8	Genome	Ts37°C		Ts42°C		RGR
	Clade	BAPS	ST	PFGE					Amp	Van	type																														size (Kb)	RG	σ	RG	σ	42°C/37°C
EFM14s	B	1.2	178	ASEF-19	Blood	Hospital	ESP	1999	S	S	-											7; 150; 195; 280																				2550.84	0.01434	0.00027	**0.01833***	0.00481	-27,8%
EFM9s	B	1.2	85	ASEF-17	Blood	Hospital	ESP	1997	S	S	-											7; 15; 150; 260																				2700.97	0.01464	0.00025	**0.01703***	0.00032	-16,3%
EFM10s	B	1.2	178	ASEF-18	Blood	Hospital	ESP	1997	S	S	-											200																				2812.18	0.01413	0.00039	**0.01160***	0.00048	17,9%
EFM6s	B	1.2	96	ASEF-32	Blood	Hospital	ESP	2002	S	S	-											5, 7, 15, 160, 420																				2368.05	0.01443	0.00043	**0.01632***	0.00053	-13,1%
BM4105SS	B	1.3	172	AS-B	ND	ND	FRA	1985	S	S	-											-																				2444.99	0.01245	0.00031	**0.01498***	0.00045	-20,3%
BM4105RF	B	1.3	172	AS-B	ND	ND	FRA	1985	S	S	-											-																				2444.99	0.01171	0.00016	**0.01403***	0.00052	-19,8%
EFM17s	A1	2.1a	442	ASEF-39	Blood	Hospital	ESP	2005	S	S	-											50, 60, 70, 100																				2377.77	0.01279	0.00024	**0.01406***	0.00066	-9,9%
CEI18.3	A1	2.1a	117	CEfm1	Feaces	Hospital	ESP	2009-2010	R	S	-											7, 10, 25, 255																				2596.00	0.01015	0.00024	0.01047	0.00031	-3,2%
EFM121	A1	2.1a	117	CEfm1	Blood	Hospital	ESP	2010	R	S	-											3, 7, 10, 25, 230																				2361.25	0.00893	0.00017	0.00855	0.00053	4,3%
EFM108	A1	2.1a	117	CEfm1	Blood	Hospital	ESP	2009	R	S	-											3, 7, 10, 25, 230																				2623.20	0.01001	0.00019	0.00957	0.00063	4,4%
EFM49	A1	2.1a	203	AREF-9	Blood	Hospital	ESP	2006	R	S	-											10; 70; 225; 375; 445																				2782.09	0.01025	0.00041	0.01117	0.00044	-9,0%
EFM51	A1	2.1a	203	AREF-9.1	Blood	Hospital	ESP	2007	R	S	-											12; 45; 70; 160; 370																				2801.63	0.01128	0.00025	0.01168	0.00039	-3,5%
EFM52	A1	2.1a	266	AREF-10	Blood	Hospital	ESP	2004	R	S	-											150																				ND	0.01261	0.00034	**0.01495***	0.00049	-18,6%
VnR20	A1	2.1a	117	CEfm1.3	Orina	Hospital	ESP	2011	R	R	-											7, 10, 25, 265, 370																				2993.50	0.01006	0.00058	**0.01114***	0.00025	-10,7%
VnR16	A1	2.1a	117	R	Wound	Hospital	ESP	2008	R	R	New											25, **55**																				2702.80	0.00864	0.00020	0.00955	0.00036	-10,5%
Lin3	A1	2.1a	203	30	ND	Hospital	USA	2004-05	R	R	F											4, 6, 12, 25, **40**, 60, 180																				3078.89	0.01120	0.00023	0.01195	0.00018	-6,7%
EI644	A1	2.1a	78	11	Catheter	Hospital	DEU	2002	R	R	A											2, 10, 25, 50, 170, **240**																				ND	0.00784	0.00034	ND	ND	ND
EFM2s	A1	3.3a1	18	ASEF-9	Blood	Hospital	ESP	1996	S	S	-											190																				2727.50	0.01272	0.00024	**0.01479***	0.00039	-16,3%
EFM25s	A1	3.3a1	18	ASEF-12	Blood	Hospital	ESP	1997	S	S	-											50, 190																				2654.60	0.01332	0.00016	**0.01555***	0.00026	-16,7%
EFM3s	A1	3.3a1	18	ASEF-24	Blood	Hospital	ESP	2000	S	S	-											160																				2103.49	0.01314	0.00028	**0.01506***	0.00042	-14,6%
EFM3	A1	3.3a1	18	AREF-D	Blood	Hospital	ESP	1997	R	S	-											50; 75; 170; 310; 355:395																				2743.85	0.01028	0.00053	0.01109	0.00036	-7,9%
EFM23	A1	3.3a1	18	AREF-D.1	Blood	Hospital	ESP	2002	R	S	-											10; 15; 75; 280; 355																				3014.30	0.01059	0.00020	**0.01170***	0.00017	-10,5%
EFM28	A1	3.3a1	18	AREF-2	Blood	Hospital	ESP	2004	R	S	-											10; 30; 75; 155; 310; 360																				2736.52	0.01057	0.00032	**0.01253***	0.00034	-18,5%
EFH1/01	A1	3.3a1	18	AREF-56	Blood	Hospital	ESP	2001	R	S	-											10, 20, 50, 135																				2735.53	0.01015	0.00031	0.01010	0.00026	0,5%
H182	A1	3.3a1	18	13	blood, hepatic fluid	Hospital	PRT	2002-2003	R	R	PP-4											40, **92**, 140, 230																				2452.78	0.01101	0.00036	**0.01208***	0.00023	-9,7%
H311	A1	3.3a1	132	54	Urine	Hospital	PRT	2002	R	R	PP-23											10, 50, 60, 200																				ND	0.01052	0.00017	ND	ND	ND
EFM34	A1	3.3a2	17	AREF-P	Blood	Hospital	ESP	1995	R	S	-											10; 60; 190; 385																				2711.27	0.01044	0.00052	**0.00695***	0.00143	33,0%
EFM39	A1	3.3a2	16	AREF-T	Blood	Hospital	ESP	2001	R	S	-											45; 80; 275																				2678.93	0.00931	0.00031	**0.01087***	0.00029	-16,8%
EFM44	A1	3.3a2	63	AREF-W.2	Blood	Hospital	ESP	2000	R	S	-											18; 35;70; 160																				ND	0.00786	0.00022	**0.00915***	0.00019	-16,4%
EFM46	A1	3.3a2	103	AREF-W	Blood	Hospital	ESP	2001	R	S	-											10; 20, 75; 160; 280;365; 390																				ND	0.00981	0.00073	0.01058	0.00026	-7,8%
EFM47	A1	3.3a2	103	AREF-W.3	Blood	Hospital	ESP	2001	R	S	-											10; 20; 75;280; 310; 345																				ND	0.00978	0.00052	**0.01090***	0.00034	-11,5%
232/09	A1	3.3a2	17	D	Blood	Hospital	ESP	2009	R	R	PP-16											4, 7, 20, 30, 70, 300																				2809.09	0.00916	0.00082	**0.01123***	0.00053	-22,6%
604/06	A1	3.3a2	17	C	Abdominal Fluid	Hospital	ESP	2006	R	R	-											10, 20, 60, 140																				2268.83	0.01078	0.00063	0.01094	0.00055	-1,5%
EI651	A1	3.3a2	16	9**	wound	Hospital	NLD	2002	R	R	X											4, 20, 45, 60, **85**, 135, 170																				2690.19	0.01034	0.00034	**0.00914***	0.00063	11,6%
EFM7s	A2	2.1b	69	ASEF-35	Blood	Hospital	ESP	2003	S	S	-											10; 30; 55; 300																				ND	0.00788	0.00075	**0.00688***	0.00037	12,7%
EFM8s	A2	2.1b	69	ASEF-44	Blood	Hospital	ESP	2005	S	S	-											200																				ND	0.01292	0.00031	**0.01482***	0.00039	-14,7%
EFH10/95	A2	2.1b	26	AREF-57	Blood	Hospital	ESP	1995	S	S	-											20, 115																				2447.86	0.01331	0.00051	**0.01506***	0.00104	-13,1%
C1726	A2	2.1b	9	MLCC9-A	Chicken meat	Food	ESP	1997	S	R	A											25; **40**; **200**																				2362.11	0.01145	0.00025	**0.01352***	0.00033	-18,1%
C1706	A2	2.1b	10	MLCC9-C	Faeces	Chicken	ESP	1996	S	R	III											50;190																				2070.23	0.01284	0.00020	0.01344	0.00033	-4,7%
H305	A2	2.1b	5	12	Wound	Hospital	PRT	2002	S	R	D											150																				ND	0.01310	0.00029	ND	ND	ND
EFM26s	A2	2.3a	21	ASEF-13	Blood	Hospital	ESP	1997	S	S	-											-																				ND	0.01305	0.00049	**0.01577***	0.00041	-20,8%
64/3	A2	2.3a	21	AS-C	ND	ND	ND	ND	S	S	-											-																				ND	0.01136	0.00025	**0.01296***	0.00036	-14,1%
EFM21s	A2	2.3a	71	ASEF-3	Blood	Hospital	ESP	1995	S	S	-											150																				2330.62	0.01379	0.00036	**0.01641***	0.00049	-19,0%
EFM13s	A2	2.3a	420	ASEF-38	Blood	Hospital	ESP	2007	S	S	-											15, 20																				ND	0.01270	0.00036	**0.01503***	0.00052	-18,3%
EFH2/96	A2	2.3a	25	ASEF-8	Blood	Hospital	ESP	1996	S	S	-											25																				2185.90	0.01259	0.00072	**0.01540***	0.00047	-22,3%
EFM30s	A2	2.3a	25	ASEF-26	Blood	Hospital	ESP	2000	S	S	-											50, 70, 200																				2166.27	0.01183	0.00055	**0.01294***	0.00084	-9,4%
BM4165	A2	2.3a	25	2	Faeces	Hospital	FRA	1987	S	R	A											40, 60, 135																				2052.42	0.01155	0.00036	**0.01296***	0.00046	-12,2%
BM4147	A2	2.3a	25	1	Faeces	Hospital	FRA	1986	S	R	A											30																				ND	0.00994	0.00079	ND	ND	ND
CEI166A	A2	2.3a	25	CEfm23	Faeces	Community	ESP	2009	R	S	-											20, 145																				2173.20	0.01298	0.00035	0.01354	0.00026	-4,3%
GEI-RF	A2	2.3b	515	AS-A	ND	ND	ND	S	S	-												-																				2375.50	0.01100	0.00030	0.01181	0.00032	-7,4%
EFM4s	A2	3.1	22	ASEF-2	Blood	Hospital	ESP	1995	S	S	-											240																				ND	0.01271	0.00031	**0.01518***	0.00025	-19,4%
EFM11s	A2	3.1	22	ASEF-36	Blood	Hospital	ESP	2006	S	S	-											55;270																				ND	0.01051	0.00070	**0.00921***	0.00052	12,4%
EFM22s	A2	3.1	32	ASEF-5	Blood	Hospital	ESP	1995	S	S	-											-																				ND	0.01238	0.00023	**0.01437***	0.00064	-16,1%
EFM12s	A2	3.1	214	ASEF-37	Blood	Hospital	ESP	2006	S	S	-											10; 100; 205; 320																				ND	0.00839	0.00037	**0.01081***	0.00027	-28,8%
EFM5s	A2	3.2	29	ASEF-34	Blood	Hospital	ESP	2007	S	S	-											-																				2389.86	0.01065	0.00036	**0.01212***	0.00061	-13,8%
EFM1s	A2	3.2	97	ASEF-31	Blood	Hospital	ESP	2001	S	S	-											250																				2082.76	0.01307	0.00040	**0.01467***	0.00066	-12,2%
C1718	A2	3.2	29	MLCC9-G	Feaces	Hospital	ESP	1997	S	R	A											60; 155																				2860.03	0.01227	0.00034	**0.01365***	0.00064	-11,2%
C851	A2	3.2	29	MLCC9-H	ND	clinical	ESP	2006	R	R	A											55; **85**; 105																				2308.31	0.01029	0.00077	0.00987	0.00032	4,1%
EFM27s	A2	3.3b	102	ASEF-20	Blood	Hospital	ESP	1999	S	S	-											7; 15; 30; 150; 190; 280; 375																				2309.98	0.01243	0.00026	**0.00971***	0.00047	21,9%
EFM45	A2	3.3b	102	ASEF-5	Blood	Hospital	ESP	2001	R	S	-											ND																				2522.13	0.00974	0.00063	**0.01230***	0.00101	-26,3%
EFM133	A2	7	19	AREF-11.5	Blood	Hospital	ESP	2010	S	S	-											20, 120																				2076.62	0.01317	0.00027	**0.01547***	0.00025	-17,5%
CHF106.3	A2	7	19	CEfm5	Feaces	Hospital	ESP	2009	R	S	-											20, 45																				2160.05	0.01020	0.00062	**0.00736***	0.00043	27,8%
CEI137A	A2	7	19	CEfm5.1	Feaces	Community	ESP	2009	R	S	-											20, 155																				2777.72	0.01267	0.00026	**0.01519***	0.00043	-19,9%
CEI169A	A2	7	19	CEfm5.2	Feaces	Community	ESP	2009	R	S	-											ND																				2602.96	0.01056	0.00114	**0.00839***	0.00101	20,5%
CEI212A	A2	7	19	CEfm5.2	Feaces	Community	ESP	2009	R	S	-											20, 35, 145																				ND	0.00707	0.00019	0.00706	0.00029	0,1%

* in bold - statistically significant; green color in T=42ºC GR indicates a higher fitness of the strain at 42ºC compared with 37ºC; Red color in T=42ºC GR indicates a fitness loss of the strain at 42ºC compared with 37ºC. **Abbreviations:** ND - Not determined.

Name	Plasmid	Generation	Receptor	PFGE	AbR phenotype		AbR genotype		AbR reversion	Plasmid	RepA_N			Inc18				
											rep17	rel3	TA1	rep1	rep2	rel6	rel7	TA2
					VAN	ERY	*vanA*	*ermB*	phenotype (%)	size	pRUM	pRUM	Axe	Inc18	pRE25	pEF1	pRE25	ωεζ
A1	-	0	GE1	AS-A	-	-	-	-	-	-				CH				
A2	-	100	GE1	AS-A	-	-	-	-	-	-				CH				
A3	-	300	GE1	AS-A	-	-	-	-	-	-				CH				
A26	-	0	64/3	AS-C	-	-	-	-	-	-								
A27	-	100	64/3	AS-C	-	-	-	-	-	-								
A28	-	300	64/3	AS-C	-	-	-	-	-	-								
A4	pH311	0	GE1	AS-A	R	R			-	50								
A5	pH311	300	GE1	AS-A	R	R			1.33%	50								
A6	pH311	300	GE1	AS-A	S	R				50								
A29	pH311	0	64/3	AS-C	R	R			-	50								
A30	pH311	300	64/3	AS-C	R	R			0.33%	50								
A31	pH311	300	64/3	AS-C	S	R	-			50, 200								
A7	pH182	0	GE1	AS-A	R	R			-	90				CH				
A8	pH182	300	GE1	AS-A	R	R			3.33%	90				CH				
A9	pH182	300	GE1	AS-A	S	S	-	-		70				CH				
A32	pH182	0	64/3	AS-C	R	R			-	90								
A33	pH182	300	64/3	AS-C	R	R			0.33%	90								
A34	pH182	300	64/3	AS-C	S	S	-	-		60								
A10	pBM4165	0	GE1	AS-A	R	R			-	40, 60								
A11	pBM4165	100	GE1	AS-A	R	R			29.67%	40, 60								
A12	pBM4165	100	GE1	AS-A	S	R				30, 60								
A13	pBM4165	300	GE1	AS-A	S	S	-	-	100%	60								
A35	pBM4165	0	64/3	AS-C	R	R			-	40, 60								
A36	pBM4165	100	64/3	AS-C	R	R			0%	40, 60								
A37	pBM4165	300	64/3	AS-C	R	R			56.67%	40, 60								
A38	pBM4165	300	64/3	AS-C	S	S	-	-		60								
A14	pIP501	0	GE1	AS-A	S	R	-		-	30								
A15	pIP501	100	GE1	AS-A	S	R	-		14.00%	30								
A16	pIP501	100	GE1	AS-A.2	S	S	-	-		-				CH				
A17	pIP501	300	GE1	AS-A	S	R	-		79.00%	30								
A18	pIP501	300	GE1	AS-A	S	S	-	-		-				CH				
A39	pIP501	0	64/3	AS-C	S	R	-		-	30								
A40	pIP501	100	64/3	AS-C	S	R	-		1.67%	30								
A41	pIP501	100	64/3	AS-C	S	S	-	-		-								
A42	pIP501	300	64/3	AS-C	S	R	-		12.33%	30								
A43	pIP501	300	64/3	AS-C	S	S	-	-		-								
A19	pRE25	0	GE1	AS-A	S	R	-		-	50								
A20	pRE25	100	GE1	AS-A	S	R	-		3.67%	50								
A21	pRE25	100	GE1	AS-A	S	S	-	-		-				CH				
A22	pRE25	300	GE1	AS-A	S	R	-		18.33%	50								
A23	pRE25	300	GE1	AS-A	S	S	-	-		-				CH				
A44	pRE25	0	64/3	AS-C	S	R	-		-	50								
A45	pRE25	100	64/3	AS-C	S	R	-		2.33%	50								
A46	pRE25	100	64/3	AS-C	S	S	-	-		25								
A47	pRE25	300	64/3	AS-C	S	R	-		10.67%	50								
A48	pRE25	300	64/3	AS-C	S	S	-	-		30								
A24	pRUM	0	GE1	AS-A	S	R	-		-	30, 200				CH				
A25	pRUM	300	GE1	AS-A	S	R	-		0.33%	200				CH				
A49	pRUM	0	64/3	AS-C	S	R	-		-	30, 200								
A50	pRUM	300	64/3	AS-C	S	R	-		0.33%	200								

Figure S1. List of sequenced evolved and non-evolved strains with antibiotic resistance (genotypical and phenotypical) and plasmid characteristics.

Abbreviations: CH - Chromosome.

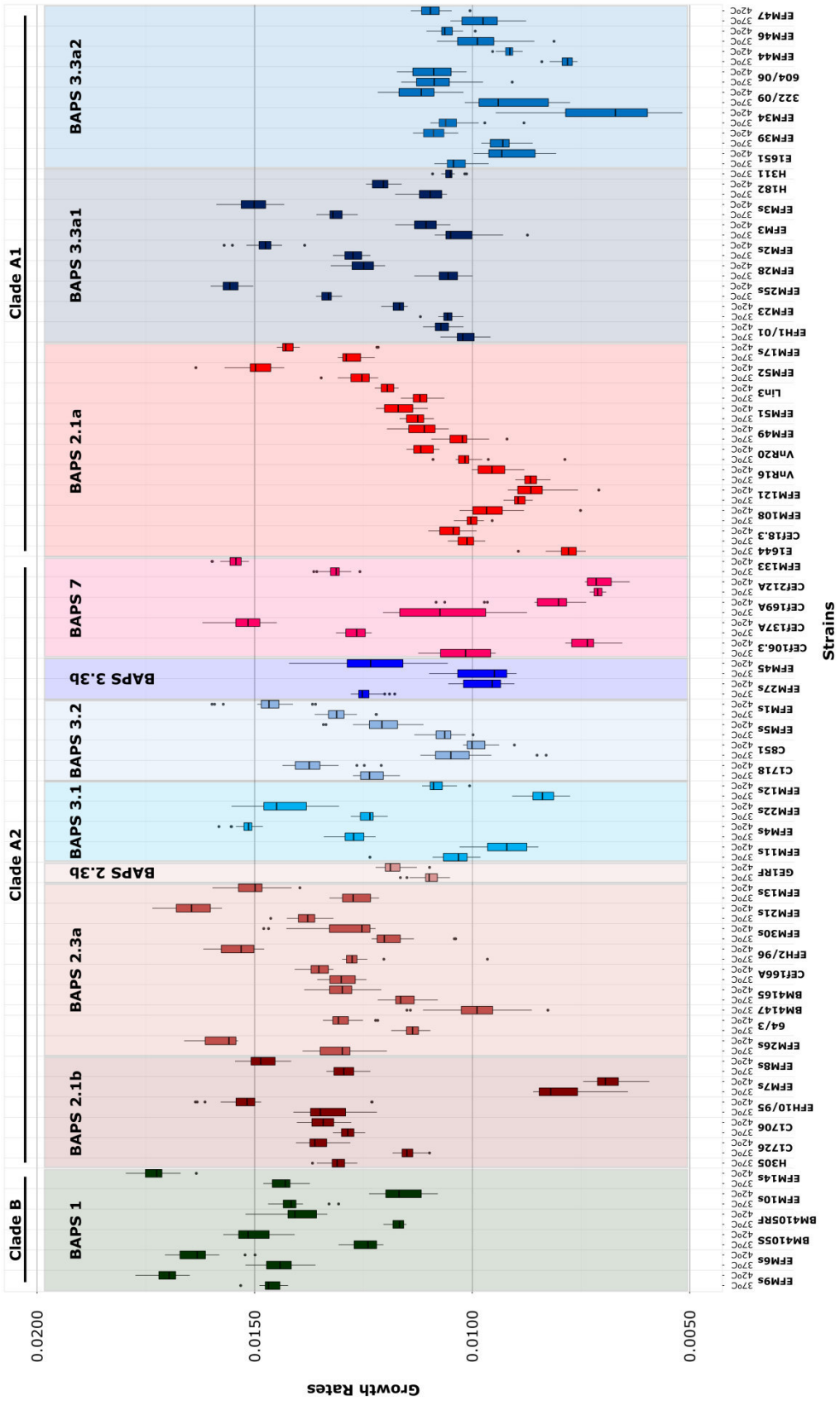
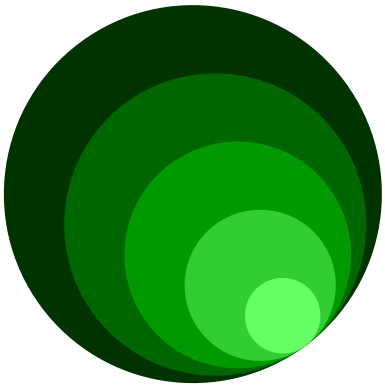


Figure S2. Box and whiskers plot of growth rates at 37ºC and 42ºC of all *E. faecium* wild type strains used in this study.

Any fool can know. The point is to understand.

Albert Einstein



Discussion

1. Population structure of *E. faecium* isolates from non-hospitalized and hospitalized humans, both colonized and infected

Enterococci have been known as members of the human gut microbiota and opportunist pathogens for more than a century. Studies regarding the population structure of enterococci are restricted to *E. faecalis* and *E. faecium*, most *E. faecium* strains being VRE strains isolated during hospital outbreaks. There are only a few studies that have addressed the diversity and population structure of vancomycin susceptible enterococci present in the human GI tract or causing nosocomial infections (1–4). Even though, *E. faecium* is the focus of this PhD dissertation there is impossible to fully understand their diversity without first discussing the total diversity of enterococci in the human gut.

The high recovery rate of enterococci in human faeces (71%-80%, of the persons analysed) in both hospitalized patients and community-based humans was similar to that obtained in previous works (2–4), although the influence of the age observed in other studies was not confirmed in our series. *E. faecalis* was consistently predominant in the faecal microbiota in young and elderly individuals with the exception of hospitalized elderly patients where *E. faecium* was predominant. The presence of other enterococcal species showed an inversely proportional trend to that of *E. faecium* indicating common functional requirements of *E. faecium* and others species and also changes in ecological conditions (diet, health and antibiotic treatment) that might shape the selection for particular clones as has been demonstrated for chicken or calves (2, 5–11).

As previously mentioned, the analysis of the population structure of *E. faecium* by ClonalFrame or BAPS analysis of MLST data allowed establishing the independent origin of major ST17, ST18 and ST78 lineages, previously located within the CC17 using goeBURST (12–15). In this PhD dissertation, we significantly extended previous studies of populations structure using BAPS at qualitative (by including a significant number of isolates from individuals of different ages and from both hospital and community settings) and quantitative levels (duplicating the number of STs included, 837 STs and 1116 STs, vs 492 STs). The two new BAPS analysis performed yield similar number of BAPS groups (8 and 9, respectively), four of them being predominant, namely BAPS 1, BAPS 2, BAPS 3 and BAPS 7 (15.2%, 39.5%, 30.9%, and 6.8%, respectively) and were validated through correlation analysis with the BAPS analysis described by Willems *et al* (13). Moreover, the correlation coefficient between the BAPS analysis described in Chapters 1 and 2 revealed little discrepancies either at BAPS group or at subgroup level indicating that, for *E. faecium*, BAPS analysis is both reproducible and robust and may accurately describe the *E. faecium* population structure. The inclusion of more STs in the second BAPS analysis performed permitted the accurate separation of two clonal lineages, ST17 and ST18, associated with antibiotic resistance and the hospital setting. The different origin for these clonal lineages had been previously suggested (13) but not confirmed. This also showed that the higher the number of STs included in the BAPS analysis, the more accurate the prediction of the *E. faecium* population structure.

Furthermore, BAPS analysis performed in chapter 1 confirmed the presence of evolutionary and functionally heterogeneous clades for the *E. faecium* species proposed by other studies (13, 16–18). *E. faecium* isolates of BAPS 1 and BAPS 3.3b, all ampicillin susceptible and mostly associated with non-hospitalized individuals, corresponding to the "community population" that seem to be enriched in pathways of complex carbohydrate utilization linked to host diet (16). These populations were highly represented in different age groups of colonized non-hospitalized individuals although its incidence was slightly reduced in the elderly (13, 16). Conversely, *E. faecium* BAPS 2.1a (lineage ST78), BAPS 3.3a1 (lineage ST18) and BAPS 3.3a2 (lineage ST17) subgroups, corresponding to Clade A1 or "hospital population" mostly comprises ampicillin and/or vancomycin resistant strains (13, 16). As it was demonstrated in Chapter 1, isolates of these BAPS groups were predominantly detected in isolates causing infections among elderly hospitalized patients. It is of note that the rates of these populations might be underestimated in both inpatients and healthy humans if you do not pre-enrich the sample, probably due to low colonization densities.

The analysis of the population structure and diversity of *E. faecium* causing BSI was performed in Chapter 2 and comprised isolates collected during a 20 year period. The observed increase in both the number of *E. faecium* BSI cases in cancer patients (gastroenterology, haematology and oncology wards) and the number of ampicillin resistant *E. faecium* high-risk clones (ST17, ST203, ST117 and ST80) in the 2006-2015 period in Hospital Universitario Ramón y Cajal, can also be indirectly inferred from the data of multicentre studies (19, 20), studies confined to a single institution (21), or studies focused on VREfm, MDR *E. faecium*, or particular groups of patients (e.g. cancer patients) (22, 23).

Previous antibiotic treatment has largely considered one of the main risk factors for acquisition of enterococci BSI (24). Therapy with broad spectrum β-lactams, including carbapenems, to treat MDR *Enterobacteriaceae* in onco-haematological patients with febrile neutropenia (22) or the use of levofloxacin to prevent such infections in these patients has dramatically increased since the mid-2000s in many hospitals including ours (23), and has been recognized as one of the main risk factors for colonization and subsequence BSI by MDR Gram positive organisms (22, 25, 26). Differences in chemotherapy or clinical practice guidelines also seem to influence acquisition and intestinal persistence of ampicillin resistant *E. faecium* (23).

The recovery from blood cultures of *E. faecium* of all 7 BAPS groups including those that are normal components of the human gut microbiota (BAPS 1 and BAPS 3.3b, being the most representative as mentioned above), suggested that bacterial translocation, colonization by contiguity (UTIs) and invasive processes, of stochastic nature, can be facilitated by either an enhanced population size (due to colonization and/or overgrowth of the antibiotic resistant clones) or by the number of patients colonized by a certain strain, classically known as "colonization pressure" (that enhance the chances of transmission) (7, 8, 27, 28). The predominance of STs of major human lineages ST17, ST18 and ST78, previously designed as HiRCCs showed a temporal trend in most clinical surveys including our BSI series

(29, 30). The apparent clonal "waves" of different HiRCCs with a recent expansion of the *E. faecium* ST78 at global level (13, 31) implies the successive selection, expansion, and evolution of certain clones, following hospital microepidemics (18, 32). Besides clonal expansion of specific strains, an increasingly high intra-clonal diversification of *E. faecium* strains belonging to ST17, ST18 and ST78 lineages, was found in this and also in other works (33), reflecting the "*ex unibus plurum*" evolutionary dynamics (34). The phenomena, initially observed for *S. aureus* (35), could be applied as a general scenario for different species.

The contemporary increase of isolates within BAPS 2.1a (ST117, ST192, ST203) and BAPS 3.3a2 (ST17) in the hospital environment is intriguing. Recent studies have highlighted the relevance of microbial inheritance in basic processes of infectious diseases (28) and the impact of antibiotic treatments in the composition and structure of microbial communities (36). Although cross transmission among individuals remains a key factor in the epidemiology of *E. faecium*, the importance of the various resident *E. faecium* populations, eventually changing in frequency with antibiotic exposure and age, comes to light in Chapters 1 and 2 of this dissertation. The apparent specialization of certain subpopulations of *E. faecium* either in colonization or infections might therefore be associated with several other host associated factors, and also differences in harboured selectable characters, as antibiotic resistance genes. Interestingly, some groups evolve independently from the acquisition of ampicillin resistance, suggesting a certain genetic isolation as seems to be the case of different lineages within BAPS 3.3b and BAPS 1. These results further confirm a population structure comprised of ecotypes representing specialization in different hosts (37, 38). It has been recently demonstrated that heterogeneity of *S. aureus* populations that colonize humans, designated as "clouds", leads to a possible global adaptive benefit for certain clones and finally for the overall species, that is enhanced by horizontal gene transfer (HGT) (39, 40). This might also explain what was observed in *E. faecium*. One of the most remarkable features of major human lineages of this species is the content of mobile genetic elements (2).

2. MGE diversity in *E. faecium*, particularly those responsible for antimicrobial resistance

Horizontal gene transfer (HGT) plays a fundamental role in the evolution of bacterial species but it was only recently that the occurrence of HGT events between ecologically distant groups of bacteria (with similar G+C content), was demonstrated (41–44). The network analysis of resistance determinants (antibiotics, heavy metals and biocides) in *Firmicutes* showed in Chapter 3 of this dissertation reveals the presence, either located in the chromosome and/or plasmids, of the same resistance determinants in different species from this phylum, which illustrates a high diversity of interactions within different bacterial communities. It still remains unknown the extent to which the HGT and specific MGE and species have contributed to the evolution of the phylum *Firmicutes* and particularly of the genera *Enterococcus*. It has been demonstrated that the association of resistance determinants with site-specific recombination systems and insertion sequences located in plasmids and/or chromosomes

favours homologous recombination through the interplay among different elements (45–49). The heterogeneous distribution of resistance determinants observed in *Firmicutes* associated with the presence of systems that protect bacteria against de acquisition of exogenous DNA (R-M systems and CRISPR) (50–52) indicates that HGT may indeed play an important role in bacterial ecology and evolution.

It had been suggested, and was further observed in work presented in chapter 3, that the number of MGEs greatly varies among different bacterial species. Nevertheless, consistent patterns, particularly regarding plasmids, have been found among the different member strains from the same species (53–55). This suggest an evolutionary co-adaptation between plasmids and bacteria, issue that has been well studied in *Enterococcus*, particularly in *E. faecium* and *E. faecalis*, due to their clinical relevance and frequent acquisition of resistance determinants.

Enterococci frequently carry species-specific RCR plasmids (e. g. pRI1 in *E. faecium*) and RepA_N plasmids (pLG1-like megaplasmids in *E. faecium*) (53, 54). RepA_N plasmids seem to contribute to the evolvability of enterococci species as they enhance their ability to colonize and invade (54, 56, 57). Aside from these narrow host rage plasmids, enterococci also carry Inc18 broad host range plasmids, originally identified in streptococci. The heterogeneous Inc18 plasmid family has greatly contributed to the spread of resistance to different antibiotics including vancomycin. Nevertheless, genes encoding antibiotic resistance in enterococci are also been located on plasmids containing RIPs of other plasmid families, plasmid chimeras with more than one RIP being frequently detected. The presence of more than one RIP might be associated with recombination between narrow and broad host plasmids, like Inc18 plasmids in order to stabilize the incoming useful trait (53). Some of these chimeric plasmids have largely contributed to the worldwide dissemination of aminoglycoside (e*rmB*) and vancomycin (*vanA*) resistance among *E. faecium* of human and animal origin. Aside from these chimeras, narrow host rage plasmids have contributed for the local dissemination of gentamicin (Tn*4001*) and β-lactams (*blaZ*) resistance among *E. faecium*.

The emergence of *E. faecium* (ST17, ST18, ST78) hospital adapted lineages carrying several antibiotic resistance plasmids highlights the role that these elements might play in the adaptation of these species to a particular ecological conditions (13, 58, 59). Chapter 4 and Chapter 5 described the influence of both clonal and plasmid backgrounds in the spread and persistence of vancomycin resistance among *E. faecium*. Predominant local AREfm of major human lineages ST17, ST18 and ST78 appear to have contributed to the high rates of vancomycin resistance observed nowadays after acquisition of *vanA* or *vanB* transposons. This is reflected by the emblematic clonal VREfm “bloomings” that have occurred in the US, Australia (ST203), Sweden (ST192), Portugal (ST18/ST132), Brazil and Canada (ST412) or The Netherlands (ST117) (54, 60–70). However, events of HGT among distinct clonal backgrounds also occurred when the prevalence of VREfm rapidly increase or when VRE became endemic (31, 71). Chapter 5 analyses VREfm from Portugal and shows how isolates of the major human lineages ST18

(BAPS 3.3a1), ST78 (BAPS 2.1a) and to a lesser extent ST17 (BAPS 3.3a2) influence the transmission of Tn*1546* in a region (72, 73). The results indicate the importance of local clonal evolution due to variation, drift and short-distance migration. This evolution diving forces can lead to changes in colonization ability, pathogenicity or even host range, the fittest clonal variants being able to facilitate the local and eventually international transmission that was revised in Chapter 4, (74–78).

The diversity of plasmids able to acquire *van* operons and their ability to recombine in chimeric plasmids is noteworthy and played a critical role in the spread of Tn*1546-vanA* both at local (Chapter 5) and at international levels (Chapter 4). The predominant recovery of pRUM::Tn*1546* highly related to the original pRUM plasmid recovered in an early outbreak VREfm isolate (79) from the USA and the frequent isolation of Inc18::Tn*1546* similar to the first *vanA* plasmid pIP816 (80, 81) among VREfm from humans and animals in Europe since late 1980s to date (82–85), revealed the contribution of pRUM and pIP186 to the evolvability of Tn*1546* in different continents.

Even though these pRUM and Inc18 plasmid types appear to have greatly contribute to the dissemination of vancomycin resistance in *E. faecium*, any enterococcal plasmid can act as a vehicle for Tn*1546* or even CTn*5382*, eventually causing self-limited outbreaks. They include pLG1-like megaplasmids (57, 60, 86–89), or pHTß-like plasmids (responsible for early outbreaks in Japan and the USA) (90, 91). Finally, chimeras of plasmids from *E. faecium*, *E. faecalis* (pheromone responsive plasmids) or *S. aureus* (pRUM) (79) reflect the relevance of HGT events between different species and genera of Firmicutes (Freitas et al., 2009a).

In vitro transfer of *E. faecium* Tn*1546-vanA* plasmids to *E. faecalis* was infrequent (Chapter 6) and highly dependent on the modules present in the plasmid backbone. More specifically, it only occurred when RIPs of the Inc18 broad host range family ($rep_{1/pIP501}$) were present in the *E. faecium* plasmids in agreement with previous studies (93). This might explain, up to some extent, the higher prevalence of vancomycin resistance in *E. faecium* compared with that of other pathogens (31, 59).

3. To determine the influence of MGE encoding antibiotic resistance on the fitness of bacterial populations.

In Chapter 6 of this PhD dissertation, transferability of a large chromosomal region containing *pbp5*, associated with ampicillin resistance in *E. faecium*, was confirmed and the chromosomal platform described as well as several new mutations in PBP5.

The role PBP5 plays in *E. faecium* resistance to cephalosporins and penicillins resistance might have facilitated the selection of subpopulations able to cause infections in humans (94). A steady increase of the number of *E. faecium* isolates with reduced susceptibility to β-lactams has been detected in clinical settings since the 1940s, and such increase parallels the number of infections caused by this species (95). In agreement with previous studies, seven changes (A68T, E85D, S204G, 466´S/D, M485A/T, E629V and P667S) were consistently observed in most AREfm. Also, several mutations were identified in

different *E. faecium* groups with different ampicillin susceptibility that indicate that a variety of evolutionary routes, and intermediate steps were possible similarly to what observed for different β-lactamase enzymes of Gram negative organisms (96).

Only two *E. faecium* strains lacking *pbp5* have been documented, GE1 and D344S. The D344S strain is susceptible to cephalosporin and ampicillin due to the spontaneous deletion of a 160 kb genome region that includes *pbp5* and other genes including some encoding other PBPs. This deletion occurred due to the interaction of CTn*5386* (a 60kb element that comprises both *pbp5* and *vanB2* genes) with Tn*916* (97). The causes for the loss of the region in which is *pbp5* is located in the GE1 laboratory strain are unknown but it might be due to the fitness cost of this region in a non-specialized environment (laboratory conditions) (98).

The mechanism responsible to the transfer of the ≈300kb region carrying *pbp5* was not evident from the genetic analysis obtained due to the lack of sequences related to conjugative transposon(s) or even ISs that could be part of a composite transposon. A possible transference of chromosomal regions by an Hfr-like (High frequency recombination) mechanisms involving specific plasmids (as for most transconjugants plasmids were also present) cannot be discarded (56). We cannot exclude acquisition of this DNA fragments through transformation neither.

The transfer of a chromosomal region containing not only the *pbp5* but also traits that enhance survival in the gastrointestinal tract of mammals as the resistance to stress by acids and bile or the ability to use substrates produced by the host as maltodextrines and sialic acids in all available *E. faecium* isolates but GE1RF, suggests a possible ancient acquisition of the platform to adapt to these hosts. This was further confirmed by the experimental conjugative transfer of *pbp5* between isolates of different *E. faecium* clades (clade A1, A2 and B) and by comparative genomic analysis.

In chapter 7, Tn*1546*-*van*A carrying plasmids conferred medium to high fitness cost in different *E. faecium* backgrounds as had been previously suggested for newly transferred plasmids or for plasmids that are not adapted to the host cell (99, 100). Such fitness differences seem to demonstrate that some *E. faecium* clades or lineages might be better adapted to the acquisition of mobile genetic elements than others (16) and/or that some plasmids are better adapted to some species than others, as happen with the megaplasmids belonging to the RepA_N family ($rep_{22/pLG1}$), that are almost exclusively found in *E. faecium* (89).

Induction of the expression of Tn*1546*-*vanA* showed a high fitness cost in both filed isolates and laboratory isogenic background. Previous studies performed in vancomycin resistant *S. aureus* also showed that the induction of vancomycin resistance had a high fitness cost for the host strain. This high fitness cost was attributed to the metabolic changes that the vancomycin resistance mechanism induces in the cell and that leads to the elimination of chromosomal encoded peptidoglycan precursors (D-Ala-D-Ala) to Tn*1546* encoded precursors (D-Ala-D-Lac) (99, 101). Fitness cost of Tn*1546* was also dependent

on the Tn*1546* variant in which the *van* gene is located. These differences might be due to the efficiency of expression of enzymes encoded by Tn*1546* due to the presence of ISs in the transposon, as the original Tn*1546* onfer a low cost compared with other truncated Tn*1546* variants studied.

In spite of the lack of fitness that Tn*1546-vanA* carrying plasmids confer to the host, most of the plasmids were stably maintained during 300 generations without selective pressure. Some of these plasmids carry known, highly effective, toxin-antitoxin systems (79, 102, 103) which might account for their persistence even when vancomycin resistance phenotype and/or genotype were eventually lost. Nonetheless, reversion of vancomycin resistance was seldom observed for most of the Tn*1546-vanA* plasmids studied similarly to what was previously described in other studies (104).

The majority of mutations found in evolved strains compared to non-evolved were related with DNA replication and repair indicating that high mutation rates might be important for a rapid host-plasmid adaptation (105, 106). Alterations in the global transcription regulation, a remarkable change in *E. faecium* 64/3 evolved strains, have also been often documented in evolved strains of Gram-negative species. In these studies, the presence of the resistance plasmid led to an increase in the transcription rates and afterwards to values similar to the non-evolved strains due to compensatory mutations in the two component regulatory system *galA*/*galS* (104, 106, 107). Thus, compensatory mutations in transcription regulation would contribute to a reduced expression of plasmid genes might play a fundamental role in host-plasmid adaptation (106). Changes found in carbohydrate and amino acid metabolism, several PST systems and inorganic ion transport systems might also contribute to the metabolic burden imposed by the plasmid due to o high uptake and process of nutrients (106). Finally, even though mutations in similar genes were found in *E. faecium* GE1 and *E. faecium* 64/3 carrying the same plasmids, most of them were disparate indicating that they followed different evolutionary pathways after acquisition of foreign DNA (104).

Also in Chapter 7, differences in the genome size of isolates in clades A1, A2 and B are described, clade A1 having the largest genome followed by clade B and then, clade A2 (16, 108). These observations are consistent with the high number of adaptive acquired genes (antibiotic resistance, virulence) in clade A1 strains compared with clade A2 and clade B. The differences in genome size and the content in acquired genetic material might have also contributed to the differences in fitness of strains of Clade B (ASEfm) and clade A1 (AREfm), the former ones having a best fitness values as is described in chapter 7. It has always been stated that de acquisition of an antibiotic resistance trait (point mutation or by horizontal gene transfer) is associated with the loss of fitness by the bacterial host (99, 100, 109–112).. We could hypothesize that the loss of fitness was due to ampicillin resistance as we did not find any significant differences in fitness between VSE and VRE in this study. The absence of significant differences between VSE and VRE wild type strains might be related with the tight regulation of Tn*1546* by the two component system *vanS-vanR* impeding the expression of vancomycin resistance when no inductor is

present and also by the fact that the plasmids carrying Tn*1546* seem to be highly adapted to certain *E. faecium* clonal backgrounds (99, 100)

Finally, another indicative of *E. faecium* ubiquitousness and adaptability is its better fitness when growth at 42ºC when compared at 37ºC. Even though *E. faecium* has been mostly recovered from the human gut, this species is also a member of the gut flora of animals with higher body temperatures than humans as such as pigs (38.8ºC) and chickens (41.8ºC) (113, 114).

References

1. Thiercelin M, Jouhaud L. 1899. *Sur un diplococque saprophyte de l'intestin susceptible de devenir pathogene.* CR French Soc Biol 5:269–271.

2. Lebreton F, Willems RJL, Gilmore MS. 2014. *Enterococcus Diversity, Origins in Nature, and Gut Colonization. In* Gilmore, MS, Clewell, DB, Ike, Y, Shankar, N (eds.), *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection.* Massachusetts Eye and Ear Infirmary, Boston: Massachusetts Eye and Ear Infirmary.

3. Mundt JO. 1963. *Occurrence of enterococci in animals in a wild environment*. Appl Microbiol 11:136–40.

4. Coque TM, Arduino RC, Murray BE. 1995. *High-level resistance to aminoglycosides: comparison of community and nosocomial fecal isolates of enterococci.* Clin Infect Dis 20:1048–51.

5. Donskey CJ. 2004. *The role of the intestinal tract as a reservoir and source for transmission of nosocomial pathogens.* Clin Infect Dis 39:219–26.

6. Woodmansey EJ. 2007. *Intestinal bacteria and ageing*. J Appl Microbiol 102:1178–86.

7. Ubeda C, Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, Viale A, Socci ND, van den Brink MRM, Kamboj M, Pamer EG. 2010. *Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans*. J Clin Invest 120:4332–41.

8. Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, Fleisher M, Schnabl B, DeMatteo RP, Pamer EG. 2008. *Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits*. Nature 455:804–7.

9. O'Sullivan O, Coakley M, Lakshminarayanan B, Conde S, Claesson MJ, Cusack S, Fitzgerald AP, O'Toole PW, Stanton C, Ross RP. 2013. *Alterations in intestinal microbiota of elderly Irish subjects post-antibiotic therapy.* J Antimicrob Chemother 68:214–21.

10. Devriese LA, Laurier L, De Herdt P, Haesebrouck F. 1992. *Enterococcal and streptococcal species isolated from faeces of calves, young cattle and dairy cows*. J Appl Bacteriol 72:29–31.

11. Aarestrup FM, Butaye P, Witte W. 2002. *Non-human reservoirs of enterococci*, p. 55–99. *In Enterococci : Pathogenesis, Molecular Biology, and Antibiotic Resistance.* American Society for Microbiology, Washington, DC, USA.

12. Guttman DS, Stavrinides J. 2010. *Population Genomics of Bacteria*, p. 121–152. *In* Robinson, DA, Falush, D, Feil, EJ (eds.), *Bacterial Population Genetics in Infectious Disease*. Wiley-Blackwell, Hoboken, NJ, USA.

13. Willems RJL, Top J, van Schaik W, Leavis H, Bonten M, Sirén J, Hanage WP, Corander J. 2012. *Restricted gene flow among hospital subpopulations of Enterococcus faecium*. MBio 3:e00151–12.

14. Tang J, Hanage WP, Fraser C, Corander J. 2009. *Identifying currents in the gene pool for bacterial populations using an integrative approach*. PLoS Comput Biol 5:e1000455.

15. Didelot X, Falush D. 2007. *Inference of bacterial microevolution using multilocus sequence data*. Genetics 175:1251–66.

16. Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems RJL, Earl AM, Gilmore MS. 2013. *Emergence of epidemic multidrug-resistant Enterococcus faecium from animal and commensal strains*. MBio 4:e00534–13.

17. Galloway-Peña J, Roh JH, Latorre M, Qin X, Murray BE. 2012. *Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of Enterococcus faecium*. PLoS One 7:e30187.

18. Howden BP, Holt KE, Lam MMC, Seemann T, Ballard S, Coombs GW, Tong SYC, Grayson ML, Johnson PDR, Stinear TP, Lindsay Grayson M, Johnson PDR, Stinear TP, Grayson ML, Johnson PDR, Stinear TP. 2013. *Genomic insights to control the emergence of vancomycin-resistant enterococci.* MBio 4:e00412–13.

19. Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock D a, Fridkin SK. 2008. *NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007.* Infect Control Hosp Epidemiol 29:996–1011.

20. European Centre for Disease Prevention and Control. 2014. *Antimicrobial resistance surveillance in Europe 2013.* Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm.

21. Johnson PDR, Ballard SA, Grabsch EA, Stinear TP, Seemann T, Young HL, Grayson ML, Howden BP. 2010. *A sustained hospital outbreak of vancomycin-resistant Enterococcus faecium bacteremia due to emergence of vanB E. faecium sequence type 203*. J Infect Dis 202:1278–86.

22. Gudiol C, Ayats J, Camoez M, Domínguez MÁ, García-Vidal C, Bodro M, Ardanuy C, Obed M, Arnan M, Antonio M, Carratalà J. 2013. *Increase in bloodstream infection due to vancomycin-susceptible Enterococcus faecium in cancer patients: risk factors, molecular epidemiology and outcomes*. PLoS One 8:e74734.

23. Sánchez-Díaz AM, Cuartero C, Rodríguez JD, Lozano S, Alonso JM, Rodríguez-Domínguez M, Tedim AP, Del Campo R, López J, Cantón R, Ruiz-Garbajosa P. 2016. *The rise of ampicillin-resistant Enterococcus faecium high-risk clones as a frequent intestinal colonizer in oncohaematological neutropenic patients on levofloxacin prophylaxis: a risk for bacteraemia?* Clin Microbiol Infect 22:59.e1–8.

24. Rice LB. 2005. *Antibiotics and gastrointestinal colonization by vancomycin-resistant enterococci.* Eur J Clin Microbiol Infect Dis 24:804–814.

25. Holland T, Fowler VG, Shelburne SA. 2014. *Invasive gram-positive bacterial infection in cancer patients.* Clin Infect Dis 59 Suppl 5:S331–4.

26. Taur Y, Xavier JB, Lipuma L, Ubeda C, Goldberg J, Gobourne A, Lee YJ, Dubin KA, Socci ND, Viale A, Perales M-A, Jenq RR, van den Brink MRM, Pamer EG. 2012. *Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation*. Clin Infect Dis 55:905–14.

27. Bonten MJ, Willems R, Weinstein RA. 2001. *Vancomycin-resistant enterococci: why are they here, and where do they come from?* Lancet Infect Dis 1:314–25.

28. Faith JJ, Colombel J-F, Gordon JI. 2015. *Identifying strains that contribute to complex diseases through the study of microbial inheritance*. Proc Natl Acad Sci U S A 112:633–40.

29. Baquero F, Coque TM. 2011. *Multilevel population genetics in antibiotic resistance*. FEMS Microbiol Rev 35:705–6.

30. Willems RJL, Hanage WP, Bessen DE, Feil EJ. 2011. *Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance.* FEMS Microbiol Rev 35:872–900.

31. Werner G, Coque TM, Hammerum AM, Hope R, Hryniewicz W, Johnson A, Klare I, Kristinsson KG, Leclercq R, Lester CH, Lillie M, Novais C, Olsson-Liljequist B, Peixe L V, Sadowy E, Simonsen GS, Top J, Vuopio-Varkila J, Willems RJ, Witte W, Woodford N. 2008. *Emergence and spread of vancomycin resistance among enterococci in Europe.* Euro Surveill 13:pii: 19046.

32. Arias CA, Murray BE. 2012. *The rise of the Enterococcus: beyond vancomycin resistance.* Nat Rev Microbiol 10:266–78.

33. Lam MMC, Seemann T, Tobias NJ, Chen H, Haring V, Moore RJ, Ballard S, Grayson LM, Johnson PDR, Howden BP, Stinear TP. 2013. *Comparative analysis of the complete genome of an epidemic hospital sequence type 203 clone of vancomycin-resistant Enterococcus faecium*. BMC Genomics 14:595.

34. Baquero F. 2011. *The 2010 Garrod Lecture: the dimensions of evolution in antibiotic resistance: ex unibus plurum et ex pluribus unum.* J Antimicrob Chemother 66:1659–72.

35. Chambers HF, Deleo FR. 2009. *Waves of resistance: Staphylococcus aureus in the antibiotic era.* Nat Rev Microbiol 7:629–41.

36. Nobel YR, Cox LM, Kirigin FF, Bokulich NA, Yamanishi S, Teitler I, Chung J, Sohn J, Barber CM, Goldfarb DS, Raju K, Abubucker S, Zhou Y, Ruiz VE, Li H, Mitreva M, Alekseyenko A V, Weinstock GM, Sodergren E, Blaser MJ. 2015. *Metabolic and metagenomic outcomes from early-life pulsed antibiotic treatment*. Nat Commun 6:7486.

37. Willems RJ, Top J, van Den Braak N, van Belkum A, Endtz H, Mevius D, Stobberingh E, van Den Bogaard A, van Embden JD. 2000. *Host specificity of vancomycin-resistant Enterococcus faecium.* J Infect Dis 182:816–23.

38. Homan WL, Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, Van Embden JDA, Willems RJL. 2002. *Multilocus sequence typing scheme for Enterococcus faecium.* J Clin Microbiol 40:1963–71.

39. Golubchik T, Batty EM, Miller RR, Farr H, Young BC, Larner-Svensson H, Fung R, Godwin H, Knox K, Votintseva A, Everitt RG, Street T, Cule M, Ip CLC, Didelot X, Peto TEA, Harding RM, Wilson DJ, Crook DW, Bowden R. 2013. *Within-host evolution of Staphylococcus aureus during asymptomatic carriage.* PLoS One 8:e61319.

40. Stanczak-Mrozek KI, Manne A, Knight GM, Gould K, Witney AA, Lindsay JA. 2015. *Within-host diversity of MRSA antimicrobial resistances.* J Antimicrob Chemother 70:2191–8.

41. Beiko RG, Harlow TJ, Ragan M a. 2005. *Highways of gene sharing in prokaryotes.* Proc Natl Acad Sci U S A 102:14332–7.

42. Halary S, Leigh JW, Cheaib B, Lopez P, Bapteste E. 2010. *Network analyses structure genetic diversity in independent genetic worlds.* Proc Natl Acad Sci 107:127–32.

43. Popa O, Dagan T. 2011. *Trends and barriers to lateral gene transfer in prokaryotes.* Curr Opin Microbiol 14:615–23.

44. Popa O, Hazkani-Covo E, Landan G, Martin W, Dagan T. 2011. *Directed networks reveal genomic barriers and DNA repair bypasses to lateral gene transfer among prokaryotes.* Genome Res 21:599–609.

45. DeMaere MZ, Williams TJ, Allen MA, Brown M V, Gibson JAE, Rich J, Lauro FM, Dyall-Smith M, Davenport KW, Woyke T, Kyrpides NC, Tringe SG, Cavicchioli R. 2013. *High level of intergenera gene exchange shapes the evolution of haloarchaea in an isolated Antarctic lake.* Proc Natl Acad Sci U S A 110:16939–44.

46. Toussaint A, Chandler M. 2012. *Bacterial Molecular Networks*. *Methods in molecular biology* (Clifton, N.J.). Springer New York, New York, NY.

47. Shen J, Wang Y, Schwarz S. 2013. *Presence and dissemination of the multiresistance gene cfr in Gram-positive and Gram-negative bacteria.* J Antimicrob Chemother 68:1697–706.

48. Bonafede ME, Carias LL, Rice LB. 1997. *Enterococcal transposon Tn5384: evolution of a composite transposon through cointegration of enterococcal and staphylococcal plasmids.* Antimicrob Agents Chemother 41:1854–8.

49. Hung W-C, Takano T, Higuchi W, Iwao Y, Khokhlova O, Teng L-J, Yamamoto T. 2012. *Comparative genomics of community-acquired ST59 methicillin-resistant Staphylococcus aureus in Taiwan: novel mobile resistance structures with IS1216V.* PLoS One 7:e46987.

50. Lindsay JA. 2014. *Staphylococcus aureus genomics and the impact of horizontal gene transfer.* Int J Med Microbiol 304:103–9.

51. Marraffini LA, Sontheimer EJ. 2010. *CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea*. Nat Rev Genet 11:181–90.

52. Johnston C, Martin B, Polard P, Claverys J-P. 2013. *Postreplication targeting of transformants by bacterial immune systems?* Trends Microbiol 21:516–21.

53. Clewell DB, Weaver KE, Coque TM, Dunny GM, Francia MV, Hayes F. 2014. *Extrachromosomal and Mobile Elements in Enterococci: Transmission, Maintenance, and Epidemiology*. *In* Gilmore, MS, Clewell, DB, Ike, Y, Shankar, N (eds.), *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary, Boston.

54. Freitas AR, Novais C, Tedim AP, Francia MVMV, Baquero F, Coque TM, Peixe L, Coque TM. 2013. *Microevolutionary Events Involving Narrow Host Plasmid Influences Local Fixation of Vancomycin-Resistance in Enterococcus.* PLoS One e60589.

55. Wardal E, Gawryszewska I, Hryniewicz W, Sadowy E. 2013. *Abundance and diversity of plasmid-associated genes among clinical isolates of Enterococcus faecalis.* Plasmid 70:329–42.

56. Manson JM, Hancock LE, Gilmore MS. 2010. *Mechanism of chromosomal transfer of Enterococcus faecalis pathogenicity island, capsule, antimicrobial resistance, and other traits*. Proc Natl Acad Sci U S A 107:12269–74.

57. Arias CA, Panesso D, Singh K V, Rice LB, Murray BE. 2009. *Cotransfer of antibiotic resistance genes and a hylEfm-containing virulence plasmid in Enterococcus faecium.* Antimicrob Agents Chemother 53:4240–6.

58. McBride SM, Fischetti VA, Leblanc DJ, Moellering RC, Gilmore MS. 2007. *Genetic diversity among Enterococcus faecalis.* PLoS One 2:e582.

59. Kuch A, Willems RJL, Werner G, Coque TM, Hammerum AM, Sundsfjord A, Klare I, Ruiz-Garbajosa P, Simonsen GS, van Luit-Asbroek M, Hryniewicz W, Sadowy E. 2012. *Insight into antimicrobial susceptibility and population structure of contemporary human Enterococcus faecalis isolates from Europe.* J Antimicrob Chemother 67:551–8.

60. Galloway-Peña JR, Nallapareddy SR, Arias CA, Eliopoulos GM, Murray BE. 2009. *Analysis of clonality and antibiotic resistance among early clinical isolates of Enterococcus faecium in the United States.* J Infect Dis 200:1566–73.

61. Mato R, de Lencastre H, Roberts RB, Tomasz A. 1996. *Multiplicity of genetic backgrounds among vancomycin-resistant Enterococcus faecium isolates recovered from an outbreak in a New York City hospital.* Microb Drug Resist 2:309–17.

62. Dunne WM, Wang W. 1997. *Clonal dissemination and colony morphotype variation of vancomycin-resistant Enterococcus faecium isolates in metropolitan Detroit, Michigan.* J Clin Microbiol 35:388–92.

63. Moreno F, Grota P, Crisp C, Magnon K, Melcher GP, Jorgensen JH, Patterson JE. 1995. *Clinical and molecular epidemiology of vancomycin-resistant Enterococcus faecium during its emergence in a city in southern Texas.* Clin Infect Dis 21:1234–7.

64. Donskey CJ, Schreiber JR, Jacobs MR, Shekar R, Salata RA, Gordon S, Whalen CC, Smith F, Rice LB. 1999. *A polyclonal outbreak of predominantly VanB vancomycin-resistant enterococci in northeast Ohio. Northeast Ohio Vancomycin-Resistant Enterococcus Surveillance Program.* Clin Infect Dis 29:573–9.

65. Bonten MJ, Hayden MK, Nathan C, van Voorhis J, Matushek M, Slaughter S, Rice T, Weinstein RA. 1996. *Epidemiology of colonisation of patients and environment with vancomycin-resistant enterococci.* Lancet (London, England) 348:1615–9.

66. Dobbs TE, Patel M, Waites KB, Moser SA, Stamm AM, Hoesley CJ. 2006. *Nosocomial spread of Enterococcus faecium resistant to vancomycin and linezolid in a tertiary care medical center*. J Clin Microbiol 44:3368–70.

67. Gordts B, Van Landuyt H, Ieven M, Vandamme P, Goossens H. 1995. *Vancomycin-resistant enterococci colonizing the intestinal tracts of hospitalized patients*. J Clin Microbiol 33:2842–6.

68. Sivertsen A, Billström H, Melefors Ö, Liljequist BO, Wisell KT, Ullberg M, Özenci V, Sundsfjord A, Hegstad K. 2014. *A multicentre hospital outbreak in Sweden caused by introduction of a vanB2 transposon into a stably maintained pRUM-plasmid in an Enterococcus faecium ST192 clone*. PLoS One 9:e103274.

69. McCracken M, Wong A, Mitchell R, Gravel D, Conly J, Embil J, Johnston L, Matlow A, Ormiston D, Simor AE, Smith S, Du T, Hizon R, Mulvey MR. 2013. *Molecular epidemiology of vancomycin-resistant enterococcal bacteraemia: results from the Canadian Nosocomial Infection Surveillance Program, 1999-2009.* J Antimicrob Chemother 68:1505–9.

70. Willems RJL. 2014. *Genetic Evolution of E. faecium and E. faecalis: Similarities and Differences4th ASM Conference on Enterococci.* American Society for Microbiology, Cartagena, Colombia.

71. Kim WJ, Weinstein RA, Hayden MK. 1999. *The changing molecular epidemiology and establishment of endemicity of vancomycin resistance in enterococci at one hospital over a 6-year period.* J Infect Dis 179:163–71.

72. Freitas AR, Novais C, Ruiz-Garbajosa P, Coque TM, Peixe L. 2009. *Dispersion of multidrug-resistant Enterococcus faecium isolates belonging to major clonal complexes in different Portuguese settings.* Appl Environ Microbiol 75:4904–8.

73. Mato R, Almeida F, Pires R, Rodrigues P, Ferreira T, Santos-Sanches I. 2009. *Assessment of high-level gentamicin and glycopeptide-resistant Enterococcus faecalis and E. faecium clonal structure in a Portuguese hospital over a 3-year period.* Eur J Clin Microbiol Infect Dis 28:855–859.

74. Wyllie D, Paul J, Crook D. 2011. *Waves of trouble: MRSA strain dynamics and assessment of the impact of infection control.* J Antimicrob Chemother 66:2685–8.

75. Price LB, Stegger M, Hasman H, Aziz M, Larsen J, Andersen PS, Pearson T, Waters AE, Foster JT, Schupp J, Gillece J, Driebe E, Liu CM, Springer B, Zdovc I, Battisti A, Franco A, Zmudzki J, Schwarz S, Butaye P, Jouy E, Pomba C, Porrero MC, Ruimy R, Smith TC, Robinson DA, Weese JS, Arriola CS, Yu F, Laurent F, Keim P, Skov R, Aarestrup FM. 2012. *Staphylococcus aureus CC398: host adaptation and emergence of methicillin resistance in livestock*. MBio 3:e00305–11–.

76. Robinson DA, Kearns AM, Holmes A, Morrison D, Grundmann H, Edwards G, O'Brien FG, Tenover FC, McDougal LK, Monk AB, Enright MC. 2005. *Re-emergence of early pandemic Staphylococcus aureus as a community-acquired meticillin-resistant clone.* Lancet 365:1256–1258.

77. de Regt MJA, van Schaik W, van Luit-Asbroek M, Dekker HAT, van Duijkeren E, Koning CJM, Bonten MJM, Willems RJL. 2012. *Hospital and community ampicillin-resistant Enterococcus faecium are evolutionarily closely linked but have diversified through niche adaptation.* PLoS One 7:e30319.

78. Li M, Du X, Villaruz AE, Diep BA, Wang D, Song Y, Tian Y, Hu J, Yu F, Lu Y, Otto M. 2012. *MRSA epidemic linked to a quickly spreading colonization and virulence determinant.* Nat Med 18:816–9.

79. Grady R, Hayes F. 2003. *Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multidrug-resistant, clinical isolate of Enterococcus faecium.* Mol Microbiol 47:1419–32.

80. Leclercq R, Derlot E, Duval J, Courvalin P. 1988. *Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium.* N Engl J Med 319:157–61.

81. Sletvold H, Johnsen PJ, Wikmark O-G, Simonsen GS, Sundsfjord A, Nielsen KM. 2010. *Tn1546 is part of a larger plasmid-encoded genetic unit horizontally disseminated among clonal Enterococcus faecium lineages*. J Antimicrob Chemother 65:1894–906.

82. Rosvoll TCS, Pedersen T, Sletvold H, Johnsen PJ, Sollid JE, Simonsen GS, Jensen LB, Nielsen KM, Sundsfjord A. 2010. *PCR-based plasmid typing in Enterococcus faecium strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHTbeta-related replicons associated with glycopeptide resistance and stabilizing toxin-antitoxin systems.* FEMS Immunol Med Microbiol 58:254–68.

83. Garcia-Migura L, Sanchez-Valenzuela AJ, Jensen LB. 2011. *Presence of glycopeptide-encoding plasmids in enterococcal isolates from food and humans in Denmark.* Foodborne Pathog Dis 8:1191–7.

84. Sletvold H, Johnsen PJ, Simonsen GS, Aasnaes B, Sundsfjord A, Nielsen KM. 2007. *Comparative DNA analysis of two vanA plasmids from Enterococcus faecium strains isolated from poultry and a poultry farmer in Norway.* Antimicrob Agents Chemother 51:736–9.

85. Johnsen PJ, Østerhus JI, Sletvold H, Sørum M, Kruse H, Nielsen K, Simonsen GS, Sundsfjord A. 2005. *Persistence of animal and human glycopeptide-resistant enterococci on two Norwegian poultry farms formerly exposed to avoparcin is associated with a widespread plasmid-mediated vanA element within a polyclonal enterococcus faecium population.* Appl Environ Microbiol 71:159–68.

86. Wardal E, Markowska K, Zabicka D, Wróblewska M, Giemza M, Mik E, Połowniak-Pracka H, Woźniak A, Hryniewicz W, Sadowy E. 2014. *Molecular analysis of vanA outbreak of Enterococcus faecium in two Warsaw hospitals: the importance of mobile genetic elements*. Biomed Res Int 2014:575367.

87. Freitas AR, Tedim AP, Novais C, Ruiz-Garbajosa P, Werner G, Laverde-Gomez JA, Cantón R, Peixe L, Baquero F, Coque TM. 2010. *Global spread of the hyl(Efm) colonization-virulence gene in megaplasmids of the Enterococcus faecium CC17 polyclonal subcluster*. Antimicrob Agents Chemother 54:2660–5.

88. Rosvoll TCS, Lindstad BL, Lunde TM, Hegstad K, Aasnaes B, Hammerum AM, Lester CH, Simonsen GS, Sundsfjord A, Pedersen T. 2012. *Increased high-level gentamicin resistance in invasive Enterococcus faecium is associated with aac(6')Ie-aph(2")Ia-encoding transferable megaplasmids hosted by major hospital-adapted lineages.* FEMS Immunol Med Microbiol 66:166–76.

89. Laverde Gomez JA, van Schaik W, Freitas AR, Coque TM, Weaver KE, Francia MV, Witte W, Werner G. 2011. *A multiresistance megaplasmid pLG1 bearing a hylEfm genomic island in hospital Enterococcus faecium isolates*. Int J Med Microbiol 301:165–75.

90. Tomita H, Pierson C, Lim SK, Clewell DB, Ike Y. 2002. *Possible connection between a widely disseminated conjugative gentamicin resistance (pMG1-like) plasmid and the emergence of vancomycin resistance in Enterococcus faecium.* J Clin Microbiol 40:3326–33.

91. Tomita H, Tanimoto K, Hayakawa S, Morinaga K, Ezaki K, Oshima H, Ike Y. 2003. *Highly conjugative pMG1-like plasmids carrying Tn1546-like transposons that encode vancomycin resistance in Enterococcus faecium.* J Bacteriol 185:7024–8.

92. Freitas AR, Novais C, Ruiz-Garbajosa P, Coque TM, Peixe L. 2009. *Clonal expansion within clonal complex 2 and spread of vancomycin-resistant plasmids among different genetic lineages of Enterococcus faecalis from Portugal.* J Antimicrob Chemother 63:1104–11.

93. Werner G, Freitas AR, Coque TM, Sollid JE, Lester C, Hammerum AM, Garcia-Migura L, Jensen LB, Francia M V., Witte W, Willems RJ, Sundsfjord A. 2011. *Host range of enterococcal vanA plasmids among Gram-positive intestinal bacteria.* J Antimicrob Chemother 66:273–282.

94. Galloway-Peña JR, Rice LB, Murray BE. 2011. *Analysis of PBP5 of early U.S. isolates of Enterococcus faecium: sequence variation alone does not explain increasing ampicillin resistance over time.* Antimicrob Agents Chemother 55:3272–7.

95. Grayson ML, Eliopoulos GM, Wennersten CB, Ruoff KL, De Girolami PC, Ferraro MJ, Moellering RC. 1991. *Increasing resistance to beta-lactam antibiotics among clinical isolates of Enterococcus faecium: a 22-year review at one institution.* Antimicrob Agents Chemother 35:2180–4.

96. Novais A, Comas I, Baquero F, Cantón R, Coque TM, Moya A, González-Candelas F, Galán J-C. 2010. *Evolutionary trajectories of beta-lactamase CTX-M-1 cluster enzymes: predicting antibiotic resistance.* PLoS Pathog 6:e1000735.

97. Rice LB, Carias LL, Hutton-Thomas R, Rudin S. 2007. *Interaction of related Tn916-like transposons: analysis of excision events promoted by Tn916 and Tn5386 integrases*. J Bacteriol 189:3909–17.

98. Nogueira T, Rankin DJ, Touchon M, Taddei F, Brown SP, Rocha EPCC. 2009. *Horizontal gene transfer of the secretome drives the evolution of bacterial cooperation and virulence.* Curr Biol 19:1683–1691.

99. Foucault M-L, Courvalin P, Grillot-Courvalin C. 2009. *Fitness cost of VanA-type vancomycin resistance in methicillin-resistant Staphylococcus aureus.* Antimicrob Agents Chemother 53:2354–9.

100. Starikova I, Al-Haroni M, Werner G, Roberts AP, Sørum V, Nielsen KM, Johnsen PJ. 2013. *Fitness costs of various mobile genetic elements in Enterococcus faecium and Enterococcus faecalis.* J Antimicrob Chemother 68:2755–65.

101. Courvalin P. 2006. *Vancomycin resistance in gram-positive cocci*. Clin Infect Dis 42 Suppl 1:S25–34.

102. Zielenkiewicz U, Ceglowski P. 2005. *The toxin-antitoxin system of the streptococcal plasmid pSM19035.* J Bacteriol 187:6094–105.

103. Lioy VS, Pratto F, de la Hoz AB, Ayora S, Alonso JC. 2010. *Plasmid pSM19035, a model to study stable maintenance in Firmicutes*. Plasmid 64:1–17.

104. Andersson DI, Hughes D. 2010. *Antibiotic resistance and its cost: is it possible to reverse resistance?* Nat Rev Microbiol 8:260–71.

105. Björkholm B, Sjölund M, Falk PG, Berg OG, Engstrand L, Andersson DI. 2001. *Mutation frequency and biological cost of antibiotic resistance in Helicobacter pylori.* Proc Natl Acad Sci U S A 98:14607–12.

106. Harrison E, Guymer D, Spiers AJ, Paterson S, Brockhurst MA. 2015. *Parallel compensatory evolution stabilizes plasmids across the parasitism-mutualism continuum.* Curr Biol 25:2034–9.

107. Maisnier-Patin S, Berg OG, Liljas L, Andersson DI. 2002. *Compensatory adaptation to the deleterious effect of antibiotic resistance in Salmonella typhimurium*. Mol Microbiol 46:355–366.

108. Kim EB, Jin G-D, Lee J-Y, Choi Y-J. 2016. *Genomic Features and Niche-Adaptation of Enterococcus faecium Strains from Korean Soybean-Fermented Foods*. PLoS One 11:e0153279.

109. Andersson DI, Levin BR. 1999. *The biological cost of antibiotic resistance*. Curr Opin Microbiol 2:489–93.

110. Björkman J, Andersson DI. 2000. *The cost of antibiotic resistance from a bacterial perspective.* Drug Resist Updat 3:237–245.

111. Johnsen PJ, Townsend JP, Bøhn T, Simonsen GS, Sundsfjord A, Nielsen KM. 2009. *Factors affecting the reversal of antimicrobial-drug resistance*. Lancet Infect Dis 9:357–64.

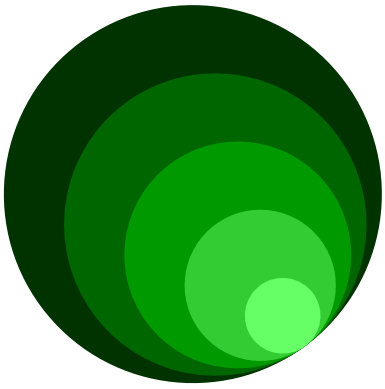
112. Johnsen PJ, Townsend JP, Bøhn T, Simonsen GS, Sundsfjord A, Nielsen KM. 2011. *Retrospective evidence for a biological cost of vancomycin resistance determinants in the absence of glycopeptide selective pressures*. J Antimicrob Chemother 66:608–10.

113. Ingram DL, Legge KF. 1970. *Variations in deep body temperature in the young unrestrained pig over the 24 hour period*. J Physiol 210:989–98.

114. Bolzani R, Ruggeri F, Olivo OM. 1979. [*Average normal temperature of the chicken in the morning and after 1-2 days of fasting*]. Boll della Soc Ital di Biol Sper 55:1618–22.

No thief, however skillful, can rob one of knowledge, and that is why knowledge is the best and safest treasure to acquire.

L. Frank Baum



Conclusions

1. Different enterococcal populations were predominant in the microbiota of hospitalized and non-hospitalized individuals of different age groups, *E. faecium* being the most common one in elderly. Intraspecies diversity was also demonstrated; ampicillin susceptible *E. faecium* of BAPS 1 and BAPS 3.3b were associated with non-hospitalized individuals while ampicillin resistant *E. faecium* of BAPS groups 2.1a and BAPS 3.3a were common among hospitalized patients (Chapter 1).

2. The high clonal diversity of *E. faecium* able to cause BSI pointed out the human gut as the origin of such infections. Changes in the GI tract microbiota of hospitalized patients due to host conditions (e.g. age) or external factors (e.g. antibiotic treatment and/or other selective pressures in the hospital setting), appears to have facilitated the selection and the consequent increase in the population size of antibiotic resistant *E. faecium* clones, leading to a shift in the composition of *E. faecium* populations that increased the chances to be infected by MDR strains and to transmit MDR strains in the hospital setting (Chapter 2).

3. The variable occurrence of certain *E. faecium* clones in individuals of different age and origin in a kind of source-sink dynamics, with frequent cases of coexistence, and preservation of rare clonal populations, suggested a frequency-dependent evolution of enterococcal populations, which could prevent the extinction of genotypes with specific ecological roles in the bacterial community that exploited particular environments (Chapters 1 and 2).

4. The asymmetric number and type of plasmids associated with disparate populations of *E. faecium* suggested an evolutionary co-adaptation between plasmids and their clonal background. The high number of plasmids in specialized *E. faecium* lineages that are most frequently recovered from hospitalized patients highlights its influence in the rapid adaptation, selection and persistence of specialized populations under changing conditions as antibiotic treatments (Chapter 3).

5. Both convergent evolution and horizontal gene transfer of *pbp5* seem to be responsible for the blooming of ampicillin resistant *E. faecium.* Fluctuations in the population size of ampicillin resistant *E. faecium* lineages due to selective pressure exerted by the increased use of β-lactams in hospitals, and further fixation of different ampicillin resistant *E. faecium* clonal lineages within dynamic landscapes, may explain their quantitative success (Chapter 6).

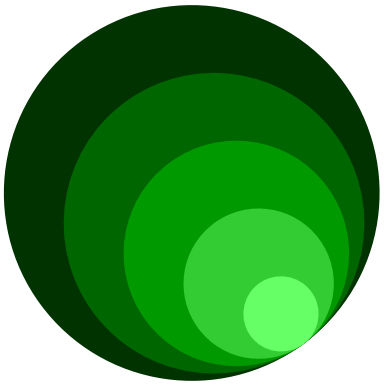
6. The spread of vancomycin resistance in *E. faecium* is mainly due to narrow (pRUM) and broad host (Inc18) plasmids in the US and Europe, respectively. Moreover, the diversity of Tn*1546-vanA* carrying plasmids, mostly chimeras of narrow and broad host range plasmids, reflects the frequent HGT events between populations of enterococci and other *Firmicutes*. The ability of Tn*1546-vanA* carrying plasmids to transfer but also to recombine provides a key-variability component in the basic scenario of coevolvability between plasmids and hosts. In this interactive landscape HGT played a relevant role in shaping the transmission dynamics of vancomycin-resistance determinants among highly related “central” clonal backgrounds that have already

been circulating for decades. Such transmission dynamics would also explain the unexpected diversity of plasmids and plasmid chimeras even in isolates belonging to the same clone, reflecting secondary micro-evolutionary events (Chapters 4 and 5).

7. The narrow host range of the predominant Tn*1546*-harbouring plasmids in combination with the fact that the majority of them confer a significant burden to the host strain and a remarkable stability in the absence of selective pressure, might explain, up to some extent, their long-term maintenance and continuous transfer among *E. faecium* populations, perpetuating the presence of VRE worldwide (Chapter 7).

Write what you know. That should leave you with a lot of free time.

Howard Nemerov



Annex

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Population Biology of Intestinal *Enterococcus* Isolates from Hospitalized and Nonhospitalized Individuals in Different Age Groups

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The diversity of enterococcal populations from fecal samples from hospitalized ($n = 133$) and nonhospitalized individuals ($n = 173$) of different age groups (group I, ages 0 to 19 years; group II, ages 20 to 59 years; group III, ages ≥60 years) was analyzed. Enterococci were recovered at similar rates from hospitalized and nonhospitalized persons (77.44% to 79.77%) of all age groups (75.0% to 82.61%). *Enterococcus faecalis* and *Enterococcus faecium* were predominant, although seven other *Enterococcus* species were identified. *E. faecalis* and *E. faecium* (including ampicillin-resistant *E. faecium*) colonization rates in nonhospitalized persons were age independent. For inpatients, *E. faecalis* colonization rates were age independent, but *E. faecium* colonization rates (particularly the rates of ampicillin-resistant *E. faecium* colonization) significantly increased with age. The population structure of *E. faecium* and *E. faecalis* was determined by superimposing goeBURST and Bayesian analysis of the population structure (BAPS). Most *E. faecium* sequence types (STs; 150 isolates belonging to 75 STs) were linked to BAPS groups 1 (22.0%), 2 (31.3%), and 3 (36.7%). A positive association between hospital isolates and BAPS subgroups 2.1a and 3.3a (which included major ampicillin-resistant *E. faecium* human lineages) and between community-based ampicillin-resistant *E. faecium* isolates and BAPS subgroups 1.2 and 3.3b was found. Most *E. faecalis* isolates (130 isolates belonging to 58 STs) were grouped into 3 BAPS groups, BAPS groups 1 (36.9%), 2 (40.0%), and 3 (23.1%), with each one comprising widespread lineages. No positive associations with age or hospitalization were established. The diversity and dynamics of enterococcal populations in the fecal microbiota of healthy humans are largely unexplored, with the available knowledge being fragmented and contradictory. The study offers a novel and comprehensive analysis of enterococcal population landscapes and suggests that *E. faecium* populations from hospitalized patients and from community-based individuals differ, with a predominance of certain clonal lineages, often in association with elderly individuals, occurring in the hospital setting.

Enterococci are relatively minor constituents of the human gastrointestinal microbiota (less than 1%) but are able to cause a wide diversity of infections, mostly in patients with underlying diseases (1, 2). High-density colonization by antibiotic-resistant enterococci increases the risk of bacteremia and transmission; however, the population structure and ecological and evolutionary forces influencing the population dynamics of gut colonizers largely remain unknown (3–5). Next-generation sequencing has provided a wealth of data about the influence of characteristics of the host (age, diet, health status, and antibiotic treatment) on the diversity and population frequency of different bacterial groups, including enterococci (6–10). However, the information provided by current metagenomic analysis, based on 16S rRNA (11, 12), or by the traditional culture-based studies (1, 13, 14) precludes any possible analysis of enterococci at the subspecies level. Furthermore, the available information about the frequency and diversity of enterococcal species in the fecal microbiota by host age is fragmented and contradictory (1, 15).

Different methods based on multilocus sequence typing (MLST), comparative genomic hybridization, and whole-genome sequencing revealed intraspecies diversity for *Enterococcus faecalis* and *Enterococcus faecium*, which are the predominant enterococcal species colonizing the human gastrointestinal tract (16–25). *E. faecium* has a population structure that has split into two major phylogenomic clusters, designated clade B, which includes community-based human isolates, and clade A, which comprises isolates from humans and animals, with a clade A1 being enriched with isolates from hospitalized patients. Specifically, strains belonging to the sequence type (ST) 17 (ST17), ST18, and ST78 lineages, which are found within clade A1, are often resistant to antibiotics and are the most frequently associated with the hospital environment (22, 26, 27). *E. faecalis*, on the other hand, seems to lack such a clear clade structure, probably because this species occupies a larger variety of ecological microniches, thus having access to a more heterogeneous spectrum of alleles than *E. faecium* (28). As a result, no clear genotypic differences are observed between hospital and community isolates (25, 28, 29), even though

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some clonal complexes (CCs) are more prevalent either among hospitalized patients, e.g., CC6-ST6, CC9-ST9, CC28-ST87, and CC40-ST40, or among community healthy volunteers, e.g., ST16 and CC58 (30–33). Recombination, which was previously detected in enterococci (17, 34, 35), may have a considerable impact on patterns of evolutionary descent, as displayed by sequence-based gene trees or even by popular allele-based population snapshots provided by eBURST analysis. This may obscure the genetic relatedness of strains and clones and, as such, interfere with epidemiological and clinical investigations, in particular, when strains are assigned to specific CCs. In addition, knowledge about the population structure of enterococcal species is biased by an overrepresentation of contemporary multidrug-resistant (MDR) clinical isolates belonging to a few high-risk clonal complexes often associated with nosocomial outbreaks and frequently associated with elderly individuals (36–38). Studies analyzing early isolates have documented a more diverse enterococcal population able to cause disease acquired either nosocomially or in the community and often associated with nonelderly adults and children. Isolates causing infections or colonizing these populations have less frequently been analyzed at the molecular level (33, 39–41).

The objective of this study was to assess for the first time the population structure of enterococci in the feces of both hospitalized and nonhospitalized individuals within different age groups. In addition, Bayesian analysis of the population structure (BAPS), a nonphylogenetic method able to find the best partition of a set of isolates into subpopulations, was applied, broadening former results obtained for *E. faecium* and providing the first analysis to probabilistically assign *E. faecalis* strains to evolutionary groups.

MATERIALS AND METHODS

Bacterial samples. Three hundred six fecal samples were collected between April 2009 and April 2011 at the Ramón y Cajal University Hospital (HRyC) and its community care area of influence. HRyC is a tertiary care public hospital with 1,155 beds that provides specialized attention to a population of about 600,000 habitants in the northern area of Madrid, Spain, who primarily attend 20 primary health centers (PHCs) of the Madrid Health Service (SERMAS). This study was conducted according to applicable government regulations and approved studies by institutional research policies (e.g., reference CEIC-106/09 [A. M. Sánchez-Díaz, C. Cuartero, J. D. Rodríguez, S. Lozano, J. M. Alonso, M. J. Rodríguez-Domínguez, A. P. Tedim, R. del Campo, J. López, R. Cantón, and P. Ruiz-Garbajosa, unpublished data]).

The samples analyzed were recovered from 173 patients with nonsevere diseases that attended a PHC or had a consultation at HRyC (and for whom no hospitalization was registered in the 6 months prior to sample collection) and from 133 hospitalized patients admitted to HRyC. The fecal samples were submitted to HRyC for stool culture with or without a specific request for *Clostridium difficile* or parasite detection and were anonymously processed so that the patients' demographic information was kept confidential. Hospitalized patients were mostly located in medical wards (78.2%), surgical wards (8.3%), and intensive care units (ICUs; 9.8%). All but 20 samples from hospitalized patients were collected after more than 48 h of hospital admission. However, these 20 patients had a history of several recent previous hospitalizations (see Tables S1 and S2 in the supplemental material).

Samples were also classified into three age groups according to the host's age. These three groups are designated with roman numerals as group I (young people 0 to 19 years old, $n = 92$ [30%], 57 nonhospitalized persons and 35 hospitalized patients), group II (adults 20 to 59 years old, $n = 108$ [35%], 62 nonhospitalized persons and 46 hospitalized patients), and group III (elderly individuals ≥60 years old, $n = 106$ [35%], 54 nonhospitalized persons and 52 hospitalized patients). Only one sample per patient was analyzed (see Tables S1 and S2 in the supplemental material).

Sample processing. About 0.5 g of each fecal sample was suspended in 1 ml of saline solution, plated on plain m-*Enterococcus* agar (Difco, Detroit, MI, USA) or m-*Enterococcus* agar supplemented with either ampicillin (10 μg/ml) or vancomycin (6 μg/ml), and incubated for 48 h at 37°C. For each sample, one colony per morphology type and plate was selected (28) for further studies. In order to enhance the recovery of minority populations of ampicillin-resistant enterococci and vancomycin-resistant enterococci (VRE), 0.1 ml of the original suspension of each sample was preenriched in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) supplemented with 2 μg/ml of ampicillin or 2 μg/ml of vancomycin, incubated for 24 h at 37°C, and subsequently plated on m-*Enterococcus* agar (Difco, Detroit, MI, USA) containing ampicillin (10 μg/ml) or vancomycin (6 μg/ml), respectively.

Identification, antibiotic susceptibility, and virulence traits. Bacterial identification was performed by the amplification of species-specific genes, D-alanine–D-alanine ligase (*ddl*) for *E. faecalis* and *aac(6′)-Ii* for *E. faecium*, as previously described (42, 43), and by matrix-assisted laser desorption ionization–time of flight mass spectrometry (Bruker, Daltonics, Bremen, Germany). Susceptibility to ampicillin, vancomycin, teicoplanin, streptomycin, gentamicin, ciprofloxacin, levofloxacin, erythromycin, tetracycline, and chloramphenicol (Oxoid, Basingstoke, United Kingdom) was determined by the disc diffusion method according to CLSI guidelines (44).

The presence of putative virulence genes encoding the *E. faecium* enterococcal surface protein (*esp*), glycosyl hydrolase ($hyl_{E.\ faecium}$), and collagen-binding adhesin (*acm*) and the *E. faecalis* enterococcal surface protein (*esp*), hyaluronidase ($hyl_{E.\ faecalis}$), cytolysin/hemolysin (*cylA*), gelatinase (*gelE*), and aggregation substance (*asa1*) was investigated by PCR and sequencing, as described before (45, 46).

Clonal relatedness. The clonal relationship among isolates of each enterococcal species was established by pulsed-field gel electrophoresis (PFGE) and MLST as previously described (16, 47), and the relationships are detailed in Tables S1 and S2 in the supplemental material. Clusters of related STs for *E. faecalis* (differing in no more than two of the seven MLST loci) were considered to belong to the same CC using the goeBURST algorithm (48, 49). CCs were defined on the basis of goeBURST analysis of the 524 STs present in the *E. faecalis* MLST database (http://efaecalis.mlst.net/).

Analysis of population structure. A BAPS software was used to probabilistically assign *E. faecalis* and *E. faecium* STs to nonoverlapping evolutionary groups (27, 50). BAPS clustering was performed with the second-order Markov model and the standard MLST data input option in a hierarchical manner. For *E. faecium*, the major clusters identified at the first stage were reanalyzed after excluding the remaining data. The rationale for this approach is to increase the statistical power to detect the more fine-scale genetic structure of a population when analyzing particular lineages separately from the remaining population. In all BAPS analyses, 10 runs of the estimation algorithm were performed using *a priori* upper bounds (10 to 30 for the major group analysis and 2 to 10 for the subgroup analysis) for the number of clusters over the interval, and in each case the runs converged to a nearly identical partition of the data in question, indicating a high level of peakedness of the posterior distribution (estimated $P = 1.000$).

The accuracy of BAPS for establishing the *E. faecium* population structure was determined using different sample sizes and discarding the inclusion of *E. faecalis* as the outgroup (see Fig. S1 to S3 in the supplemental material) (27). Correlation analysis was performed for each of the comparisons mentioned above using Microsoft Excel 2010 software. This study constitutes the first application of BAPS to investigate *E. faecalis* population and evolutionary genetics by the same approach that was previously used for *E. faecium* (27).

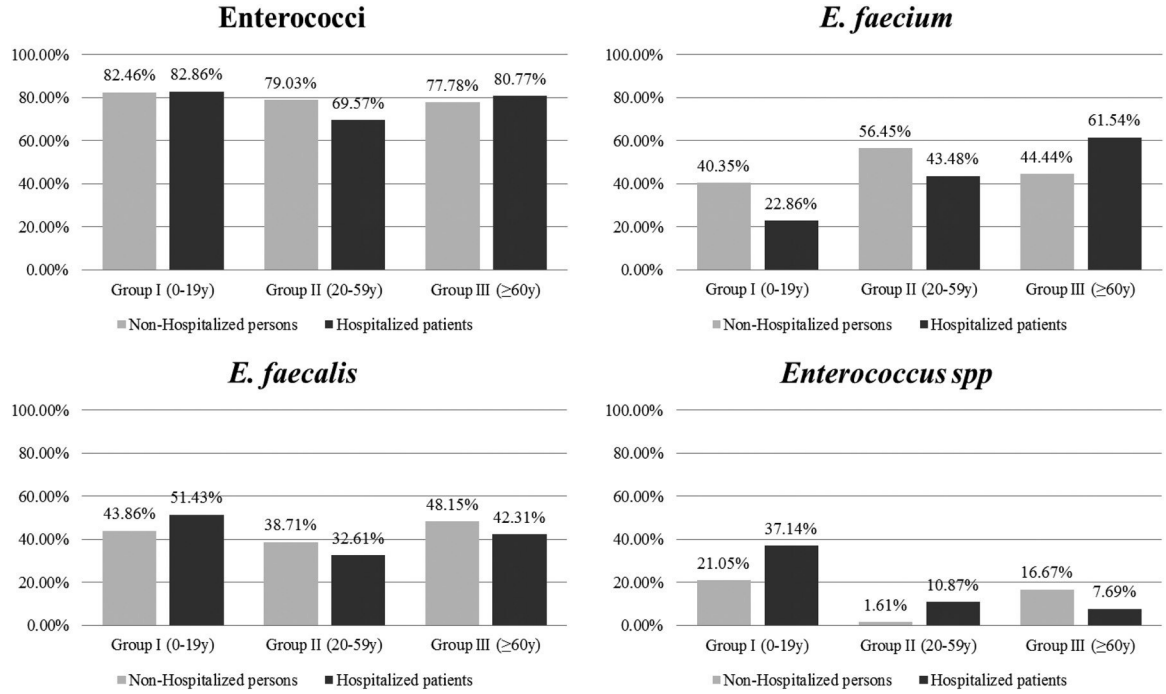

FIG 1 Proportion of nonhospitalized and hospitalized individuals colonized with enterococci by age group. The *E. faecalis*/*E. faecium* colonization ratios in nonhospitalized persons by age group were as follows: group I (0 to 19 years old), 1.08; group II (20 to 59 years old), 0.68; and group III (≥60 years old), 1.08. The *E. faecalis*/*E. faecium* colonization ratios in hospitalized patients were as follows: group I (0 to 19 years old), 2.25; group II (20 to 59 years old), 0.75; and group III (≥60 years old), 0.68.

Statistical analysis. The statistical significance of the results was calculated by the chi-square test; *P* values of <0.05 were considered statically significant.

The STATA generalized estimating equations (GEE) model (which takes into account clone-related data) (51) was used for calculating odd ratios (ORs) and 95% confidence intervals (CIs) related to the colonization isolates. The analyses were done by comparison with major BAPS subgroup 3.3a (BAPS 3.3a) for *E. faecium* and BAPS group 1 (BAPS 1) for *E. faecalis*.

For the analysis of all isolates available in MLST databases, ORs between BAPS groups and different sources (hospitalized patients, nonhospitalized persons, and animals) were calculated. Environmental, food, and other sources were also considered, but due to the low number of isolates in these categories, OR analysis was not performed.

RESULTS

Prevalence and diversity of enterococcal species in human fecal samples. Enterococci were recovered by culture from 78.8% of the individuals analyzed ($n = 241/306$) and at similar rates between hospitalized and nonhospitalized individuals (77.4% versus 79.8%) and among all age groups (75.0 to 82.6%). The enterococci recovered corresponded to three of the five groups of enterococci previously described by Facklam et al. (52) on the basis of phenotypic and genotypic characteristics, which used to be designated by roman numerals (1). The rate at which individuals were colonized by different species varied in each age group, with *E. faecalis* and *E. faecium* being the predominant species identified (Fig. 1 and 2). Among nonhospitalized persons, *E. faecalis* and *E. faecium* colonization rates were age independent (*E. faecalis*/*E. faecium* ratios, 1.14, 0.71, 1.12 for age groups I, II, and III, respectively). The *E. faecalis* colonization rate among hospitalized patients was also age independent, but the *E. faecium* colonization rate and particularly the ampicillin-resistant *E. faecium* colonization rate significantly ($P < 0.01$) increased with age (*E. faecalis*/*E. faecium* ratios, 1.90, 0.71, and 0.65 for age groups I, II, and III, respectively) (Fig. 1 and 3). Besides *E. faecium* and *E. faecalis*, both classified

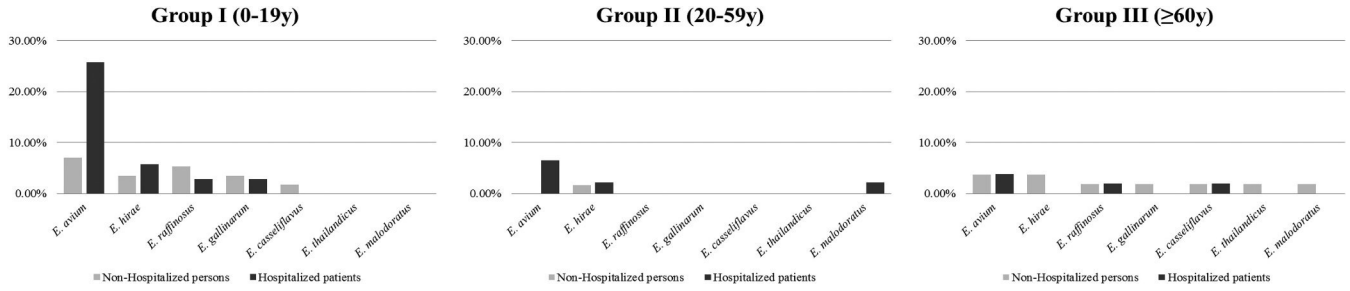

FIG 2 Proportion of nonhospitalized and hospitalized individuals colonized by different *Enterococcus* spp. by age group.

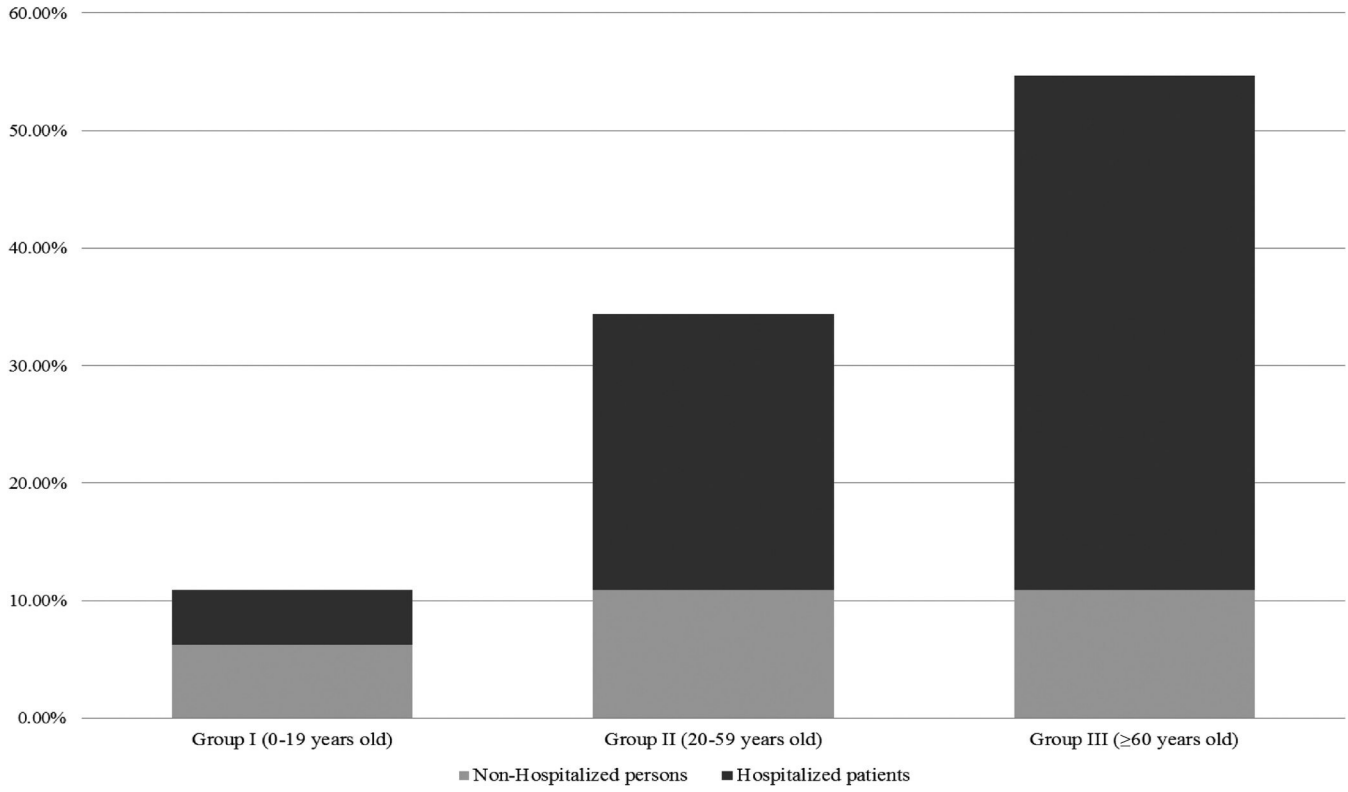

FIG 3 Proportion of nonhospitalized and hospitalized individuals colonized by ampicillin-resistant *E. faecium* by age group. The proportions of hospitalized/nonhospitalized individuals colonized by ampicillin-resistant *E. faecium* were as follows for the different age groups: group I (0 to 19 years old), 0.75; group II (20 to 59 years old), 2.14; and group III (≥60 years old), 4.

within enterococcal group II described by Facklam et al. (52), other species within enterococcal groups I (20 *E. avium*, 7 *E. raffinosus*, 2 *E. malodoratus* isolates), II (4 *E. casseliflavus* isolates, 3 *E. gallinarum* isolates, 1 *E. thailandicus* isolate), and group III (8 *E. hirae* isolates) were identified (Fig. 2).

Colonization by more than one enterococcal species was a frequent event. The simultaneous recovery of both *E. faecalis* and *E. faecium* (13.7%, $n = 42/306$) was increasingly observed as age increased ($P < 0.01$) (for age groups I, II, and III, 6.5% [$n = 6/92$], 13.9% [$n = 15/108$], and 19.8% [$n = 21/106$], respectively), suggesting that the increased rate of *E. faecium* colonization in hospitalized patients in age groups II and III did not interfere with the *E. faecalis* colonization rate, although it might have influenced the *E. faecalis* clonal composition. Low rates of cocolonization by *E. faecalis* and *Enterococcus* spp. (5.23%, 16/306), *E. faecium* and *Enterococcus* spp. (4.25%, 13/306), or *E. faecalis*, *E. faecium*, and other enterococcal species (0.65%, 2/306) were also detected.

Ampicillin resistance (22.2%, $n = 68/306$) was detected among *E. faecium* (94.1%, $n = 64/68$) and *E. raffinosus* (5.9%, $n = 4/68$) isolates. Ampicillin-resistant *E. faecium* isolates were more significantly associated with hospitalized patients (44.7%, $n = 46/103$) than with nonhospitalized individuals (13.0%, $n = 18/138$). A low number of individuals colonized with VRE, all of which were identified to be vancomycin-resistant *E. faecalis* isolates, was also detected (1.6%, $n = 5/306$, consisting of 2 nonhospitalized and 3 hospitalized individuals of different ages) (see Table S2 in the supplemental material).

The population structure of the *E. faecium* and *E. faecalis* isolates is detailed below. For other enterococci, isolates of the same species exhibited different PFGE types, with the exception of some *E. avium* isolates (data not shown). All these species were resistant to quinupristin-dalfopristin, were often resistant to erythromycin (*E. avium*, *E. hirae*, *E. raffinosus*, *E. gallinarum*, *E. casseliflavus*) and tetracycline (*E. raffinosus*), and eventually became resistant to levofloxacin (*E. raffinosus*, *E. gallinarum*) and high concentrations of streptomycin (*E. avium*, *E. raffinosus*, *E. gallinarum*) and gentamicin (*E. avium*, *E. raffinosus*).

BAPS analysis of *E. faecium* population structure. A BAPS analysis was used to infer the population structure of the *E. faecium* isolates according to previous findings that demonstrated that eBURST analysis is not sufficient to reliably delineate the patterns of recent evolutionary descent of *E. faecium* (27, 53). The analysis was repeated by taking into account the significant enlargement of the MLST database (http://efaecium.mlst.net/), in which the number of STs increased from 492 to 837 in the 2 years since the time of publication of the original 2012 study (27).

A hierarchical BAPS clustering analysis of the currently available 837 *E. faecium* STs representing 2,402 isolates of different origins yielded 8 BAPS groups. The majority of STs grouped in BAPS 1, BAPS 2, BAPS 3, and BAPS 7 (15.1%, 39.7%, 31.5%, and 8.5%, respectively), while BAPS 4, BAPS 5, BAPS 6, and BAPS 8 were much more infrequently detected (1.3%, 1.9%, 0.8% and 1.2%, respectively). BAPS nested analysis subdivided BAPS 1 into six subgroups (BAPSs 1.1 to 1.6) and BAPS 2 (BAPSs 2.1a, 2.1b, 2.3a, 2.3b), BAPS 3 (BAPSs 3.1, 3.2, 3.3a, 3.3b), and BAPS 7 (BAPSs 7.1 to 7.4) into four subgroups each (Table 1). The origi-

TABLE 1 *E. faecium* BAPS analysis data

BAPS group	BAPS subgroup	No. of STs	% STs	No. of isolates
BAPS 1	1.1	9	1.08	11
	1.2	61	7.29	100
	1.3	12	1.43	16
	1.4	2	0.24	2
	1.5	36	4.30	41
	1.6	6	0.72	6
	Subtotal	126	15.05	176
BAPS 2	2.1a	88	10.51	577
	2.1b	133	15.89	321
	2.3a	78	9.32	135
	2.3b	33	3.94	49
	Subtotal	332	39.67	1,082
BAPS 3	3.1	72	8.60	122
	3.2	28	3.35	59
	3.3a	107	12.78	679
	3.3b	57	6.81	92
	Subtotal	264	31.54	952
BAPS 4		11	1.31	11
BAPS 5		16	1.91	19
BAPS 6		7	0.84	9
BAPS 7	7.1	54	6.45	120
	7.2	6	0.72	6
	7.3	10	1.19	14
	7.4	1	0.12	2
	Subtotal	71	8.48	142
BAPS 8		10	1.19	11
Total		837		2,402

nal BAPS subgroups 2.1, 2.3, and 3.3 described by Willems et al. (27) have now been split into two subgroups each, and here these are arbitrarily designated BAPSs 2.1a and 2.1b, BAPSs 2.3a and 2.3b, and BAPSs 3.3a and 3.3b, for backwards compatibility (see Fig. S3 in the supplemental material).

Next, we analyzed the congruence between the BAPS grouping of the 492 STs using the BAPS assignment previously described by Willems et al. (27) and the BAPS grouping from this study. A correlation coefficient of 0.5958 indicates some discrepancies between the partitioning of the 492 STs. These discrepancies, probably related to the presence of an *E. faecalis* outgroup in the BAPS analysis of Willems et al. (27), are mostly due to STs that moved from BAPS 2 (15 STs), BAPS 3 (25 STs), and BAPS 5 (1 ST) in the 2012 study (27) to BAPS 7 in our study (see Fig. S1 in the supplemental material). Subsequently, the 492 *E. faecium* STs included in the work of Willems et al. (27) were compared to the BAPS grouping of the same 492 STs obtained using the extended *E. faecium* MLST database of 837 STs in order to infer the influence of the sample size on BAPS assignment. The correlation coefficient analysis revealed almost perfect correlations for the classification of BAPS groups (0.9996) and BAPS subgroups (0.9988) on the basis of 492 and 837 STs (see Fig. S2 and S3 in the supplemental material) and that only a small number of changes in BAPS assignment (44/837 STs, 5.2%) occurred at either the group or the subgroup level when the number of STs analyzed was significantly increased. This further indicates that, for *E. faecium*, BAPS analysis is both reproducible and robust and may accurately describe the *E. faecium* population structure.

Since the extended data set of 837 STs slightly changed the BAPS grouping of STs, we decided to recalculate the ORs to assess the significance of the association between the BAPS groups and the origins of the isolates (see Table S3 in the supplemental material). This revealed that isolates from hospitalized individuals were positively associated with BAPSs 2.1a and 3.3a and negatively associated with all other BAPS groups. Conversely, isolates of all BAPS groups from nonhospitalized individuals were negatively associated with BAPSs 2.1a and 3.3a but positively associated with BAPS 1.2 and BAPS 3.3b (Fig. 4).

Isolates of animal origin were negatively associated with BAPS 3.3a and BAPS 1.2 but showed a positive association with BAPSs

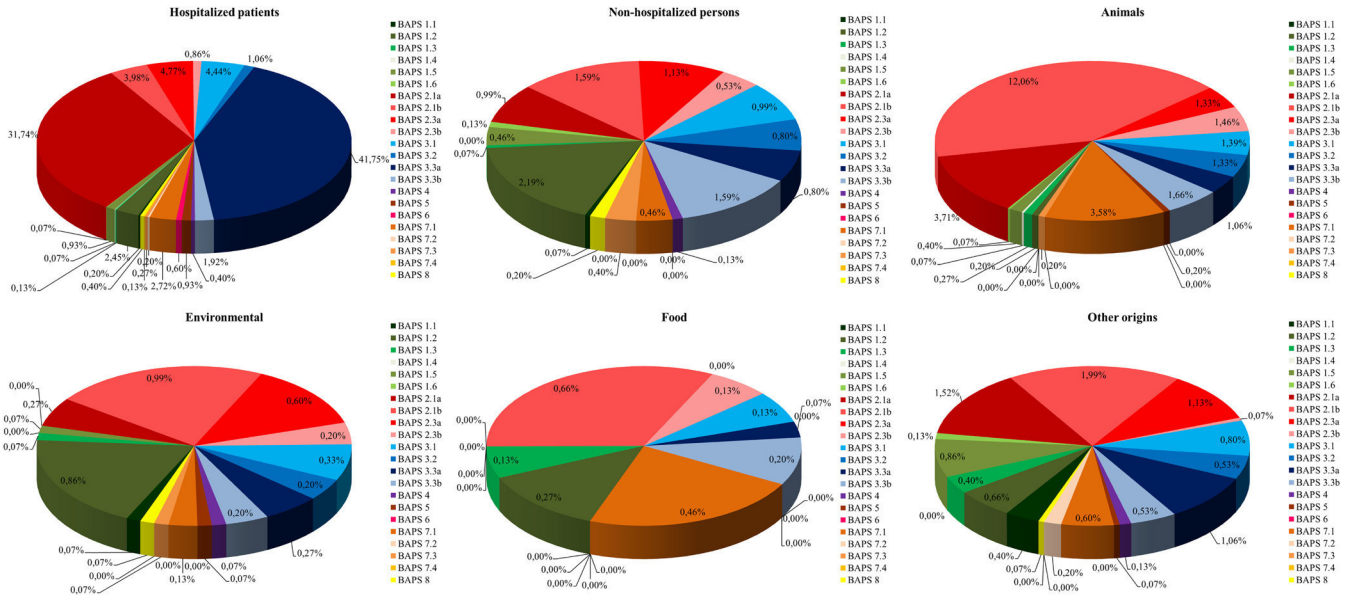

FIG 4 *E. faecium* BAPS group distribution by isolate origin. This distribution by isolate origin was calculated by inclusion of all isolates present in the *E. faecium* MLST database (http://efaecium.mlst.net/) in August 2013.

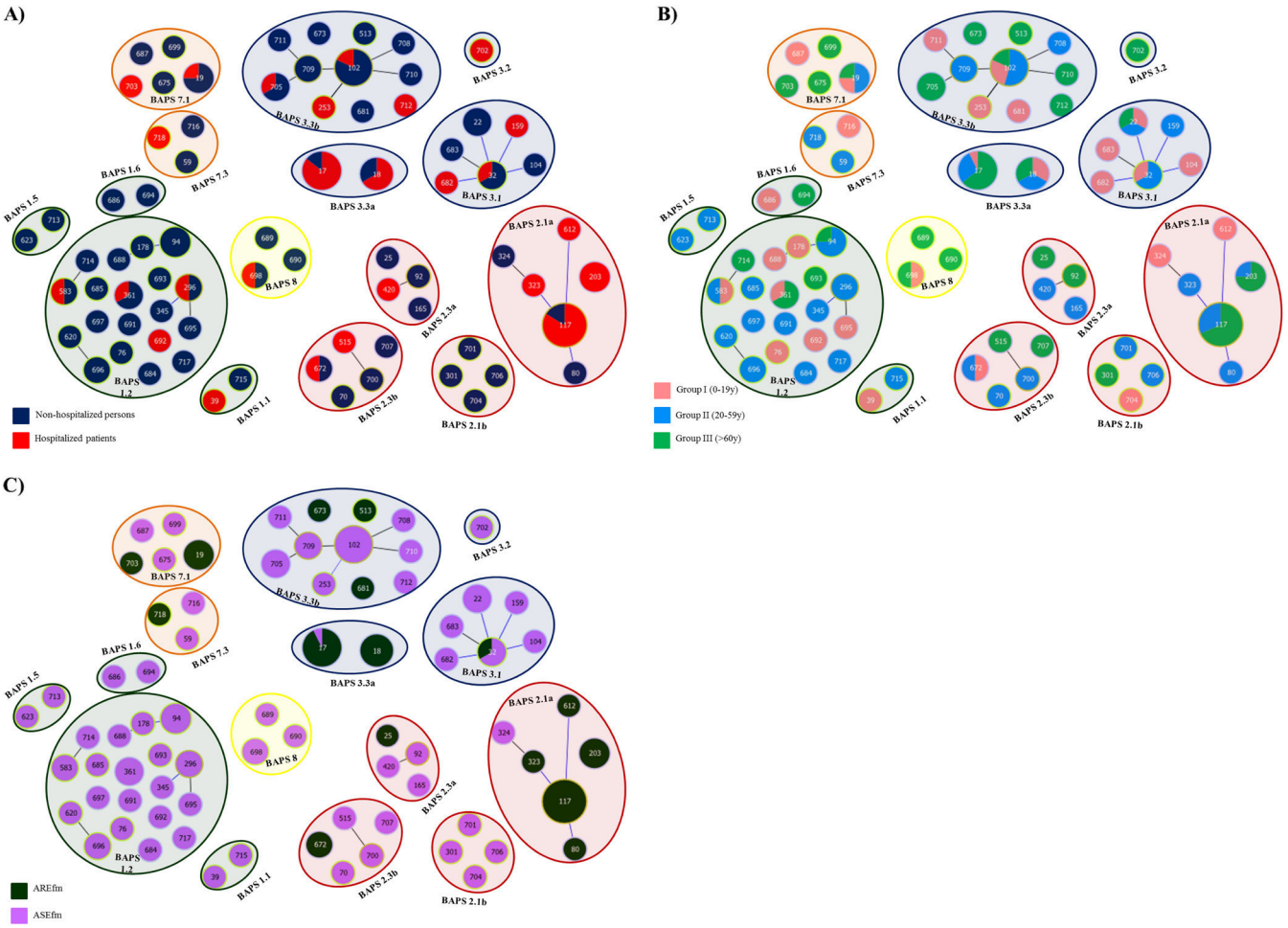

FIG 5 Population structure of *E. faecium* colonization by origin (A), by age group (B), and by susceptibility to ampicillin (C). AREfm, ampicillin-resistant *E. faecium*; ASEfm, ampicillin-susceptible *E. faecium*.

1.5, 2.1a, 2.1b, 2.3a, 2.3b, 3.1, 3.2, and 7.1 (Fig. 4; see also Table S3 in the supplemental material).

Genotypic relatedness of *E. faecium* isolates colonizing different age groups. The 150 *E. faecium* isolates obtained from 142 samples in this study corresponded to 75 distinct STs. Forty-seven STs, representing 62.7% of the studied isolates, were STs first reported here (see Table S1 in the supplemental material). The remaining ones corresponded to globally spread STs, like ST78 (n = 34, 7 STs), ST17 (n = 14, 1 ST), and ST18 (n = 6, 1 ST), and also ST102 (n = 20, 7 STs), ST22 (n = 13, 9 STs), ST94 (n = 12, 7 STs), ST9 (2, 2 STs), and ST5 (1 ST), which were previously detected among community-based isolates (see Table S1 in the supplemental material). The 75 STs were partitioned into BAPS 1 (24 STs, 22.0% of isolates), BAPS 2 (19 STs, 31.3% of isolates), BAPS 3 (20 STs, 36.7% of isolates), BAPS 7 (8 STs, 7.3% of isolates), and BAPS 8 (3 STs, 2.7% of isolates) (Fig. 5).

STs classified as BAPS 1 mainly corresponded to subgroup 1.2 (n = 27 [81.2%], 19 STs). The proportion of isolates with STs that grouped in BAPS 1 steadily decreased with age (Fig. 5 and 6), but isolates of this group were still prevalent among the adults of

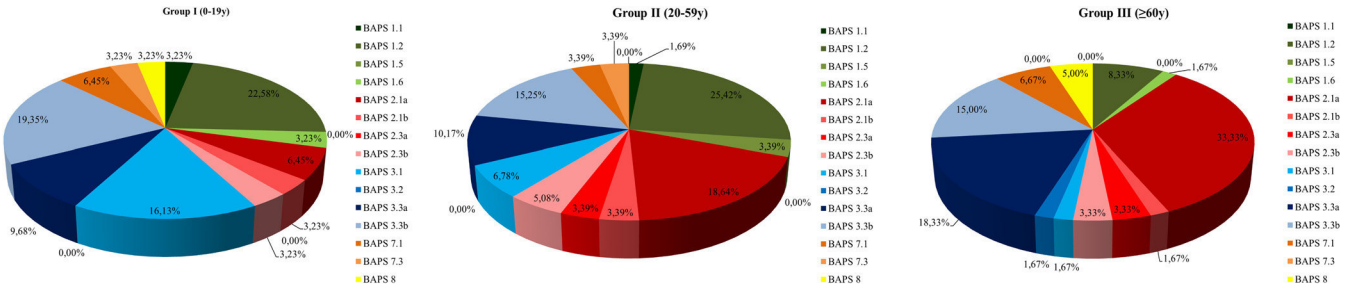

FIG 6 *E. faecium* BAPS group distribution in human colonization by age group.

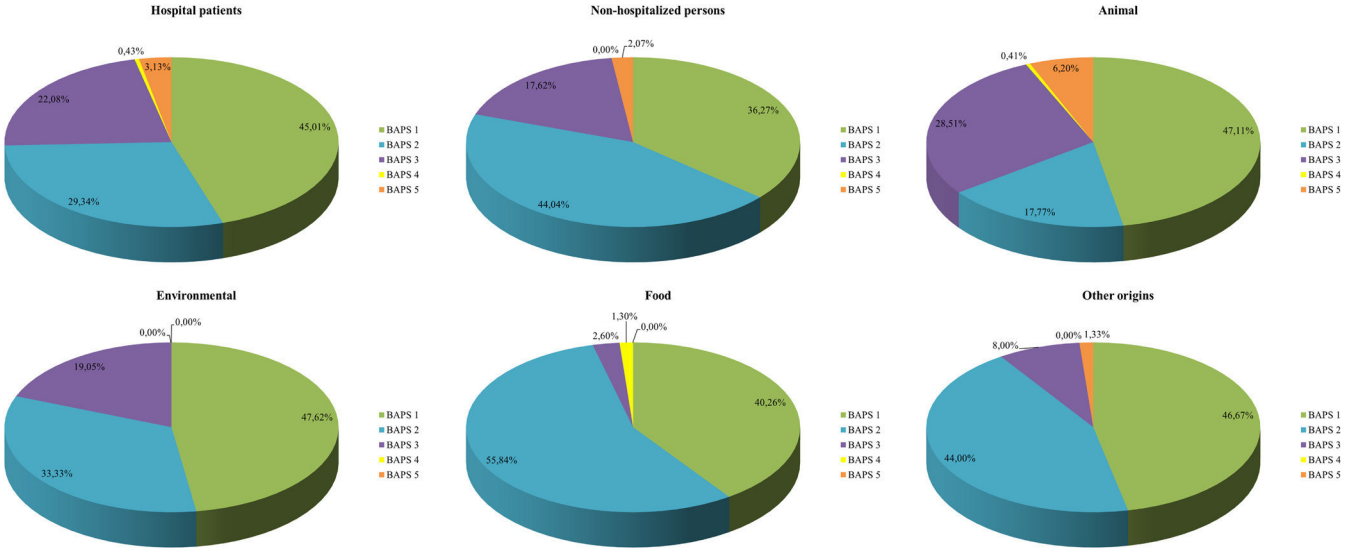

FIG 7 *E. faecalis* BAPS group distribution by origin. This analysis of the distribution by origin included all isolates present in the *E. faecalis* MLST database (http://efaecalis.mlst.net/) in August 2013.

group II (15/27). All strains within BAPS 1 were ampicillin susceptible and were mainly recovered from nonhospitalized persons (23/27, $P < 0.01$).

Within BAPS 2, subgroup 2.1a was predominant (70.2%, 33/47) and increasingly detected with age, constituting the predominant group in elderly patients (Fig. 5 and 6). Most isolates were recovered in hospitals, exhibited ampicillin resistance, and harbored genes encoding adhesive surface protein (Esp) and collagen-adhesin (Acm) (27/33 and 31/33, respectively), which are associated with colonization and pathogenicity (see Table S1 in the supplemental material). STs contained within BAPS 2.1a were ST117 ($n = 25$), ST203 ($n = 4$), ST80 ($n = 1$), ST323 ($n = 1$), ST324 ($n = 1$), and ST612 ($n = 1$). *E. faecium* ST117 (strain showing PFGE type CEfm1), apart from predominantly being a colonizing clone, was also frequently associated with severe infections in patients in HRyC (54). The other BAPS 2 subgroups, namely, BAPS 2.3b ($n = 6$ [12.8%], 5 STs), BAPS 2.3a ($n = 4$ [8.5%], 4 STs), and BAPS 2.1b ($n = 4$ [8.5%], 4 STs) were detected in both hospitalized and nonhospitalized individuals (Fig. 5 and 6).

BAPS 3 was represented by subgroups 3.1, 3.2, 3.3a, and 3.3b. Most isolates in BAPS 3.1 (6 STs, 18.2% of isolates) were ampicillin-susceptible *E. faecium* isolates (9/10) from nonhospitalized individuals (7/10) ($P < 0.01$) in age groups I and II (Fig. 5 and 6; see also Table S1 in the supplemental material). Isolates in BAPS 3.3a and BAPS 3.3b, previously described to be BAPS 3.3, differed in their susceptibility to ampicillin. BAPS 3.3a ($n = 20$ [36.4%], 14 ST17 and 6 ST18 isolates) comprised ampicillin-resistant *E. faecium* isolates (19/20, $P < 0.01$) that contained *hyl* from *E. faecium* (16/20) and that were predominantly recovered from hospitalized patients (16/20, $P < 0.01$). Conversely, BAPS 3.3b (11 STs, 43.6% of isolates) was significantly associated with ampicillin-susceptible *E. faecium* isolates (21/24, $P < 0.01$), mostly from nonhospitalized persons (18/24, $P < 0.01$).

BAPS 7.1 was the most predominant subgroup within BAPS 7 and comprised 5 STs representing 8 isolates. Finally, 3 STs comprising BAPS 8 and representing 4 isolates (2 ST698 isolates, 1 ST689 isolate, and 1 ST690 isolate), all of which were ampicillin-susceptible *E. faecium* isolates (of which 3 were *esp*$^+$), were recovered from nonhospitalized persons (see Table S1 in the supplemental material).

Differences in the rates of recovery of ampicillin-resistant enterococci (56.3%, 36/64) but not in the rates of recovery of vancomycin-resistant enterococci (100%, 5/5) were noticed when samples were cultured without or with selective enrichment. Ampicillin-resistant *E. faecium* isolates that were cultured only after enrichment mostly belonged to BAPS 2.1a ($n = 14$; 9 ST117 isolates, 3 ST203 isolates, 1 ST80 isolate, and 1 ST323 isolate) and BAPS 3.3a ($n = 8$; 6 ST17 and 2 ST18 isolates), and the majority of these isolates were recovered from hospitalized patients. Isolates of other BAPS groups were also found and are described in Table S1 in the supplemental material.

BAPS analysis of *E. faecalis* population structure. Previous studies based on MLST have suggested that recombination may play an important role in the diversification of *E. faecalis* (17, 20, 21). As methods to infer evolutionary descent are highly influenced by recombination, we analyzed the *E. faecalis* population structure using Bayesian-based population genetic modeling implemented in BAPS software, in addition to goeBURST analysis. The sample included 1,310 isolates corresponding to 523 STs available in a public database (http://efaecalis.mlst.net/).

A maximum likelihood-based phylogenetic reconstruction of STs using concatenated MLST gene sequences placed ST80 far apart from all other STs. When ST80 (amounting to only 1 isolate from the MLST database) was excluded from the analysis to better observe differences among tree features, practically all clades showed low bootstrap support, which supports previous analyses indicating that recombination may obscure the phylogenetic signal in nucleotide-based phylogenetic reconstructions in *E. faecalis*. A hierarchical BAPS clustering analysis subdivided the *E. faecalis* population into 5 BAPS groups (Fig. 7). Most of the STs and isolates were distributed among BAPSs 1, 2, and 3 (44.7%, 27.5%, and 20.6%, respectively), while BAPSs 4 (1.0%) and 5 (6.1%) represented only a small fraction of the STs analyzed (see Table S4 in the supplemental material).

OR calculations revealed that isolates from hospitalized patients were not significantly associated with any of the BAPS groups, while BAPS 2 was positively associated with isolates from nonhospitalized persons (OR = 1.8507, $P < 0.01$) and negatively associated with animal isolates (OR = 0.4659, $P < 0.01$) (Fig. 7; see also Table S5 in the supplemental material). Although signals of microevolutionary hospital specialization within the different BAPS groups were not found, some STs were enriched in isolates from hospitalized patients: ST6 (107/123), ST64 (12/18), ST9 (22/25), ST28 (16/17), ST87 (15/16), ST49 (4/4), ST88 (4/4), and ST159 (4/4). Furthermore, isolates from animals were frequently found to be ST58 (8/8), ST82 (25/27), and ST174 (11/11).

We also analyzed the *E. faecalis* population for traces of significant admixtures, as recombination is the driving force of admixture dynamics and it might influence the evolvability of specific amplified lineages. Admixtures were significantly present in some STs from animal and community-based hosts. However, additional analyses revealed that admixtures were not significantly found in STs that were unique or shared between hosts, STs from hospital or nonhospital origin, STs from human and nonhuman origin, or STs that represented antibiotic-resistant isolates (data not shown). The combination of these results suggests that the majority of *E. faecalis* isolates seem to belong to a single recombining population that exchanges alleles regardless of the genetic background (BAPS groups), ecological origin (isolation source, hospital or nonhospital, human or nonhuman), or antibiotic resistance phenotype.

The influence of the sample size (and, therefore, the underlying diversity) in the accuracy of BAPS for establishing the *E. faecalis* population structure was assessed using two data sets (see Fig. S4 in the supplemental material). The first data set consisted of 433 STs available in the MLST database (http://efaecalis.mlst.net/) before, including the new *E. faecalis* STs found in this study. The second data set included 523 STs available in the MLST database at the end of 2013. In both analyses, ST80 was excluded. The negative correlation coefficient of −0.6439 obtained when comparing ST assignments to BAPS groups of the set with 433 STs and those to BAPS groups of the set with 523 STs was due to the split of BAPS 1 and BAPS 2 and the existence of three more BAPS groups when using the second, larger data set (see Fig. S4 in the supplemental material). These results indicate that in *E. faecalis*, BAPS analysis is highly influenced by the sample size, as larger samples contain a higher diversity of strains of different spatial-temporal origins.

Genotypic relatedness of *E. faecalis* isolates colonizing different age groups. The 130 *E. faecalis* isolates identified in this study represented 58 STs (see Table S2 in the supplemental material) that were partitioned into *E. faecalis* BAPS 1 (36.9%), BAPS 2 (40.0%), and BAPS 3 (23.1%). OR calculations revealed that none of the three BAPS groups were significantly associated with a particular source or age group, as all the BAPS groups contained isolates from both hospitalized and nonhospitalized individuals of all ages in more or less equal numbers (Fig. 8).

Within BAPS 1 ($n = 48/130$ [36.9%], 20 STs), ST6 ($n = 16$) was predominant and mainly comprised isolates from hospitalized patients (13/16) and elderly individuals (11/13) (Fig. 8; see also Table S2 in the supplemental material). All were multidrug resistant (MDR), showing high levels of resistance to gentamicin or streptomycin and also to erythromycin (100% of isolates), tetracycline (93.8%, 15/16), and levofloxacin (87.5%, 14/16) and exhibiting a highly similar PFGE profile (the ST6-H10 profile) identical to that of the widespread international mid-Atlantic clone, which also causes bacteremia in HRyC (55). The 5 vancomycin-resistant *E. faecalis* isolates (*vanA*; data not shown) found in this study were also ST6-H10. Putative virulence factors *asa1* (100%) and *gelE* (81.3%) were identified in most ST6 isolates, while *cylA* (56.3%) and *esp* (37.5%) were less frequently identified. Other STs were represented by a very small number of isolates, which were usually susceptible to antibiotics and which had a highly variable presence of virulence factors.

Within BAPS 2 ($n = 52/130$ [40.0%], 26 STs), ST40 isolates ($n = 15$) were predominant. These isolates were recovered from both nonhospitalized and hospitalized individuals of different ages, often harbored *gelE* (88.2%) and, less frequently, *asa1* (41.2%) and *esp* (47.1%), and were resistant to tetracycline (70.6%) and erythromycin (47.1%). Similar to the findings for BAPS 1, other STs were represented by a single isolate or very few isolates that often contained *esp* (see Table S2 in the supplemental material). Among them were STs that were identified over several decades to be ST55, ST30, or ST19 (31, 33).

Finally, BAPS 3 ($n = 30/130$, 12 STs) was predominantly comprised of ST16 and ST179, previously classified to be CC16 by goeBURST analysis (7 ST16 and 11 ST179 isolates). These STs also included isolates from both nonhospitalized and hospitalized individuals of different ages that often harbored *asa1* (99.4%), *esp* (77.8%), or *gelE* (61.1%) and that were often resistant to different antibiotics (see Table S2 in the supplemental material). Other STs classified before as CC28 by goeBURST analysis (1 isolate each of ST333, ST518, and ST519) and recovered from adults or elderly hospitalized patients were also enriched in isolates that harbored putative virulence factors (all harbored *asa1*, *gelE*, *esp*, and *cylA*) and that were also MDR (with all isolates showing high levels of resistance to gentamicin and streptomycin, tetracycline, erythromycin, and levofloxacin) (see Table S2 in the supplemental material).

DISCUSSION

This study describes a consistently high rate of recovery of enterococci from feces from both hospitalized and nonhospitalized individuals and individuals in different age groups, similar to the findings reported in other studies, in which the rates of recovery ranged from 71% to 80% (1, 56, 57). These equilibrated constant rates of colonization indicate a major resiliency of the genus *Enterococcus* under heterogeneous conditions imposed by age, changing environments, and highly variable host niches. Previous studies (6, 58–60) have described changes in the rates of recovery of the genus *Enterococcus* in fecal microbiota with aging, which was not confirmed in our work, and a consistent predominance of the species *E. faecalis* in the fecal flora of young and elderly individuals, which is essentially consistent with our findings, with the important exception of the growing predominance of *E. faecium* in elderly individuals, particularly hospitalized elderly patients. Other studies yielded contradictory information about the frequency and diversity of *E. faecium* and other enterococcal species in the fecal microbiota (1, 15). Shifts in the prevalence of *Enterococcus* populations might result from fluctuating changes in the environmental conditions over time as a result of changes in diet (10) or changes in health status or antibiotic treatment (1, 5, 61–64), all of which delineate particular selective landscapes in hospitals (58, 61). Aging interacts with these conditions, and age-dependent enterococcal colonization dynamics have also been

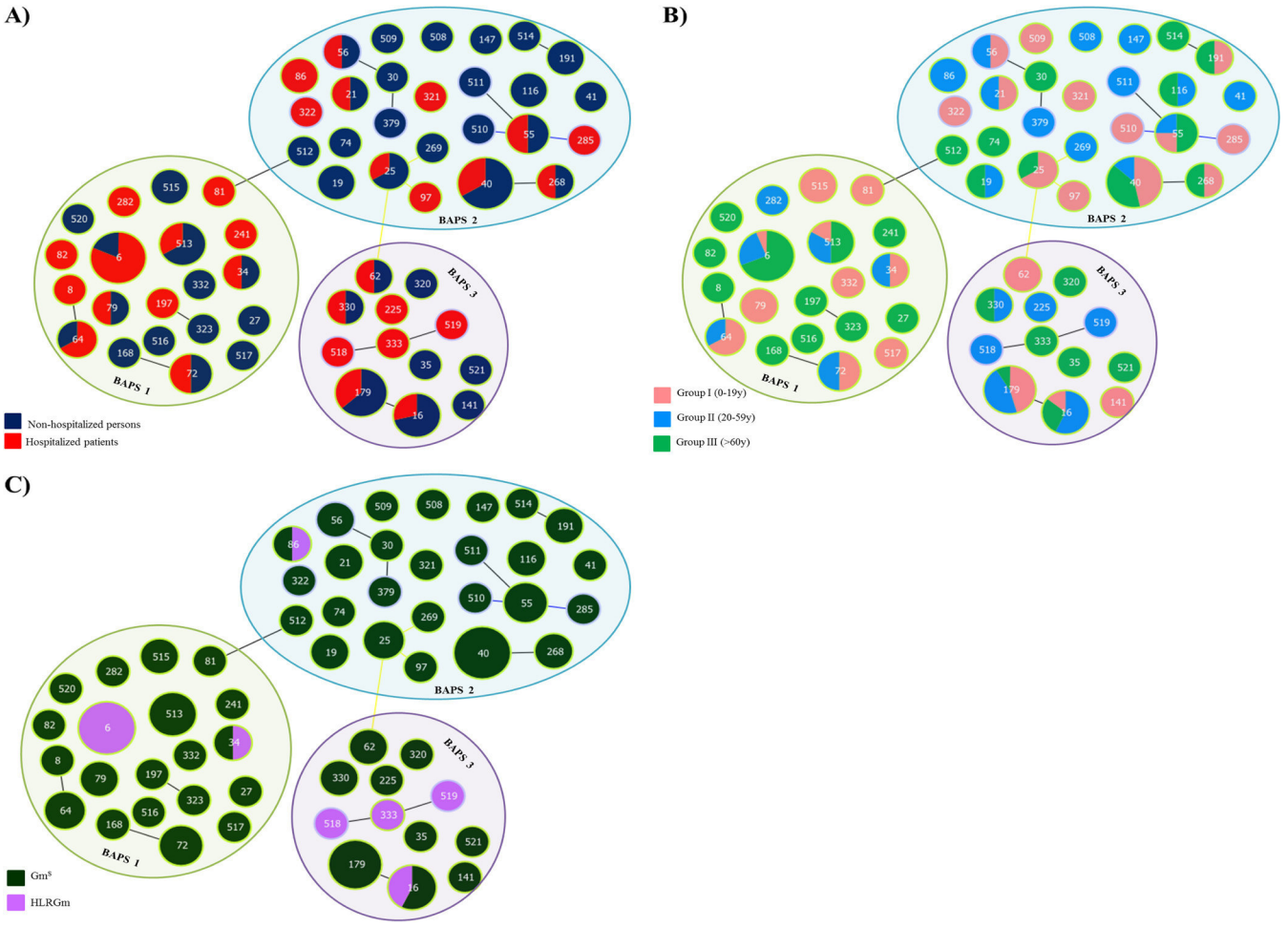

FIG 8 Population structure of *E. faecalis* colonization by origin (A), by age group (B), and by susceptibility to gentamicin (C). Gm^S, gentamicin susceptible; HLRGm, high-level resistance to gentamicin.

demonstrated for chickens and calves (1, 65), probably as an interaction with antibiotic consumption (1, 66, 67).

Considering the currently available diversity of known genotypes, the superimposing of goeBURST analysis of the clonal relationship among multiple isolates with BAPS allowed the detection of a low number of presumptive evolutionarily and functionally heterogeneous clades for the *E. faecium* species (22, 25–27). BAPS 1 *E. faecium* isolates, associated with the clade B phylogenetic lineage (a clade with isolates with pathways of complex carbohydrate utilization linked to host diet and with a majority of ampicillin-susceptible *E. faecium* strains), were highly represented in the different age groups, although their incidence was slightly reduced in the elderly (26, 27). Conversely, BAPS 2.1a and BAPS 3.3a (containing most of the ampicillin-resistant *E. faecium* strains), associated with clade A1 (26, 27) and mostly found in elderly hospitalized patients, represented *E. faecium* strains that are spreading in hospitals and causing clinical infections. The rates of these populations in the nosocomial and the community settings might be underestimated, as we have demonstrated here that if you do not preenrich the sample, some of the clones that are even more widespread might escape screening, probably due to low colonization densities. The observed population structure of *E. faecium* indicates a certain specialization of subpopulations for the colonization of particular age groups, which is usually associated with several other host-associated factors and also differences in the selectable characteristics harbored by the isolates, such as antibiotic resistance genes. Interestingly, some groups evolve independently from the acquisition of ampicillin resistance, suggesting a certain genetic isolation, which seems to be the case for different lineages within BAPS 3.3b, BAPS 1, and BAPS 2. These results further confirm a population structure comprised of ecotypes representing specialization in different hosts (16, 68).

E. faecalis populations showed a considerable level of genetic diversity. Because of that and in contrast to the findings for *E. faecium*, no BAPS groups were significantly associated with aging, hospital exposure, or host species, and with the exception of BAPS 2, none of the BAPS groups showed a positive association with nonhospitalized individuals. The wide recovery of certain STs (e.g., ST6, ST16, ST40, or ST55) able to colonize hospitalized and nonhospitalized humans (this study) and also animals (30, 31, 69) may be related to the more generalist lifestyle of this enterococcal species, which weakens the possibility of the recognition of ecotypes associated with a particular environment, at least by using the same approach that was so useful with *E. faecium*. However, despite possible limitations in the methods available for analysis of the *E. faecalis* population structure, it is now clear that

certain multihost *E. faecalis* subpopulations, such as ST6 or ST16, have developed different strategies of adaptation to harsh and fluctuating habitats (31, 33). Among these are the lack of loci for cluster regulatory interspaced short palindromic repeats (CRISPR; a bacterial defense system against foreign DNA that facilitates the acquisition of foreign DNA, such as antibiotic resistance and virulence genes) (70) and the frequent acquisition of phages (71).

Other enterococcal species have largely been recognized to be part of the human fecal microbiota (1), and this was confirmed in our study. The inverse parallel trends in the frequencies of populations of these species and the frequency of *E. faecium* are of particular interest. The dynamics of colonization by these species might reflect differences in the functional requirements of the host with age and deserve further analysis.

This study provides a novel, integrated, and comprehensive image of the landscape of *Enterococcus* populations in a balanced amount of nonhospitalized and hospitalized individuals of different ages and suggests that a number of enterococcal lineages might be predominant in certain age groups and/or hospital environments. However, a number of clones are spread in different types of individuals and their prevalence is reduced in others in a kind of source-sink dynamics (72–74), with frequent cases of coexistence and the preservation of rare clonal populations being found. This suggests a frequency-dependent evolution of enterococcal populations which prevents the extinction of different genotypes that do not play equivalent ecological roles (75–78).

The work also illustrates the high degree of plasticity of *E. faecium* and *E. faecalis* genomes, as reflected by admixture analysis (27; this study), which showed variable intraclonal PFGE patterns (31, 63) (see Tables S1 and S2 in the supplemental material) and recombination of large fragments of the chromosome (79–83; our unpublished results). The consequences of such a high degree of variability have scarcely been explored from a population-based perspective. However, it can be expected that genome plasticity would contribute to the variation and selection of genes from a common intraspecies genetic pool needed for adaptation to environments imposing different stress conditions. Future progress in understanding enterococcal population biology will require a global analysis combining many ecological features, population dynamics, and population genetics (78, 84, 85).

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REFERENCES

1. **Lebreton F, Willems RJL, Gilmore MS, Lebreton F, Willems RJL.** 2014. Enterococcus diversity, origins in nature, and gut colonization. *In*Gilmore MS, Clewell DB, Ike Y, Shankar N (ed), Enterococci: from commensals to leading causes of drug resistant infection. Massachusetts Eye and Ear Infirmary, Boston, MA. http://www.ncbi.nlm.nih.gov/books/NBK190427/.
2. **Arias CA, Murray BE.** 2012. The rise of the Enterococcus: beyond vancomycin resistance. Nat Rev Microbiol **10:**266–278. http://dx.doi.org/10.1038/nrmicro2761.
3. **Taur Y, Xavier JB, Lipuma L, Ubeda C, Goldberg J, Gobourne A, Lee YJ, Dubin KA, Socci ND, Viale A, Perales M-A, Jenq RR, van den Brink MRM, Pamer EG.** 2012. Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. Clin Infect Dis **55:**905–914. http://dx.doi.org/10.1093/cid/cis580.
4. **Bonten MJ, Willems R, Weinstein RA.** 2001. Vancomycin-resistant enterococci: why are they here, and where do they come from? Lancet Infect Dis **1:**314–325. http://dx.doi.org/10.1016/S1473-3099(01)00145-1.
5. **Donskey CJ.** 2004. The role of the intestinal tract as a reservoir and source for transmission of nosocomial pathogens. Clin Infect Dis **39:**219–226. http://dx.doi.org/10.1086/422002.
6. **Woodmansey EJ, McMurdo MET, Macfarlane GT, Macfarlane S.** 2004. Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. Appl Environ Microbiol **70:**6113–6122. http://dx.doi.org/10.1128/AEM.70.10.6113-6122.2004.
7. **Enck P, Zimmermann K, Rusch K, Schwiertz A, Klosterhalfen S, Frick J-S.** 2009. The effects of ageing on the colonic bacterial microflora in adults. Z Gastroenterol **47:**653–658. http://dx.doi.org/10.1055/s-0028-1109055.
8. **Tiihonen K, Ouwehand AC, Rautonen N.** 2010. Human intestinal microbiota and healthy ageing. Ageing Res Rev **9:**107–116. http://dx.doi.org/10.1016/j.arr.2009.10.004.
9. **Petersen C, Round JL.** 2014. Defining dysbiosis and its influence on host immunity and disease. Cell Microbiol **16:**1024–1033. http://dx.doi.org/10.1111/cmi.12308.
10. **Firmesse O, Rabot S, Bermúdez-Humarán LG, Corthier G, Furet J-P.** 2007. Consumption of Camembert cheese stimulates commensal enterococci in healthy human intestinal microbiota. FEMS Microbiol Lett **276:**189–192. http://dx.doi.org/10.1111/j.1574-6968.2007.00933.x.
11. **Medini D, Donati C, Tettelin H, Masignani V, Rappuoli R.** 2005. The microbial pan-genome. Curr Opin Genet Dev **15:**589–594. http://dx.doi.org/10.1016/j.gde.2005.09.006.
12. **Dobrindt U, Hacker J.** 2001. Whole genome plasticity in pathogenic bacteria. Curr Opin Microbiol **4:**550–557. http://dx.doi.org/10.1016/S1369-5274(00)00250-2.
13. **Finegold SM, Sutter VL, Methisen GE.** 1983. Normal indigenous intestinal flora, p 3–32. *In* Hentges D (ed), Human intestinal microflora in health and disease. Academic Press, New York, NY.
14. **Tannock GW, Cook G.** 2002. Enterococci as members of the intestinal microflora of humans, p 101–132. *In* Gilmore MS, Clewell DB, Courvalin P, Dunny GM, Murray BE, Rice LB (ed), The enterococci: pathogenesis, molecular biology, and antibiotic resistance. ASM Press, Washington, DC.
15. **Silva N, Igrejas G, Gonçalves A, Poeta P.** 2011. Commensal gut bacteria: distribution of Enterococcus species and prevalence of Escherichia coli phylogenetic groups in animals and humans in Portugal. Ann Microbiol **62:**449–459. http://dx.doi.org/10.1007/s13213-011-0308-4.
16. **Homan WL, Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, Van Embden JDA, Willems RJL.** 2002. Multilocus sequence typing scheme for Enterococcus faecium. J Clin Microbiol **40:**1963–1971. http://dx.doi.org/10.1128/JCM.40.6.1963-1971.2002.
17. **Ruiz-Garbajosa P, Bonten MJM, Robinson DA, Top J, Nallapareddy SR, Torres C, Coque TM, Cantón R, Baquero F, Murray BE, del Campo R, Willems RJL.** 2006. Multilocus sequence typing scheme for Enterococcus faecalis reveals hospital-adapted genetic complexes in a background of high rates of recombination. J Clin Microbiol **44:**2220–2228. http://dx.doi.org/10.1128/JCM.02596-05.
18. **Leavis HL, Willems RJL, van Wamel WJB, Schuren FH, Caspers MPM, Bonten MJM.** 2007. Insertion sequence-driven diversification creates a globally dispersed emerging multiresistant subspecies of E. faecium. PLoS Pathog **3:**e7. http://dx.doi.org/10.1371/journal.ppat.0030007.
19. **Van Schaik W, Willems RJL.** 2010. Genome-based insights into the evolution of enterococci. Clin Microbiol Infect **16:**527–532. http://dx.doi.org/10.1111/j.1469-0691.2010.03201.x.
20. **Nallapareddy SR, Duh R-W, Singh KV, Murray BE.** 2002. Molecular typing of selected Enterococcus faecalis isolates: pilot study using multi-

locus sequence typing and pulsed-field gel electrophoresis. J Clin Microbiol **40:**868–876. http://dx.doi.org/10.1128/JCM.40.3.868-876.2002.
21. **Nallapareddy SR, Wenxiang H, Weinstock GM, Murray BE.** 2005. Molecular characterization of a widespread, pathogenic, and antibiotic resistance-receptive Enterococcus faecalis lineage and dissemination of its putative pathogenicity island. J Bacteriol **187:**5709–5718. http://dx.doi.org/10.1128/JB.187.16.5709-5718.2005.
22. **Galloway-Peña J, Roh JH, Latorre M, Qin X, Murray BE.** 2012. Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of Enterococcus faecium. PLoS One **7:**e30187. http://dx.doi.org/10.1371/journal.pone.0030187.
23. **Qin X, Galloway-Peña JR, Sillanpaa J, Roh JH, Nallapareddy SR, Chowdhury S, Bourgogne A, Choudhury T, Muzny DM, Buhay CJ, Ding Y, Dugan-Rocha S, Liu W, Kovar C, Sodergren E, Highlander S, Petrosino JF, Worley KC, Gibbs RA, Weinstock GM, Murray BE.** 2012. Complete genome sequence of Enterococcus faecium strain TX16 and comparative genomic analysis of Enterococcus faecium genomes. BMC Microbiol **12:**135. http://dx.doi.org/10.1186/1471-2180-12-135.
24. **Palmer KL, Godfrey P, Griggs A, Kos VN, Zucker J, Desjardins C, Cerqueira G, Gevers D, Walker S, Wortman J, Feldgarden M, Haas B, Birren B, Gilmore MS.** 2012. Comparative genomics of enterococci: variation in Enterococcus faecalis, clade structure in E. faecium, and defining characteristics of E. gallinarum and E. casseliflavus. mBio **3**(1): e00318-11. http://dx.doi.org/10.1128/mBio.00318-11.
25. **Howden BP, Holt KE, Lam MMC, Seemann T, Ballard S, Coombs GW, Tong SYC, Grayson ML, Johnson PDR, Stinear TP.** 2013. Genomic insights to control the emergence of vancomycin-resistant enterococci. mBio **4**(4):e00412-13. http://dx.doi.org/10.1128/mBio.00412-13.
26. **Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems RJL, Earl AM, Gilmore MS.** 2013. Emergence of epidemic multidrug-resistant Enterococcus faecium from animal and commensal strains. mBio **4**(4):e00534-13. http://dx.doi.org/10.1128/mBio.00534-13.
27. **Willems RJL, Top J, van Schaik W, Leavis H, Bonten M, Sirén J, Hanage WP, Corander J.** 2012. Restricted gene flow among hospital subpopulations of Enterococcus faecium. mBio **3**(4):e00151-12. http://dx.doi.org/10.1128/mBio.00151-12.
28. **Ruiz-Garbajosa P, Cantón R, Pintado V, Coque TM, Willems R, Baquero F, del Campo R.** 2006. Genetic and phenotypic differences among Enterococcus faecalis clones from intestinal colonisation and invasive disease. Clin Microbiol Infect **12:**1193–1198. http://dx.doi.org/10.1111/j.1469-0691.2006.01533.x.
29. **Waar K, Muscholl-Silberhorn AB, Willems RJL, Slooff MJH, Harmsen HJM, Degener JE.** 2002. Genogrouping and incidence of virulence factors of Enterococcus faecalis in liver transplant patients differ from blood culture and fecal isolates. J Infect Dis **185:**1121–1127. http://dx.doi.org/10.1086/339682.
30. **Kuch A, Willems RJL, Werner G, Coque TM, Hammerum AM, Sundsfjord A, Klare I, Ruiz-Garbajosa P, Simonsen GS, van Luit-Asbroek M, Hryniewicz W, Sadowy E.** 2012. Insight into antimicrobial susceptibility and population structure of contemporary human Enterococcus faecalis isolates from Europe. J Antimicrob Chemother **67:**551–558. http://dx.doi.org/10.1093/jac/dkr544.
31. **Freitas AR, Novais C, Ruiz-Garbajosa P, Coque TM, Peixe L.** 2009. Clonal expansion within clonal complex 2 and spread of vancomycin-resistant plasmids among different genetic lineages of Enterococcus faecalis from Portugal. J Antimicrob Chemother **63:**1104–1111. http://dx.doi.org/10.1093/jac/dkp103.
32. **Kawalec M, Pietras Z, Daniłowicz E, Jakubczak A, Gniadkowski M, Hryniewicz W, Willems RJL.** 2007. Clonal structure of Enterococcus faecalis isolated from Polish hospitals: characterization of epidemic clones. J Clin Microbiol **45:**147–153. http://dx.doi.org/10.1128/JCM.01704-06.
33. **McBride SM, Fischetti VA, Leblanc DJ, Moellering RC, Gilmore MS.** 2007. Genetic diversity among Enterococcus faecalis. PLoS One **2:**e582. http://dx.doi.org/10.1371/journal.pone.0000582.
34. **Willems RJL, Top J, van Santen M, Robinson DA, Coque TM, Baquero F, Grundmann H, Bonten MJM.** 2005. Global spread of vancomycin-resistant Enterococcus faecium from distinct nosocomial genetic complex. Emerg Infect Dis **11:**821–828. http://dx.doi.org/10.3201/1106.041204.
35. **De Been M, van Schaik W, Cheng L, Corander J, Willems RJ.** 2013. Recent recombination events in the core genome are associated with adaptive evolution in Enterococcus faecium. Genome Biol Evol **5:**1524–1535. http://dx.doi.org/10.1093/gbe/evt111.
36. **Safdar N, Maki DG.** 2002. The commonality of risk factors for nosocomial colonization and infection with antimicrobial-resistant Staphylococcus aureus, Enterococcus, gram-negative bacilli, Clostridium difficile, and Candida. Ann Intern Med **136:**834–844. http://dx.doi.org/10.7326/0003-4819-136-11-200206040-00013.
37. **Willems RJL, Hanage WP, Bessen DE, Feil EJ.** 2011. Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance. FEMS Microbiol Rev **35:**872–900. http://dx.doi.org/10.1111/j.1574-6976.2011.00284.x.
38. **Leavis HL, Bonten MJM, Willems RJL.** 2006. Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. Curr Opin Microbiol **9:**454–460. http://dx.doi.org/10.1016/j.mib.2006.07.001.
39. **Chowdhury SA, Nallapareddy SR, Arias CA, Murray BE.** 2014. The majority of a collection of U.S. endocarditis Enterococcus faecalis isolates obtained from 1974 to 2004 lack capsular genes and belong to diverse, non-hospital-associated lineages. J Clin Microbiol **52:**549–556. http://dx.doi.org/10.1128/JCM.02763-13.
40. **Galloway-Peña JR, Nallapareddy SR, Arias CA, Eliopoulos GM, Murray BE.** 2009. Analysis of clonality and antibiotic resistance among early clinical isolates of Enterococcus faecium in the United States. J Infect Dis **200:**1566–1573. http://dx.doi.org/10.1086/644790.
41. **Butler KM.** 2006. Enterococcal infection in children. Semin Pediatr Infect Dis **17:**128–139. http://dx.doi.org/10.1053/j.spid.2006.06.006.
42. **Coque TM, Murray BE.** 1995. Identification of Enterococcus faecalis strains by DNA hybridization and pulsed-field gel electrophoresis. J Clin Microbiol **33:**3368–3369.
43. **Depardieu F, Perichon B, Courvalin P.** 2004. Detection of the van alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR. J Clin Microbiol **42:**5857–5860. http://dx.doi.org/10.1128/JCM.42.12.5857-5860.2004.
44. **CLSI.** 2013. Performance standards for antimicrobial susceptibility testing; 22nd informational supplement. CLSI, Wayne, PA.
45. **Nallapareddy SR, Singh KV, Okhuysen PC, Murray BE.** 2008. A functional collagen adhesin gene, acm, in clinical isolates of Enterococcus faecium correlates with the recent success of this emerging nosocomial pathogen. Infect Immun **76:**4110–4119. http://dx.doi.org/10.1128/IAI.00375-08.
46. **Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, Jabes D, Goossens H.** 2004. Development of a multiplex PCR for the detection of asa1, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among European hospital isolates of Enterococcus faecium. J Clin Microbiol **42:**4473–4479. http://dx.doi.org/10.1128/JCM.42.10.4473-4479.2004.
47. **Coque TM, Willems RJL, Fortún J, Top J, Diz S, Loza E, Cantón R, Baquero F.** 2005. Population structure of Enterococcus faecium causing bacteremia in a Spanish university hospital: setting the scene for a future increase in vancomycin resistance? Antimicrob Agents Chemother **49:**2693–2700. http://dx.doi.org/10.1128/AAC.49.7.2693-2700.2005.
48. **Francisco AP, Bugalho M, Ramirez M, Carriço JA.** 2009. Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. BMC Bioinformatics **10:**152. http://dx.doi.org/10.1186/1471-2105-10-152.
49. **Francisco AP, Vaz C, Monteiro PT, Melo-Cristino J, Ramirez M, Carriço JA.** 2012. PHYLOViZ: phylogenetic inference and data visualization for sequence based typing methods. BMC Bioinformatics **13:**87. http://dx.doi.org/10.1186/1471-2105-13-87.
50. **Corander J, Marttinen P, Sirén J, Tang J.** 2008. Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. BMC Bioinformatics **9:**539. http://dx.doi.org/10.1186/1471-2105-9-539.
51. **Hardin JW, Hilbe JH.** 2002. Generalized estimating equations. Chapman and Hall/CRC, London, United Kingdom.
52. **Facklam RR, Carvalho MG, Teixeira LM.** 2002. Enterococcus, p 1–54. *In* Gilmore MS, Clewell DB, Courvalin P, Dunny GM, Murray BE, Rice LB (ed), The enterococci: pathogenesis, molecular biology, and antibiotic resistance. ASM Press, Washington, DC.
53. **Turner KME, Hanage WP, Fraser C, Connor TR, Spratt BG.** 2007. Assessing the reliability of eBURST using simulated populations with known ancestry. BMC Microbiol **7:**30. http://dx.doi.org/10.1186/1471-2180-7-30.

54. **Tedim AP, Ruiz-Garbajosa P, Rodriguez CM, Derdoy L, Cardenas G, Loza E, Cantón R, Baquero F, Coque TM.** 2014. Evolution of healthcare bacteraemic episodes of Enterococcus faecium (1995-2012) reflects changes in phylogenomic groups with internal clonal epidemic waves, poster P1239, p 203. Abstr 24th Eur Congr Clin Microbiol Infect Dis., Barcelona, Spain. European Society of Clinical Microbiology and Infectious Diseases, Basel, Switzerland.
55. **Tedim AP, Miranda AC, Ruiz-Garbajosa P, Freitas AR, Francia MV, Baquero F, Coque TM.** 2010. Plasmid diversity among Enterococcus faecalis clinical isolates from Spain (2001-2009). Abstr 3rd Int Am Soc Microbiol Conf Enterococci, Portland, OR. American Society for Microbiology, Washington, DC.
56. **Mundt JO.** 1963. Occurrence of enterococci in animals in a wild environment. Appl Microbiol **11:**136–140.
57. **Coque TM, Arduino RC, Murray BE.** 1995. High-level resistance to aminoglycosides: comparison of community and nosocomial fecal isolates of enterococci. Clin Infect Dis **20:**1048–1051. http://dx.doi.org/10.1093/clinids/20.4.1048.
58. **Woodmansey EJ.** 2007. Intestinal bacteria and ageing. J Appl Microbiol **102:**1178–1186. http://dx.doi.org/10.1111/j.1365-2672.2007.03400.x.
59. **Stewart CJ, Marrs ECL, Magorrian S, Nelson A, Lanyon C, Perry JD, Embleton ND, Cummings SP, Berrington JE.** 2012. The preterm gut microbiota: changes associated with necrotizing enterocolitis and infection. Acta Paediatr **101:**1121–1127. http://dx.doi.org/10.1111/j.1651-2227.2012.02801.x.
60. **Kirtzalidou EI, Mitsou EK, Pramateftaki P, Kyriacou A.** 2012. Screening fecal enterococci from Greek healthy infants for susceptibility to antimicrobial agents. Microb Drug Resist **18:**578–585. http://dx.doi.org/10.1089/mdr.2012.0028.
61. **Ubeda C, Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, Viale A, Socci ND, van den Brink MRM, Kamboj M, Pamer EG.** 2010. Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. J Clin Invest **120:**4332–4341. http://dx.doi.org/10.1172/JCI43918.
62. **Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, Fleisher M, Schnabl B, DeMatteo RP, Pamer EG.** 2008. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature **455:**804–807. http://dx.doi.org/10.1038/nature07250.
63. **Berg RD.** 1996. The indigenous gastrointestinal microflora. Trends Microbiol **4:**430–435. http://dx.doi.org/10.1016/0966-842X(96)10057-3.
64. **O'Sullivan O, Coakley M, Lakshminarayanan B, Conde S, Claesson MJ, Cusack S, Fitzgerald AP, O'Toole PW, Stanton C, Ross RP.** 2013. Alterations in intestinal microbiota of elderly Irish subjects post-antibiotic therapy. J Antimicrob Chemother **68:**214–221. http://dx.doi.org/10.1093/jac/dks348.
65. **Devriese LA, Laurier L, De Herdt P, Haesebrouck F.** 1992. Enterococcal and streptococcal species isolated from faeces of calves, young cattle and dairy cows. J Appl Bacteriol **72:**29–31. http://dx.doi.org/10.1111/j.1365-2672.1992.tb04877.x.
66. **Aarestrup FM, Butaye P, Witte W.** 2002. Non-human reservoirs of enterococci, p 55–99. *In* Gilmore MS, Clewell DB, Courvalin P, Dunny GM, Murray BE, Rice LB (ed), The enterococci: pathogenesis, molecular biology, and antibiotic resistance. ASM Press, Washington, DC.
67. **Devriese LA, Pot B, Collins MD.** 1993. Phenotypic identification of the genus Enterococcus and differentiation of phylogenetically distinct enterococcal species and species groups. J Appl Bacteriol **75:**399–408. http://dx.doi.org/10.1111/j.1365-2672.1993.tb02794.x.
68. **Willems RJ, Top J, van Den Braak N, van Belkum A, Endtz H, Mevius D, Stobberingh E, van Den Bogaard A, van Embden JD.** 2000. Host specificity of vancomycin-resistant Enterococcus faecium. J Infect Dis **182:**816–823. http://dx.doi.org/10.1086/315752.
69. **Freitas AR, Coque TM, Novais C, Hammerum AM, Lester CH, Zervos MJ, Donabedian S, Jensen LB, Francia MV, Baquero F, Peixe L.** 2011. Human and swine hosts share vancomycin-resistant Enterococcus faecium CC17 and CC5 and Enterococcus faecalis CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids. J Clin Microbiol **49:**925–931. http://dx.doi.org/10.1128/JCM.01750-10.
70. **Palmer KL, Gilmore MS.** 2010. Multidrug-resistant enterococci lack CRISPR-cas. mBio **1**(4):e00227-10. http://dx.doi.org/10.1128/mBio.00227-10.
71. **Duerkop BA, Clements CV, Rollins D, Rodrigues JLM, Hooper LV.** 2012. A composite bacteriophage alters colonization by an intestinal commensal bacterium. Proc Natl Acad Sci U S A **109:**17621–17626. http://dx.doi.org/10.1073/pnas.1206136109.
72. **Pulliam H.** 1988. Sources, sinks, and population regulation. Am Nat **132:**652–661. http://dx.doi.org/10.1086/284880.
73. **Perron GG, Gonzalez A, Buckling A.** 2007. Source-sink dynamics shape the evolution of antibiotic resistance and its pleiotropic fitness cost. Proc Biol Sci **274:**2351–2356. http://dx.doi.org/10.1098/rspb.2007.0640.
74. **Hermsen R, Hwa T.** 2010. Sources and sinks: a stochastic model of evolution in heterogeneous environments. Phys Rev Lett **105:**248104. http://dx.doi.org/10.1103/PhysRevLett.105.248104.
75. **Levin BR.** 1988. Frequency-dependent selection in bacterial populations. Philos Trans R Soc Lond B Biol Sci **319:**459–472. http://dx.doi.org/10.1098/rstb.1988.0059.
76. **Dugatkin LA, Perlin M, Lucas JS, Atlas R.** 2005. Group-beneficial traits, frequency-dependent selection and genotypic diversity: an antibiotic resistance paradigm. Proc Biol Sci **272:**79–83. http://dx.doi.org/10.1098/rspb.2004.2916.
77. **Gonzalez A, Ronce O, Ferriere R, Hochberg ME.** 2013. Evolutionary rescue: an emerging focus at the intersection between ecology and evolution. Philos Trans R Soc Lond B Biol Sci **368:**20120404. http://dx.doi.org/10.1098/rstb.2012.0404.
78. **Cordero OX, Polz MF.** 2014. Explaining microbial genomic diversity in light of evolutionary ecology. Nat Rev Microbiol **12:**263–273. http://dx.doi.org/10.1038/nrmicro3218.
79. **Quintiliani R, Courvalin P.** 1994. Conjugal transfer of the vancomycin resistance determinant vanB between enterococci involves the movement of large genetic elements from chromosome to chromosome. FEMS Microbiol Lett **119:**359–363. http://dx.doi.org/10.1111/j.1574-6968.1994.tb06913.x.
80. **Laverde Gomez JA, Hendrickx AP, Willems RJ, Top J, Sava I, Huebner J, Witte W, Werner G.** 2011. Intra- and interspecies genomic transfer of the Enterococcus faecalis pathogenicity island. PLoS One **6:**e16720. http://dx.doi.org/10.1371/journal.pone.0016720.
81. **Manson JM, Hancock LE, Gilmore MS.** 2010. Mechanism of chromosomal transfer of Enterococcus faecalis pathogenicity island, capsule, antimicrobial resistance, and other traits. Proc Natl Acad Sci U S A **107:**12269–12274. http://dx.doi.org/10.1073/pnas.1000139107.
82. **De Regt MJA, van Schaik W, van Luit-Asbroek M, Dekker HAT, van Duijkeren E, Koning CJM, Bonten MJM, Willems RJL.** 2012. Hospital and community ampicillin-resistant Enterococcus faecium are evolutionarily closely linked but have diversified through niche adaptation. PLoS One **7:**e30319. http://dx.doi.org/10.1371/journal.pone.0030319.
83. **Novais C, Escada R, Freitas AR, Silveira E, Coque TM, Peixe L.** 2011. High incidence of horizontal transfer of ampicillin resistance (pbp5) among CC17 Enterococcus faecium, p S129. Abstr 21st Eur Congr Clin Microbiol Infect Dis, Milan, Italy. European Society of Clinical Microbiology and Infectious Diseases, Basel, Switzerland.
84. **Luck GW, Daily GC, Ehrlich PR.** 2003. Population diversity and ecosystem services. Trends Ecol Evol **18:**331–336. http://dx.doi.org/10.1016/S0169-5347(03)00100-9.
85. **Urban MC, Skelly DK.** 2006. Evolving metacommunities: toward an evolutionary perspective on metacommunities. Ecology **87:**1616–1626. http://dx.doi.org/10.1890/0012-9658(2006)87[1616:EMTAEP]2.0.CO;2.

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The Plasmidome of Firmicutes: Impact on the Emergence and the Spread of Resistance to Antimicrobials

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ABSTRACT The phylum *Firmicutes* is one of the most abundant groups of prokaryotes in the microbiota of humans and animals and includes genera of outstanding relevance in biomedicine, health care, and industry. Antimicrobial drug resistance is now considered a global health security challenge of the 21st century, and this heterogeneous group of microorganisms represents a significant part of this public health issue.

The presence of the same resistant genes in unrelated bacterial genera indicates a complex history of genetic interactions. Plasmids have largely contributed to the spread of resistance genes among *Staphylococcus*, *Enterococcus*, and *Streptococcus* species, also influencing the selection and ecological variation of specific populations. However, this information is fragmented and often omits species outside these genera. To date, the antimicrobial resistance problem has been analyzed under a "single centric" perspective ("gene tracking" or "vehicle centric" in "single host-single pathogen" systems) that has greatly delayed the understanding of gene and plasmid dynamics and their role in the evolution of bacterial communities.

This work analyzes the dynamics of antimicrobial resistance genes using gene exchange networks; the role of plasmids in the emergence, dissemination, and maintenance of genes encoding resistance to antimicrobials (antibiotics, heavy metals, and biocides); and their influence on the genomic diversity of the main Gram-positive opportunistic pathogens under the light of evolutionary ecology. A revision of the approaches to categorize plasmids in this group of microorganisms is given using the 1,326 fully sequenced plasmids of Gram-positive bacteria available in the GenBank database at the time the article was written.

INTRODUCTION

Firmicutes constitutes one of the dominant bacteria phyla of human and animal gut microbiota. It comprises a number of genera of outstanding relevance in health care and industry such as *Staphylococcus*, *Listeria*, and lactic acid bacteria (LAB), a group of microorganisms that ferment carbohydrates into lactic acid and that includes the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, and

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Weisella. Furthermore, species of *Negativicutes* (*Selenomonas*, *Veillonella*) and *Clostridium* have clinical interest for humans and animals (Table 1).

Antibiotic resistance (AbR) in this heterogeneous group of organisms constitutes a significant part of the public health problem. The most recent report by the Centers for Disease Control and Prevention in the United States provides a ranking list of AbR human pathogens according to their threat level to society and the attention that such a problem requires. Gram-positive organisms were grouped in the categories of "urgent" (*Clostridium difficile*), "serious" (methicillin-resistant *Staphylococcus aureus* [MRSA], antibiotic-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus* [VRE]), and "concerning" (erythromycin-resistant *Streptococcus pyogenes* and clindamycin-

TABLE 1 Fully characterized plasmids from low G+C bacteria available in GenBank database (updated September 2014)

			Plasmid			
Phylum/class	Order	Family	Total	AbR	Met^R	AbR+Met^R
Actinobacteria			**252**	**16**	**40**	**7**
	Actynomicetales	*Streptomycetaceae*	56	2	4	2
		Corynebacteriaceae	38	10	7	3
		Nocardiaceae[a]	30	0	9	0
		Mycobacteriaceae[b]	29	2	7	2
		Micrococcaceae[c]	27	1	7	0
		Pseudonocardiaceae[d]	9	0	1	0
		Propionibacteriaceae	7	0	0	0
		Gordonaciaee	6	0	3	0
		Microbacteriaceae[e]	4	0	0	0
		Streptosporangiaceae[f]	2	0	0	0
		Nocardiopsaceae	1	0	0	0
		Nocardioidaceae	1	0	0	0
		Promicromonosporaceae[g]	1	0	0	0
		Micromonosporaceae	1	0	0	0
		Kineosporiineae	2	0	1	0
		Brevibacteriaceae	2	0	0	0
		Frankiaceae	3	0	0	0
		Tsukamurellaceae	1	0	0	0
	Bifidobacteriales	*Bifidobacteriaceae*	29	0	0	0
Firmicutes			**1,073**	**244**	**178**	**85**
Negativicutes	*Selenomonadales*		17	0	1	0
Clostridia			86	7	4	0
Erysipelotrix			1	0	0	0
Bacilli			969	237	121	85
	Lactobacillales					
		Lactobacillaceae	172	16	19	3
		Streptococcaceae	133	15	4	0
		Enterococcaceae	74	24	3	2
		Leuconostoc	29	0	9	0
		Carnobacteriaceae	6	0	1	0
		Oenococcus	6	0	0	0
		Weisella	4	0	0	0
	Bacillales	*Staphylococcaceae*	275	175	118	80
		Bacillaceae	223	5	9	0
		Listeriaceae	14	1	8	0
		Paenobacillaceae	9	0	0	0
		Macrococcus	8	1	0	0
		Planococcaceae	6	0	1	0
		Bacillales group XII	4	0	0	0
		Alicyclobacillaceae	3	0	0	0
		Bhargaceae	2	0	0	0

[a]*Nocardiaceae* (4 *Nocardia*, 25 *Rhodococcus*)
[b]*Mycobacteriaceae* (*Mycobacterium* plus *Amycolicicoccus*)
[c]*Micrococcaceae* (4 *Micrococcus*, 23 *Arthrobacter*)
[d]*Pseudonocardiaceae* (2 *Amycolatopsis*, 6 *Pseudonocardia*, 1 *Saccharomonospora*)
[e]*Microbacteriaceae* (4 *Clavibacter*)
[f]*Streptosporangiaceae* (1 *Planobispora*, 1 *Streptosporangium*)
[g]*Promicromonosporaceae* (*Xylanimonas*)

resistant *Streptococcus agalactiae*) on the basis of the limited therapeutic options to treat infections caused by these bacteria-resistant variants (1). LAB, which are used as probiotics and in the preparation of various products (dairy, fermented meat and seafood, fermented cereals and vegetables, wine), are defined as "generally regarded as safe" (GRAS) microorganisms by the U.S. Food and Drug Administration. However, the potential risk to transfer acquired AbR genes recently found in LAB species to animal and human pathogens is a cause for concern. AbR LAB may also contaminate industrial processes, leading to economic losses (2). In addition, the possibility that opportunistic or commensal bacteria and nonpathogen organisms could serve as reservoirs of AbR genes is increasingly recognized (3). Consequently, several European and American regulatory agencies have recently recommended the mandatory screening of some species such as *Enterococcus faecalis* and *Enterococcus faecium* as indicators of the presence of AbR in foods and food animals and as a mirror of the patterns of antibiotic use in veterinary medicine and agriculture (4, 5). Finally, it is worth mentioning that AbR in a context of the wide use of antibiotics favors the selection of clonal lineages of multihost species with zoonotic potential (e.g., *S. aureus*, *E. faecium*, *Clostridium perfringens*) as well as emblematic zoonotic species such as *Listeria monocytogenes* (see below).

The presence of the same AbR genes in ecologically connected (but also in unconnected) bacterial genera, mentioned above, indicates a complex history of genetic interactions in which AbR genes have parasitized the natural circuits of adaptive gene flow. Plasmids have largely contributed to the spread of AbR and other adaptive genes among members of *Staphylococcus*, *Enterococcus*, and to a lesser extent, species of the *Streptococcus* pyogenic group (6–8), thus influencing the selection of particular subspecies populations due to the acquisition of AbR (8–10). However, the global adaptive role of plasmids of other genera remains largely unexplored outside single pathogens colonizing or infecting single "relevant" hosts. The "single centric" perspective, focusing on "gene tracking" or "vehicle centric" (plasmid, transposon, or other mobile genetic elements [MGEs]) in "single host-single pathogen" systems hampers a comprehensive view of gene and plasmid dynamics and their role in the evolvability of bacterial communities. An integrative view of plasmid ecology is needed to understand community evolvability.

In this work, we analyze the development of AbR in *Firmicutes* within an ecological framework using gene exchange networks. We also discuss the role of plasmids in the emergence, spread, and maintenance of genes encoding resistance to antimicrobials (antibiotics, heavy metals, and biocides) and their influence on the genomic diversity of the main Gram-positive opportunistic pathogens in the light of evolutionary ecology. Finally, a critical revision of plasmid classifications in this group of microorganisms is also provided under this eco-evo perspective by analyzing the 1,326 fully sequenced plasmids of Gram-positive bacteria (*Firmicutes* and *Actinobacteria*) available in the GenBank database at the time this article was written.

AN ECO-EVO PERSPECTIVE TO ANALYZE HGT IN FIRMICUTES

Recent phylogenomic analyses using networks revealed a history of horizontal gene transfer (HGT) events even among highly structured and ecologically disconnected groups of bacteria (11–13). These events are more likely to occur in the case of donors and recipients with a similar G+C content (differing in <5% for 86% of connected pairs) (14) and involving plasmids able to mediate exchange of information between close or distant chromosomal backgrounds (12, 15). Although limited by the current number of available genome sequences, such studies evidenced sound differences in "betweeness" among different bacterial groups and plasmids of *Firmicutes*. LAB frequently undergo HGT events among similar species (11), with streptococci acting as a hub for interactions with more distant ecological groups (12), and some plasmids of the Inc18 family possibly contributing to the spread of AbR genes among different bacterial species (15). To analyze this situation in more detail, we constructed a gene exchange network that comprises all genes conferring resistance to antibiotics and heavy metals described in *Firmicutes* so far (Fig. 1 and 2). This network clearly shows that many resistance genes in different bacterial genera can present plasmid and/or chromosomal locations, illustrating the diversity of interactions, often plasmid mediated, within bacterial communities (Fig. 1 and 2). Available (and often fragmented) knowledge from different fields enabled us to state that the dynamics of bacterial populations are influenced by the interplay of selection processes at different levels of organization (genes, MGEs, clones, species) and their associated environments (16–20). Because of that, the complexity resulting from such interplay cannot be understood using either single centric studies or the abovementioned phylogenomic analysis of HGT networks.

The presence of the same genes in different genetic contexts implies contacts and exchanges between bac-

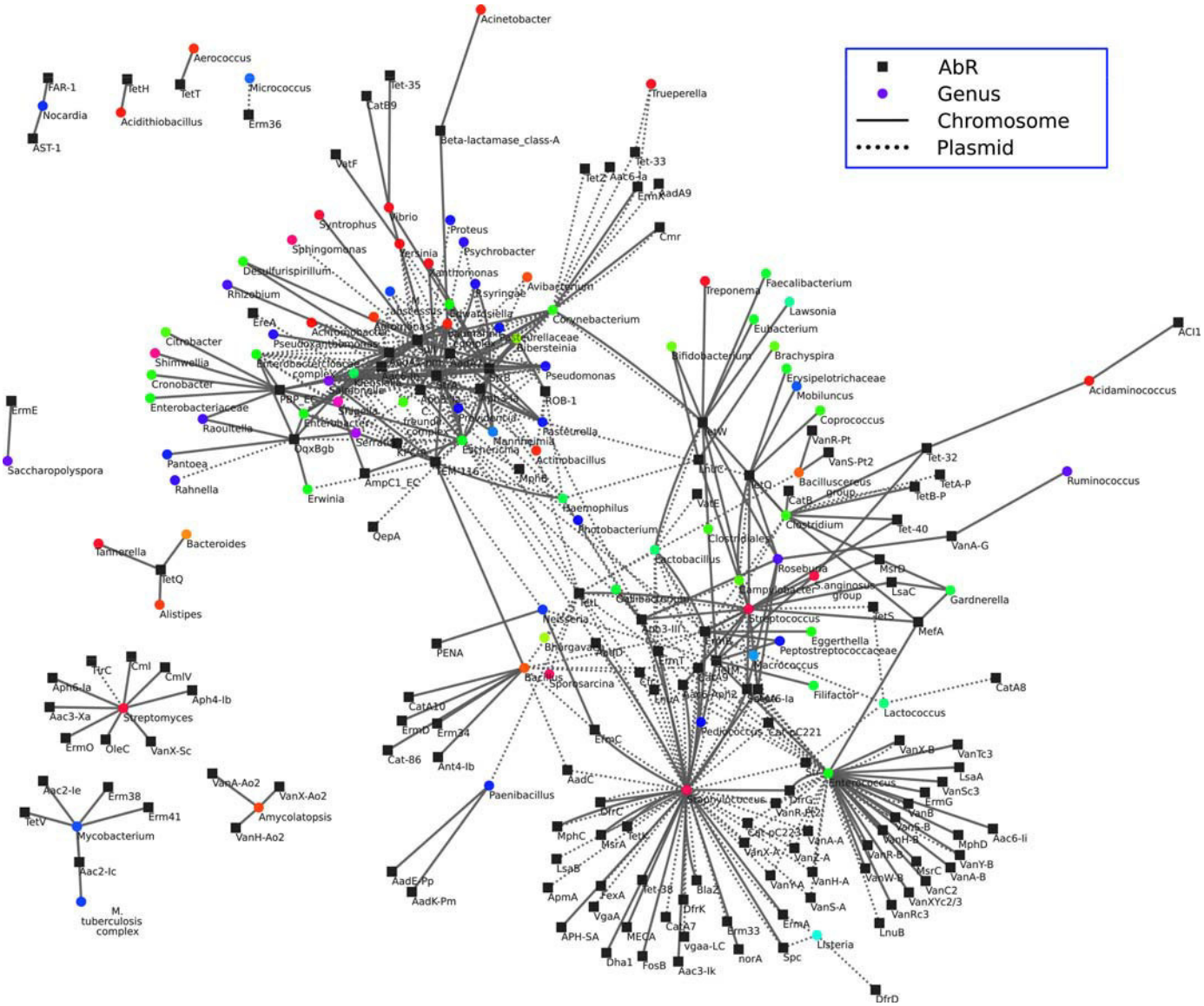


FIGURE 1 Protein content network (PCN) of AbR proteins found in plasmids and chromosomes of *Firmicutes* and *Actinobacteria*. To determine the AbR protein catalog of Gram-positive strains (chromosomes and plasmids), a Blastp search was performed of all their proteomes against the ARG-ANNOT database (http://en.mediterranee-infection.com/article.php?laref=283&titre=arg-annot) using a cut-off of 1e-30 and 85% of identity. The presence of the Gram-positive AbR proteins identified above in all bacterial species (only complete sequences, not partial) was determined using a similar Blast search (blastp, 1e-30 E-value and 85% identity) against the NCBI GenBank database. The nodes correspond to bacterial species (circular nodes; each color indicates one genus) and AbR proteins (square nodes). Nodes were connected by an edge when a positive hit between AbR proteins and one or more strains of a given species were identified. Edges further indicate the location of the AbR genes associated with each AbR protein of the Gram-positive catalog. Solid lines represent chromosomal location, and dotted lines represent plasmid location. When an AbR gene was located in both chromosomes and plasmids, both lines were plotted. doi:10.1128/microbiolspec.PLAS-0039-2014.f1

teria belonging to different genera, probably facilitated in complex biofilms and environments allowing high local bacterial densities. HGT via transduction or conjugative mechanisms has been extensively documented in *Lactobacillales* and is a prominent process for niche-specific adaptation in different genera (12, 21–23), with plasmids and conjugative transposons being the most relevant providers of communal adaptive gene pools in microbial ensembles sharing complex niches.

An important question is if resistance genes contributed to the recombination between different replicons and, consequently, to their evolvability. The frequent association of resistance genes with site-specific recombination systems and insertion sequences located either in plasmids or in chromosomes favors homologous recombination and therefore different events of integration or excision, as well as the interplay among different elements (19, 24–28). Restriction-modification (RM) systems and clustered regularly interspaced short palindromic repeats (CRISPR) are the main posttransfer barriers protecting a given host cell from invasion by foreign DNA either by conjugation transformation or transduction (9, 29, 30). Some RM systems specifically limit the acquisition of plasmids to some pathogens which may influence their clonal structure (e.g., RM types I, III, and IV in *S. aureus*) (31, 32). This may also explain the lack of plasmids in certain species such as *S. pneumoniae* or the narrow host range of plasmids from some *Clostridium* species (33, 34). Anti-RM systems such as analogues of ArdA (alleviation of restriction of DNA) proteins that act against type I restriction systems (detected in Tn*916* and CTn*6000*) or other genes predicted to be involved in methylation (e.g., in CTn*6000* and Tn*1721*) are involved in the restricted spread of certain MGEs, as well as in certain clonal expansions. There is evidence that the presence of complete CRISPR loci is inversely proportional to the presence of MGEs in *Clostridium* and *Staphylococcus* (34, 35), a situation that has also been suggested to occur for *Streptococcus* and *Enterococcus* (34, 36, 37). In agreement with this statement, Fig. 1 and 2 reflect a heterogeneous distribution of AbR and heavy metal and biocide resistance (Met^R and Bc^R) genes in different genera. Particularly interesting is the confinement of vancomycin resistance within enterococci and of some AbR, Met^R, and Bc^R genes within staphylococci and clostridia, a situation that is in part due to the barriers shaping different populations (see next sections).

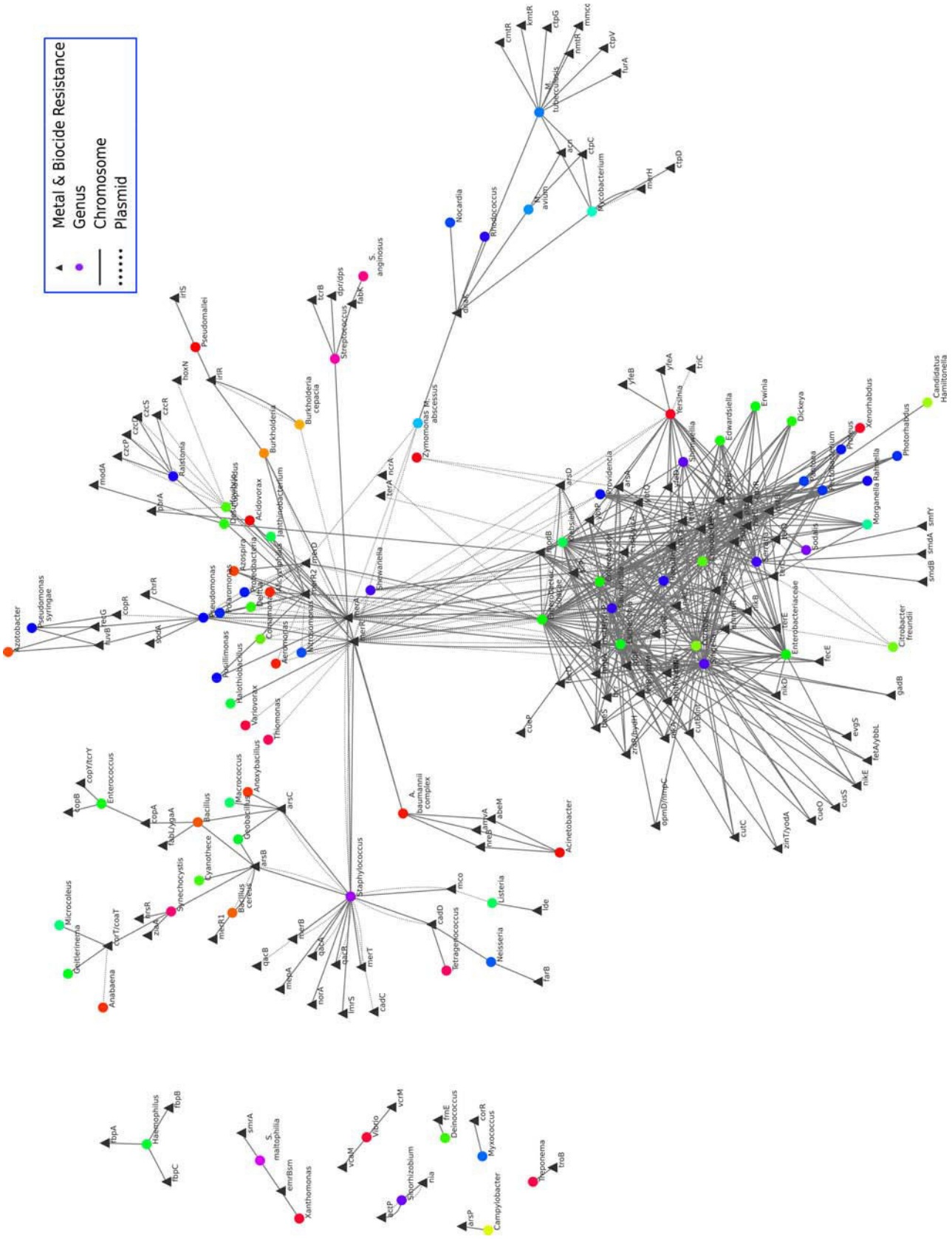
Fluctuating environments, concentration gradients, and high population sizes, all frequent in different "source-sink" ecologies such as bacterial populations under antimicrobial selective pressure, favor DNA transfer and the selection of some clonal and plasmid variants (38–42). Such selective processes favor the emergence of novel variants, resulting from genetic drift, or migration to heterogeneous environments. Recent studies demonstrated that plasmid transfer occurs *in vivo* more frequently than in experimental evolution assays (43), and that gradients in populations under selection pressure by diverse antimicrobials favor selection of multidrug resistance (MDR) plasmids (44, 45).

The influence of plasmids has been extensively discussed in the literature from different evolutionary perspectives (20, 46–48), but only limited information about plasmid ecology and the specific roles that plasmids actively play within microbial systems *in situ* is available. Mosaic MGEs have often been documented in *Firmicutes*, reflecting a different epidemiological history of contacts with strains of the same or different species. Some mosaic MGEs have been fixed, making the interpretation of results by using traditional classification schemes extremely difficult (49).

PLASMID DIVERSITY AND CLASSIFICATION SYSTEMS

Efforts in plasmid characterization and classification are justified for the understanding of plasmid biology. Nowadays, plasmid categorization is relevant from the public and environmental health perspective to follow the movement of genes coding for resistance to antimicrobials (antibiotics, heavy metals, biocides), colonization and virulence factors for humans and animals, and/or other adaptive traits that drive ecological success (bacteriocins, metabolic traits) and consequently increase the population size of bacteria harboring MGEs. In fact, only a "representative diversity" of bacterial plasmids has been systematically analyzed in a few genera of multihost opportunistic pathogens of interest in biomedicine, with a particular emphasis on species of the *Enterobacteriaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, and *Enterococcaceae* families (7, 50–53). The diversity of plasmids from *Lactococcus* (54), *Lactobacillus* (55), *C. perfringens* (56), *Micrococcus* (57), and *Bifidobacterium* (58) has also been analyzed from different perspectives.

Plasmid diversity within a particular bacterial species in the *Firmicutes* phylum started to be comprehensively analyzed in the 1960s just after the discovery of staphylococcal plasmids. These elements were initially categorized into three main classes designated by roman numerals on the basis of size, replication machinery,



ability to be transferred, phenotypic and functional characteristics, and host range (7, 51, 53, 59, 60). Class I comprised high copy number plasmids (10 to 60 copies per cell) of less than 5 kb with a rolling circle replication (RCR) mechanism that often harbored one or two AbR genes (usually conferring resistance to tetracycline, chloramphenicol, macrolides, and trimethoprim). Class II comprised low copy number plasmids (4 to 6 copies per cell) of 15 to 40 kb, with a theta replication mechanism, which typically carried resistance to antibiotics (β-lactams, aminoglycosides, and macrolides), heavy metals (arsenic, cadmium, and mercury), and/or antiseptics (quaternary ammonium compounds). Class III comprised plasmids similar to those found in class II which were transferred by conjugation (61). Afterward, Richard Novick and others classified staphylococcal plasmids in 15 incompatibility (Inc) groups based on the finding that two plasmids with the same replication (rep) proteins cannot be stably maintained in the same cell (50, 62, 63). Plasmids of most Inc groups correspond to class I (10 Inc groups of apparently closely related plasmids) and class II (diverse plasmids that belong to the same Inc group) (53). Following the same Inc numeral designation criteria, Brantl et al. categorized a few streptococcal plasmids that replicated via a theta mechanism and that were regulated by an antisense RNA that mediated transcriptional attenuation, such as the Inc18 family (64) (see below). Pheromone-responsive plasmids of enterococci were also subgrouped into different incompatibility groups on the basis of distinct responses to small peptides or pheromones which are secreted by plasmid-free donors (65).

A multiplex-PCR typing system based on the diversity of replication initiator proteins (RIPs) developed by Jensen et al. (59) has recently been applied for the characterization of *Firmicutes* plasmids, mainly staphylococci (66) and enterococci (67–71) of human, animal, and environmental origin. According to this typing system, RIP variants are designated as "rep" followed by a subindex number and are arbitrarily called Rep families. Although this system is very useful to enlarge the knowledge of scarcely explored plasmid diversity in contemporary isolates of enterococci and staphylococci, its application is limited to known plasmids, mainly AbR plasmids of these genera, as illustrated in various surveys and this study (59, 66, 68, 71).

The diversity of mobilization (MOB) systems has also recently been used to classify plasmids and other conjugative elements in different bacterial groups including *Firmicutes* (72, 73). The approach relies on the variability of relaxases (RELs), which form part of the plasmid MOB region, are involved in the initiation of DNA transfer, and that, aside from the origin of transfer (*oriT*), are present in both conjugative and mobilizable plasmids as well as in other conjugative elements (74). To date, only five (MOB_P, MOB_Q, MOB_V, MOB_C, and MOB_T) out of seven known REL families have been identified in *Firmicutes* (7, 72, 74). MOB_Q, MOB_C, and MOB_T are present in conjugative elements, and MOB_V is present in mobilizable plasmids. MOB_P has been identified in both conjugative and mobilizable elements (7, 72, 73). The application of this PCR-based classification scheme is obviously limited to the typing of known RELs. Frequent plasmid mosaicism, redundancy, and coexistence of different "core" genes, and the interplay of plasmids with other conjugative elements that contain homologs of RIPs and RELs, complicates the establishment of a robust plasmid core ontology and precludes the use of typing approaches similar to those used in Gram-negative organisms such as plasmid multilocus sequence type (http://pubmlst.org/plasmid/).

Whole-genome (plasmid/chromosome) sequencing provides accurate and nonbiased information on plasmid backbones. Although the number of fully sequenced

FIGURE 2 PCN of metal-biocide (Met^R/Bc^R) proteins found in plasmids and chromosomes of *Firmicutes* and *Actinobacteria*. To determine the Met^R/Bc^R protein catalog of Gram-positive strains (chromosomes and plasmids), a Blastp search was performed of all their proteomes against the BacMet database (http://bacmet.biomedicine.gu.se/) using a cut-off of 1e-30 and 85% of identity. The presence of the Gram-positive Met^R/Bc^R proteins identified above in all bacterial species (only complete sequences, not partial) was determined using a similar Blastp search (blastp, 1e-30 evalue and 85% identity) against the NCBI GenBank database. The nodes correspond to bacterial species (circular nodes) and Met^R/Bc^R proteins (triangular nodes). Nodes were connected by an edge when a positive hit between Met^R/Bc^R proteins on one or more strains of a given species was identified. Edges further indicate the location of the Met^R/Bc^R genes associated with each Met^R/Bc^R protein of the Gram-positive catalog. Solid lines represent chromosomal location, and dotted lines represent plasmid location. When a Met^R/Bc^R gene was located in both chromosomes and plasmids, both lines were plotted. doi:10.1128/microbiolspec.PLAS-0039-2014.f2

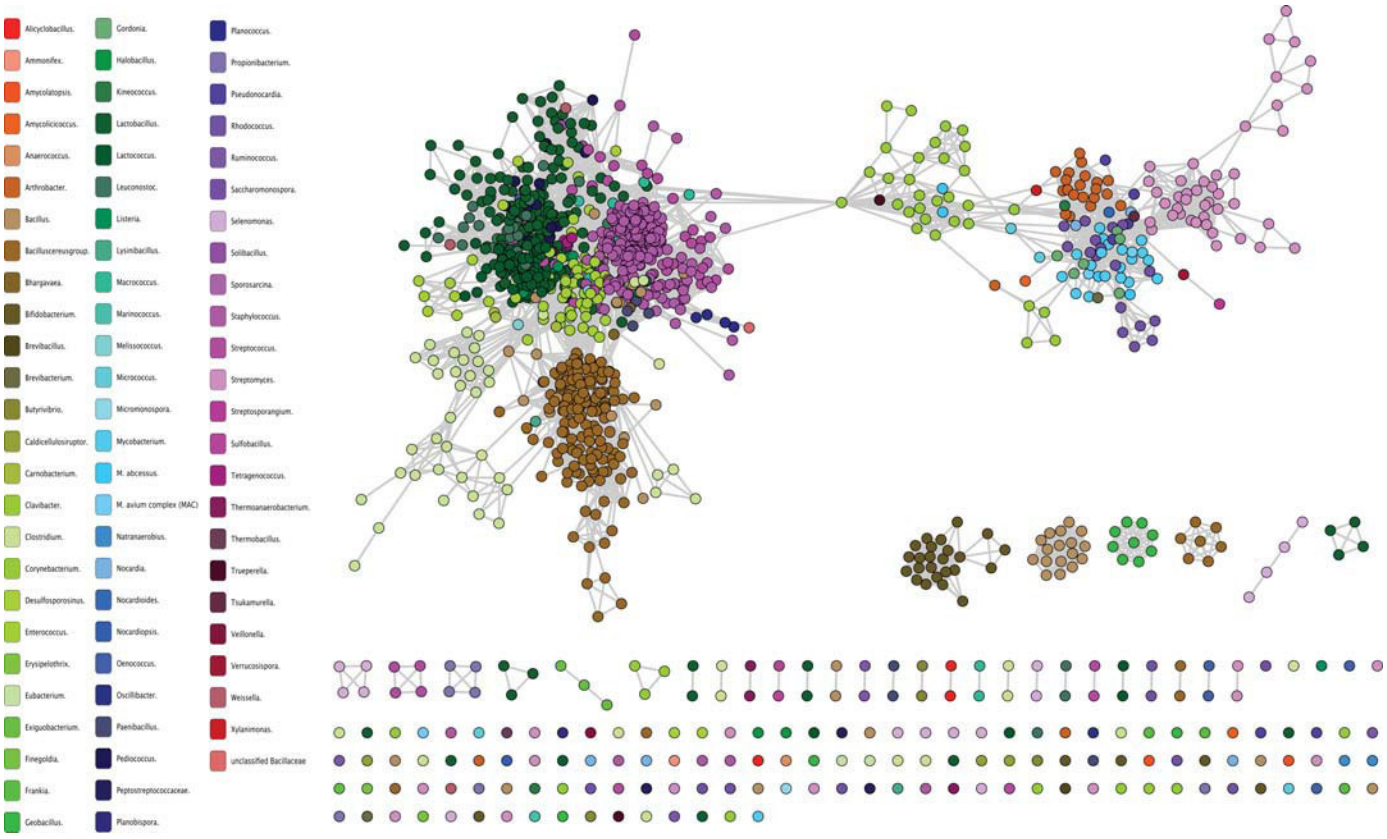
plasmids in databases is still limited, we used a plasmid homology network analysis of the 1,326 fully sequenced plasmids of *Firmicutes* and *Actinobacteria* to study the diversity of plasmids carrying genes coding for AbR and Met^R/Bc^R and the impact of plasmids in the evolvability of contemporary AbR bacterial populations of *Firmicutes*.

Figures 3 and 4 illustrate the existence of group-specific plasmid populations, with a number of plasmids being shared between *Lactobacillales* (mainly *Enterococcus* and *Streptococcus*) and *Bacillales* (*Staphylococcus*), which are greatly implicated in the spread of AbR and Met^R/Bc^R. These shared plasmids include RCR and theta-replicating plasmids of different families, which have been recently analyzed at the molecular level (7, 75, 76). Next in this section, we will analyze the diversity of these groups and highlight the usefulness of current typing systems for each group. However, it is of note that the main genera of *Firmicutes* carry a variable number of plasmids containing several replication and transfer systems, some of them being able to be transferred. The interplay between genes, plasmids, and populations will be analyzed under an ecological perspective in the section "Gene and Plasmid Flow Shapes the Evolutionary Ecology of Firmicutes."

Rolling Circle Replication Plasmids

RCR plasmids are classified in a few families according to the RIP and the double origin of replication (*dso*) (see comprehensive reviews in references 75, 77–79). Most of the RCR plasmids known to date have been found in species of *Firmicutes*, *Proteobacteria*, *Cyanobacteria*, and *Spirochaetes*, and some of them have been identified in genetically distant hosts. The production of single-stranded DNA and the mechanism of replication of these plasmids enhance their ability to recombine, by either homologous recombination or illegitimate recombination with other RCRs and theta replicating plasmids

FIGURE 3 Plasmid homology network. The genomic homology network was performed using "All-versus-All" genomic Megablast (238) of 1,326 fully sequenced plasmids from low G+C bacterial species (*Firmicutes* and *Actinobacteria* phyla) available at public gene databases. The nodes correspond to bacterial plasmids (circular nodes; different colors representing different genera). Two nodes are connected by an edge if they share homologous DNA. doi:10.1128/microbiolspec.PLAS-0039-2014.f3



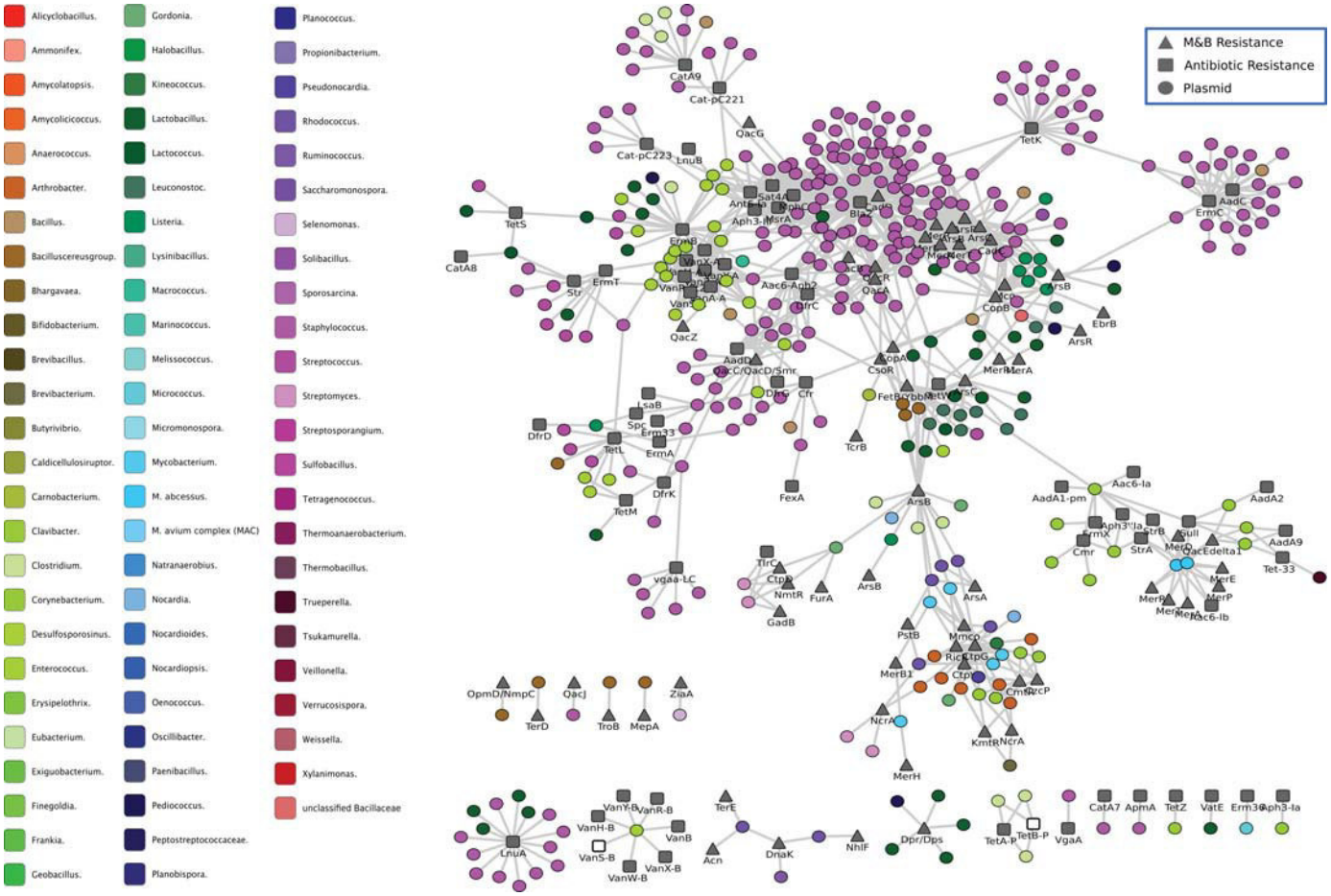


FIGURE 4 PCN of AbR and Met^R/Bc^R proteins located on plasmids of *Firmicutes* and *Actinobacteria*. PCN of AbR and Met^R proteins found in Gram-positive plasmids. We formed the PCN by representing plasmids as circular nodes, AbR as square nodes, and Met^R/Bc^R as triangular nodes, connecting two nodes (plasmid and AbR or Met^R/Bc^R) if one plasmid has this AbR or Met^R/Bc^R. The presence of the Met^R/Bc^R gene was determined by blasp of all plasmid proteins against the BacMet database. The presence of the AbR gene was determined by blasp of all plasmid proteins against the ARG-ANNOT database. The colors for the genus are the same as those in Fig. 3. doi:10.1128/microbiolspec.PLAS-0039-2014.f4

(Fig. 5 to 10 and text below). RCR plasmids are also frequently integrated into chromosomes (e.g., pUB110 within SCCmec cassettes in methicillin-resistant *S. aureus* or pC194/pUB110 [*catA*] in *S. pneumoniae* genomes) (80).

In *Firmicutes*, four groups of RCR plasmids have been defined according to RIP similarity, namely Rep_trans (PF025486), Rep_1 (PF14046), Rep_2 (PF01719), and Rep_L, which are historically represented by plasmids pT181, pUB110, pMV158, and pSN2, respectively (53, 75, 77, 81). Within these families, some members have been fixed by selection and might be maintained by the vertical expansion of certain clones, aside from HGT, with the emergence of variants from time to time. Figures 11 to 14 and Supplementary Table S1 show the similarity of genes encoding RIPs of all available fully sequenced plasmids and the correspondence to the Rep families described by Jensen et al. (59). These plasmids may contain different adaptive genes (AbR, heat shock proteins, or bacteriocins), although most of them are classified as "cryptic," without any clear adaptive function.

The Rep_1 family

The Rep_1 family comprises plasmids with RIPs of the families rep_{13} (associated with *catA7*, which encodes resistance to chloramphenicol), rep_{21} (cryptic or eventually carrying *lnuA*, coding for resistance to lincosamides), rep_{22} (carrying a variety of AbR genes), and other underrepresented members categorized as $rep_{Unique7}$. How-

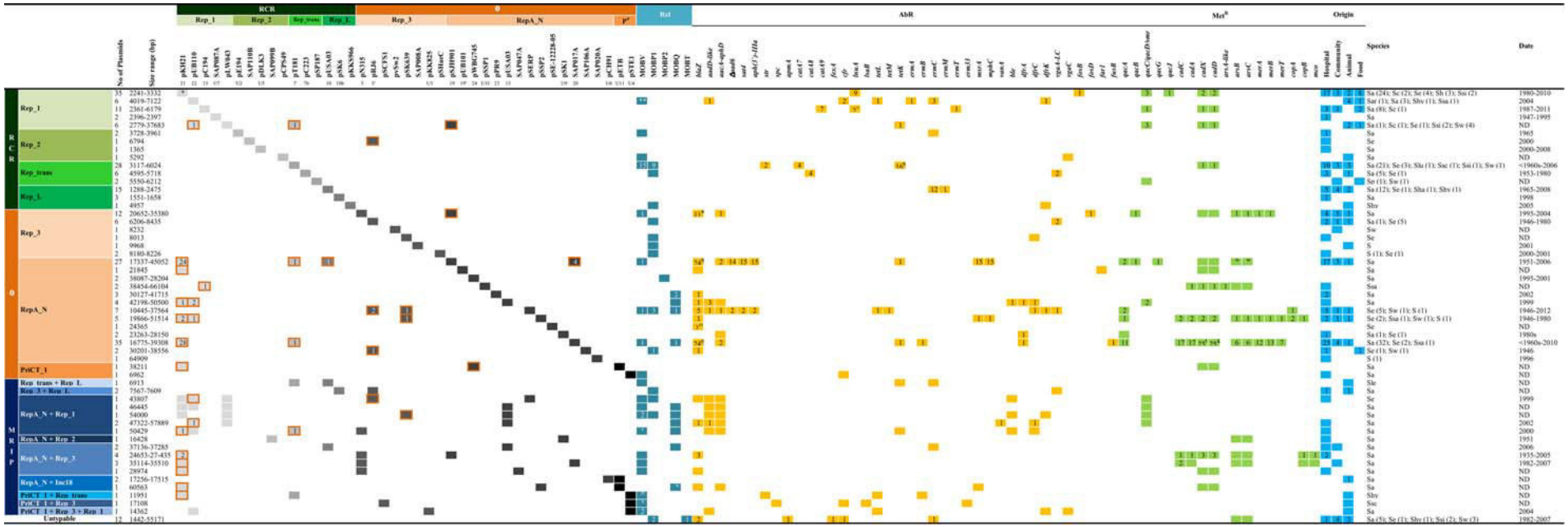


FIGURE 5 Plasmids from *Staphylococcus* spp. The presence of an orange border in the RIP family indicates that the corresponding RIP is truncated. [a]PriCT_1; *One of these plasmids (GenBank accession number NC_013381) has a truncated rep_pKH21 (rep_1) gene, and no other known RIPs were identified. **Two of these plasmids (GenBank accession number NC_016054 and NC_019144) appear to have two copies of the MOB_V gene. [+]One of these plasmids (GenBank accession number NC_008354) has two copies of the *lnuA* gene. [§]One plasmid (GenBank accession number NC_001393) has a truncated copy of the *tetK* gene. [¥]One plasmid (GenBank accession number NC_010419) has a truncated copy of the *blaZ* gene. [£]The plasmid (GenBank accession number NC_005076) appears to have two copies of the MOB_V gene. [$]One plasmid (GenBank accession number NC_018959) has a truncated copy of the *blaZ* gene. [&]Two plasmids (GenBank accession numbers NC_007931 and NC_016942) have two copies of the *arsB* and *arsC* genes. [¢]This plasmid (GenBank accession number NC_013320) appears to have two copies of the MOB_V gene. [¶]This plasmid (GenBank accession number NC_005004) has a truncated copy of the *blaZ* gene. [ϕ]Three plasmids (GenBank accession numbers NC_013321, NC_019007, and NC_018976) have a truncated copy of the *blaZ* gene. [Ж]Eleven of these plasmids have a truncated copy of the *cadD* gene (GenBank accession numbers NC_020531, NC_020538, NC_013337, NC_020534, NC_020565, NC_020567, NC_020530, NC_020539, NC_017352, NC_013323, and NC_022610). [Θ]One plasmid (GenBank accession number NC_013334) has a truncated copy of the *cadX* gene. [M]This plasmid (GenBank accession number NC_020237) appears to have two copies of the MOB_V gene. [Þ]This plasmid (GenBank accession number NC_022598) appears to have two copies of the MOB_V gene. Abbreviations: MRIP, Multi-RIP; S, *Staphylococcus* spp; Sar, *Staphylococcus arlettae*; Sa, *S. aureus*; Sc, *Staphylococcus chromogenes*; Se, *Staphylococcus epidermidis*; Sha, *Shaemolyticus haemolyticus*; Shy, *Staphylococcus hyicus*; Sle, *Staphylococcus lentus*; Slu, *Staphylococcus lugdunensis*; Sp, *Staphylococcus pasteuri*; Ssa, *Staphylococcus saprophyticus*; Ssc, *Staphylococcus sciuri*; Ssi, *Staphylococcus simulans*; Sw, *Staphylococcus warneri*. doi:10.1128/microbiolspec.PLAS-0039-2014.f5

ever, the available typing systems are unable to classify relevant Rep_1 plasmid members including plasmids containing heat shock proteins in *Streptococcus thermophilus*, plasmid-borne bacteriocins in *S. pyogenes* (82), or *S. pneumoniae* plasmids (80, 83), among others (Fig. 11). Remarkably, RIPs of this Rep_1 group are often detected in mosaic plasmids of staphylococci and enterococci (Fig. 5 and 7), some plasmid chimeras being fixed and persistently recovered for years. For example, emblematic mosaics theta/RCR plasmids of staphylococci (e.g., cointegrates of RepA_N/pSK41 and Rep_1/pUB110, which encode resistance to gentamicin) and *E. faecalis* (e.g., pAMα1) have both been selected in those lineages since the early 1970s (7, 71, 84).

The Rep_trans group

Plasmids of the Rep_trans group are clustered in two large branches (Fig. 12). One branch comprises plasmids of *Staphylococcus* that harbor *tetK* (rep_7) and *catA8/catA7* (rep_{7b}) with different MOB genes. Such plasmids have been reported in *S. aureus* since their first detection in the early 1950s (84) and were eventually described in contemporary *E. faecalis* isolates (68, 85). A second branch contains pRI1-like plasmids (rep_{14}), which correspond to plasmids of different enterococcal species (*E. faecium, Enterococcus hirae, Enterococcus mundtii)* isolated from foodborne animals and hospital patients (7, 59, 71, 86). These plasmids can be mobilized by other AbR conjugative theta replicating plasmids present in the same cell (71, 87), and it seems they are widely spread among enterococcal populations.

The Rep_2 group

The Rep_2 group (Fig. 13) comprises numerous promiscuous elements able to replicate in distant hosts which have been extensively analyzed at the molecular level by Espinosa et al. using pMV158 as a model (75). Plasmids carrying *ermT* (an inducible methylase conferring resistance to first-line macrolide-lincosamide antibiotics such as erythromycin and clindamycin), from group A Streptococci (GAS) and group B Streptococci (GBS), are the sole representatives of AbR in this group. They appear to be responsible for the rise of macrolide resistance among GAS and GBS in hospitals since the mid-1990s (8).

The Rep_L group

In contrast to the above-mentioned RCR plasmid groups, proteins within the Rep_L family (Fig. 14) are represented in public gene databases by a very few RIPs of *Staphylococcus, Selenomonas* (class *Negativicutes*), and *Butyrivibrio* (Clostridia) species, all these genera being frequent components of the oral flora of humans and the rumen of some animal species. These plasmids are responsible for the widespread *ermC* in staphylococci (rep_{10}). Interestingly, the emergence of both *ermT*-Rep_2 and *ermC*-Rep_L plasmids seems to be associated with the abusive use of tylosin in cattle, amplified by the location of these AbR genes in RCR plasmids, and further transferred to other populations of *Firmicutes* (8, 88, 89).

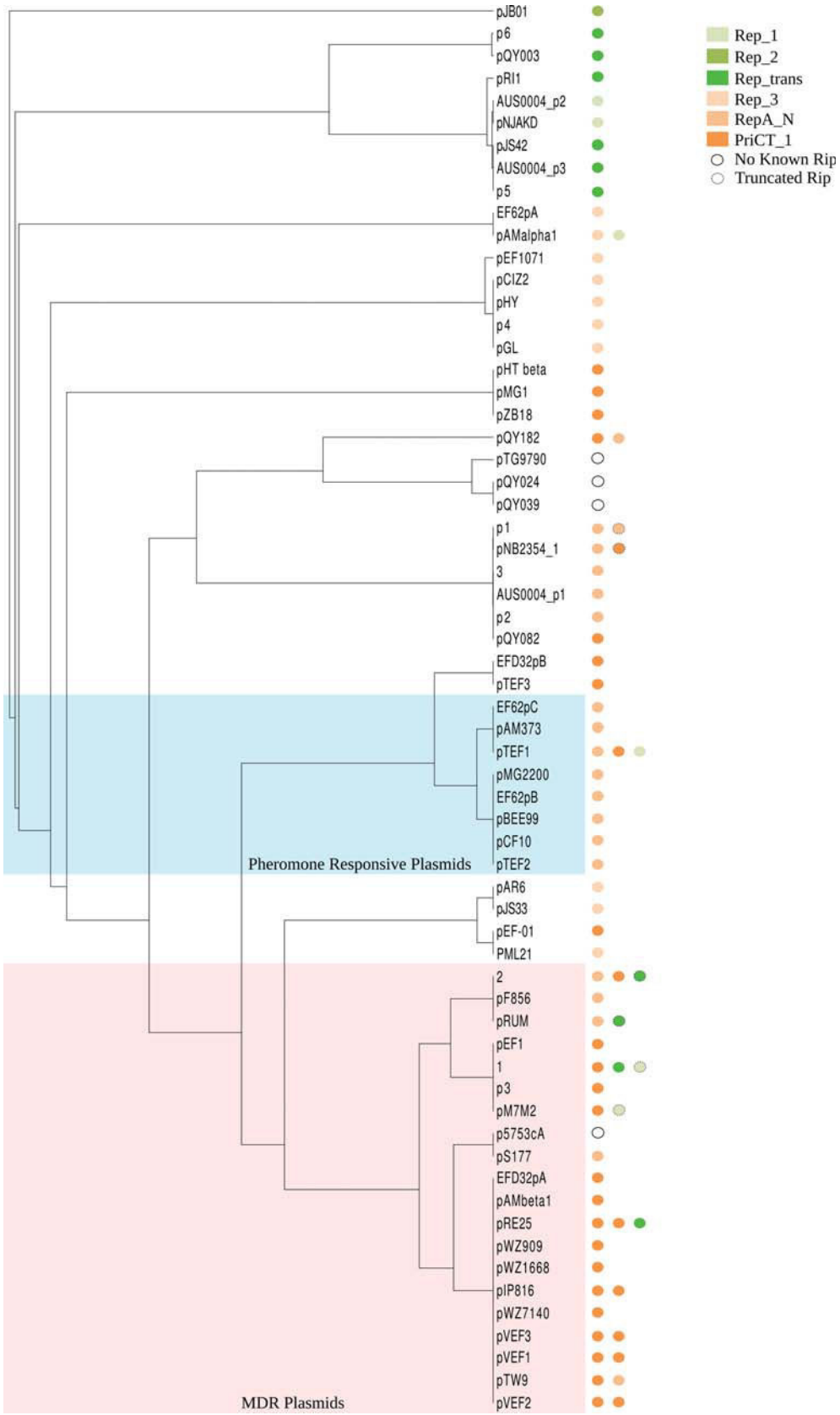
RCR plasmids were associated with REL of the group MOB_{V1} (72, 73, 75), although representatives of all the RCR groups mentioned above that lack REL were detected in databases. Interestingly, RELs of MOB_{P1} and MOB_T families were also found, and their presence is probably due to the co-integration of RCR with theta-replicating plasmids (see below).

Theta-Replicating Plasmids

Four families of plasmids that replicate by a theta mechanism, three that comprise conjugative plasmids (RepA_N, Inc18, and pMG1) and one in groups small nonconjugative elements (Rep_3), are involved in the capture, spread, and maintenance of AbR among different genera of *Firmicutes*. Members of the RepA_N and Inc18 families are often enriched in insertion sequences, mainly IS*257*, IS*256*, IS*1216*, IS*L3*, and IS*431*, that facilitate co-integration, rearrangements, and deletions among elements of *Staphylococcus*, *Enterococcus*, LAB, and *Clostridium* of different origins (6, 7, 28, 90–95). Such recombination events seem to have facilitated the origin of the great mosaicism of MDR plasmids that often carry more than one RIP, lack transfer and maintenance modules, and eventually carry more than one REL (Fig. 5 and 7). The transfer mechanisms of RepA_N pSK41-like plasmids and the Inc18-like plasmids are similar and are categorized as type IV secretion systems (96).

The Rep_3 family

Plasmids containing RIPs with the Rep_3 domain (Fig. 15) are common among disparate bacterial genera including *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Lactobacillus*, and *Enterobacteriaceae* (7). Figure 15 shows the diversity of RIPs among fully sequenced plasmids of *Firmicutes*, and Fig. 5 to 10 reflect the features of known members of this family within each genus of biomedical interest. In enterococci, Rep_3 plasmids (<15 kb) have been found in isolates recovered from hospitalized patients, animals (pigs, cows), cheese, milk, and dry-fermented sausage, frequently associated with the production of bacteriocins that are active against a



variety of Gram-positive genera (7). In *Lactobacillus* and *Lactococcus*, they harbor bacteriocins and, eventually, AbR genes. Rep_3 plasmids play a relevant role as vehicles of AbR among staphylococci. Plasmids from *S. aureus* are overrepresented by closely related variants containing Rep_5, which are associated with genes coding for penicilinase and resistance to heavy metals (cadmium and arsenic) (51, 53, 66, 84). Staphylococcal plasmids within this group include AbR plasmids from coagulase-negative strains of animal origin, some of them with RIPs that would not be detected by current typing systems (97, 98).

The Inc18 family

First described in the 1990s, the Inc18 family comprises a highly heterogeneous group of broad host range, low copy number plasmids (<10 per cell) that replicate by a theta mechanism, regulated by an antisense RNA that mediates transcriptional attenuation and that are able to conjugate on solid media at high frequencies (64, 99). The transfer system of pIP501 has been extensively studied and constitutes a paradigm of conjugation systems, showing significant similarity with the *tra* regions of RepA_N plasmids pGO1 and pSK41 from *S. aureus* and pMRC01 from *Lactococcus lactis* (96, 100).

The Inc18 group is traditionally represented by three emblematic plasmids: pSM19035 (101) and pIP501 from *S. agalactiae* and pAMβ1 from *E. faecalis* (64, 101–104). It gets its name from the apparent incompatibility of these plasmids with each other described in seminal studies in the field and following the nomenclature of Inc groups started by Richard Novick for staphylococcal plasmids (50, 60, 64, 105). Inc18 plasmids frequently carry the postsegregational killing systems, ες, and type I partition cassette *prgPprgO*, which are associated with a variety of RIPs and seem to contribute to their persistence in different populations in the absence of antibiotic selection pressure (7, 106, 107). Detailed molecular characterization of such plasmids is described elsewhere (64, 99, 108) and shows a remarkably high modular interplay among different Inc18 plasmids, leading to the high modularity observed in plasmid sequences (see Fig. 5 to 10 and text below).

Inc18 plasmids have contributed remarkably to the spread of AbR (macrolides, chloramphenicol, aminoglycosides, and glycopeptides) and Met^R (copper and mercury) among streptococci and other phylogenetically distant genera of Gram-positive (*S. aureus*, *Listeria*, *Bacillus subtilis*, *Lactobacillus*, *Leuconostoc*, various *Clostridium* species) and Gram-negative bacteria (108–111). Plasmid relatives of pAMβ1 (harboring *ermAM*, later on recognized as *ermB*, and conferring resistance to macrolides, lincosamides, and streptogramines) and pIP501 (carrying *ermB* and $catA7_{pC221}$, which confers resistance to chloramphenicol) were rapidly spread during the 1970s and have frequently been detected among streptococci of groups A, B, and D (enterococci) since then (110, 112–114) (see also Supplementary Table S1 for contemporary representatives of this plasmid group). Initially, the successful spread of intact AbR plasmids among clones of various streptococcal genera, including *S. pneumoniae*, and *S. aureus* was reported, despite the lack of stability in these last two clonal backgrounds (110, 113). Inc18 plasmids conferring resistance to aminoglycosides (kanamycin, streptomycin, and neomycin) and to macrolides were also detected in 1972, in the emblematic *Streptococcus* (*Enterococcus*) *faecalis* strain JH1 that carried pJH1 (an MDR plasmid, presumably Inc18) and pJH2 (a RepA_N pheromone-responsive plasmid carrying hemolysin and bacteriocins). pJH1 represented the first description of conjugative transfer of AbR plasmids in enterococci (114). Aminoglycoside resistance in pJH1 relatives was due to the presence of Tn*5405*, a transposon comprising three genes in tandem (an aminoglycoside 6-adenyltransferase [*aad*], a streptothricin acetyltransferase [*sat*], and an aminoglycoside-phosphotransferase [*aph3*]). These genes were identified later on in *S. pyogenes, S. agalactiae, S. aureus, Campylobacter coli, C. perfringens, and C. difficile* (now *Peptoclostridium difficile)*.

More recently, diverse Inc18 plasmids carrying Tn*1546* in enterococci and staphylococci have emerged

FIGURE 6 Hierarchical clustering dendrogram of plasmids from enterococci. The matrix distance used for building the UPGMA dendrogram is based on the Raup-Crick distance of the orthologous protein profile of each plasmid. For each plasmid, a presence/absence protein profile was made using cut-off values of 80% identity and 80% coverage. Protein clustering was made by using CD-HIT (239). Different background colors are used to emphasize branches of related plasmids and are the same as those defined in Fig. 5. Names to the left of the dendrogram indicate the RIP family. Background colors were used to point out plasmid groups frequently involved in mobility of AbR genes and *E. faecalis* pheromone-responsive plasmids. Circles indicate RIPs identified in each plasmid according to data shown in Fig. 7. doi:10.1128/microbiolspec.PLAS-0039-2014.f6

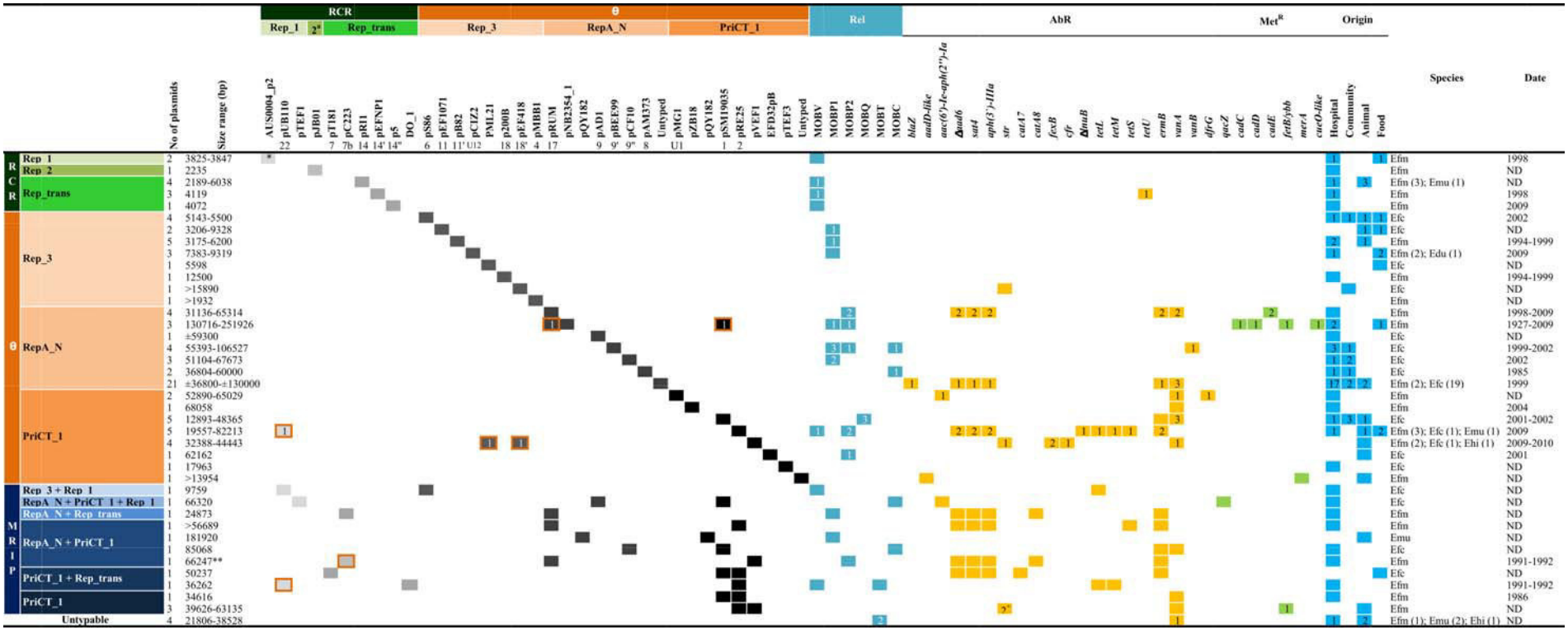


FIGURE 7 Plasmids from *Enterococcus* spp. The presence of an orange border in the RIP family indicates that the corresponding RIP is truncated. [a]Rep_2; *One of these plasmids (GenBank accession number NC_015849) has a truncated rep_AUS0004_p2 (Rep_1) gene, and no other known replication initiator proteins were found. **This plasmid (GenBank accession number NC_017962) has two copies of Tn*401*; in one of them the *add(6)* gene is not truncated; this plasmid also appears to have two copies of the MOB_{P1} gene. [+]These two plasmids (GenBank accession numbers NC_008768 and NC_008821) have a truncated copy of the *str* gene. Abbreviations: MRIP, multi-RIP; Efm, *E. faecium*; Efc, *E. faecalis*; Emu, *E. mundtii*; Edu, *E. durans*; Ehi, *E. hirae*. doi:10.1128/microbiolspec.PLAS-0039-2014.f7

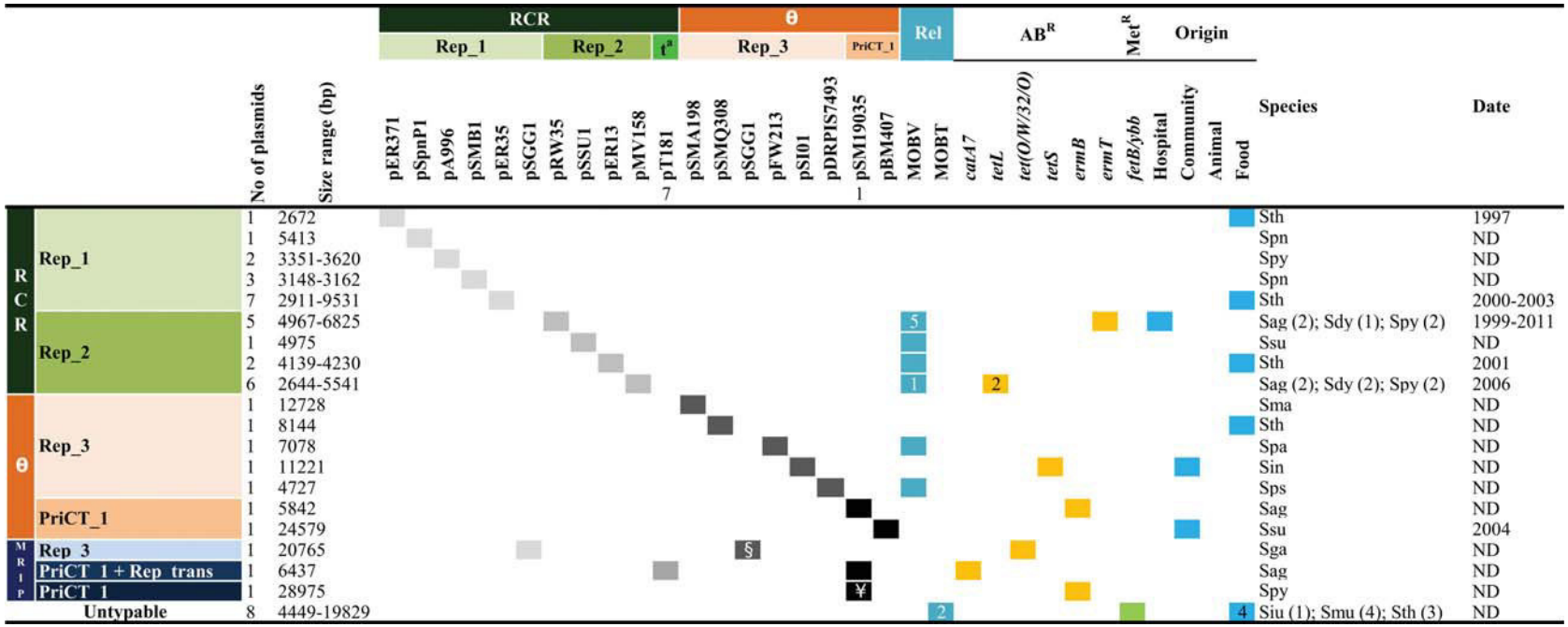


FIGURE 8 Plasmids from *Streptococcus* spp. [a]Rep_trans; [§]This plasmid (GenBank accession number NC_015219) has two similar replication genes belonging to the Rep_3 family. [¥]This plasmid (GenBank accession number NC_006979) has two similar replication genes belonging to the PriCT_1 family. Abbreviations: MRIP, Multi-RIP; Sag, *S. agalactiae*; Sdy, *Streptococcus dysgalactiae*; Sga, *Streptococcus gallolyticus*; Siu, *Streptococcus infantarius*; Sin, *Streptococcus infantis*; Sma, *Streptococcus macedonicus*; Smu, *Streptococcus mutans*; Spa, *Streptococcus parasanguinis*; Spn, *S. pneumoniae*; Sps, *Streptococcus pseudopneumoniae*; Spy, *S. pyogenes*; Ssu, *Streptococcus suis*; Sth, *Streptococcus thermophilus*. doi:10.1128/microbiolspec.PLAS-0039-2014.f8

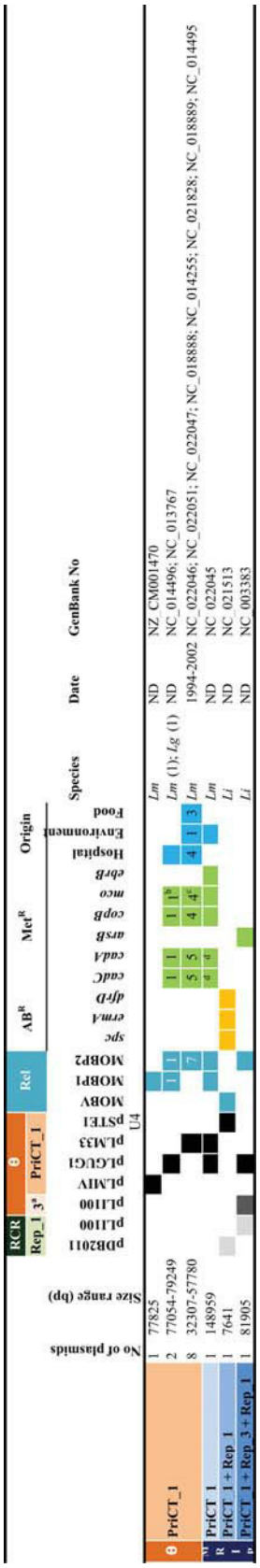


FIGURE 9 Plasmids from *Listeria* spp. [a]rep_3. [b]One plasmid (GenBank NC_013767) has two copies of the *mco* gene, one of which is truncated. [c]One plasmid (GenBank NC_018888) has a truncated copy of the *mco* gene. [d]This plasmid (GenBank NC_022045) has two copies of the *cadC-cadA* operon. Abbreviations: MRIP, multi-RIP; Lm, *L. monocytogenes*; Lg, *Listeria grayi*; Li, *Listeria innocua*. doi:10.1128/microbiolspec.PLAS-0039-2014.f9

in different locations. In Europe, Inc18::Tn*1546* plasmids (such as pVEF1, pEVF2, pVEF3, and pVEF4) seem to have evolved from pIP816 (the first Inc18::Tn*1546* was isolated in France in 1987). They lack a transfer system and appear to be confined to *E. faecium* (70, 115, 116). Inc18::Tn*1546* plasmids from the United States are linked to *E. faecalis* isolates (pWZ909, pWZ1668, pWZ7140) and contain a complete transfer system (117, 118). A plethora of multiresistant mosaic Inc18 plasmids containing up to three RIPs, including RepR of pIP501 (CAA35647.1) and RepS of pRE25 (YP_783890.1), have been described in different *Firmicutes* (70, 71, 116, 119). These plasmids have an arsenal of insertion sequences, mainly IS*1216* and IS*L3*, which facilitate genetic exchange with different genetic elements of different origins and the acquisition of different AbR (*tetS)* and Met^R (*tcrB*, *mer* operon). These ISs also facilitate the co-integration with other RCR (e.g., pC221, which is cointegrated in pRE25) or theta replication plasmids as pheromone responsive (116, 119–121) or some pSK41-like elements. Figure 16 shows the diversity of Inc18 RIPs that can be identified by typing systems. All these RIPs have a primase domain PriCT_1 that allowed their identification as belonging to the Inc18 family. Fig. 6, 7, and 9 illustrate the mosaicism of Inc18 plasmids in enterococci and *Listeria*.

The pMG1/pHT plasmids

The pMG1/pHT plasmids are related to those of the Inc18 family (RIP homolog approximately 30% identical to Inc18 initiators, including the PriCT_1 domain [Fig. 16]) (122), although they also show high homology with the pXO2 virulence plasmid from *Bacillus anthracis*. Because many open reading frames of pHT and pMG1 plasmids do not show significant homology with any reported proteins, they used to be categorized as a new type of highly efficient conjugative plasmids with a MOB$_P$ family REL. This plasmid group is represented by relatives of pHT (pHTα, pHTβ, and pHTγ) and pMG1, which have greatly facilitated the dissemination of resistance to glycopeptides (Tn*1546_vanA*) and high-level resistance to aminoglycosides (Tn*4001*-like elements) among human *E. faecium* and *E. avium* isolates from the United States and Japan (123, 124) and, to a lesser extent, European countries (7, 70, 71, 122).

The RepA_N family

This is a large family of plasmids (also including a few phages) that are widespread among the low G+C Gram-positive bacteria and which possess RIP homologs to the RepA protein of pAD1 (76). The five groups of RepA

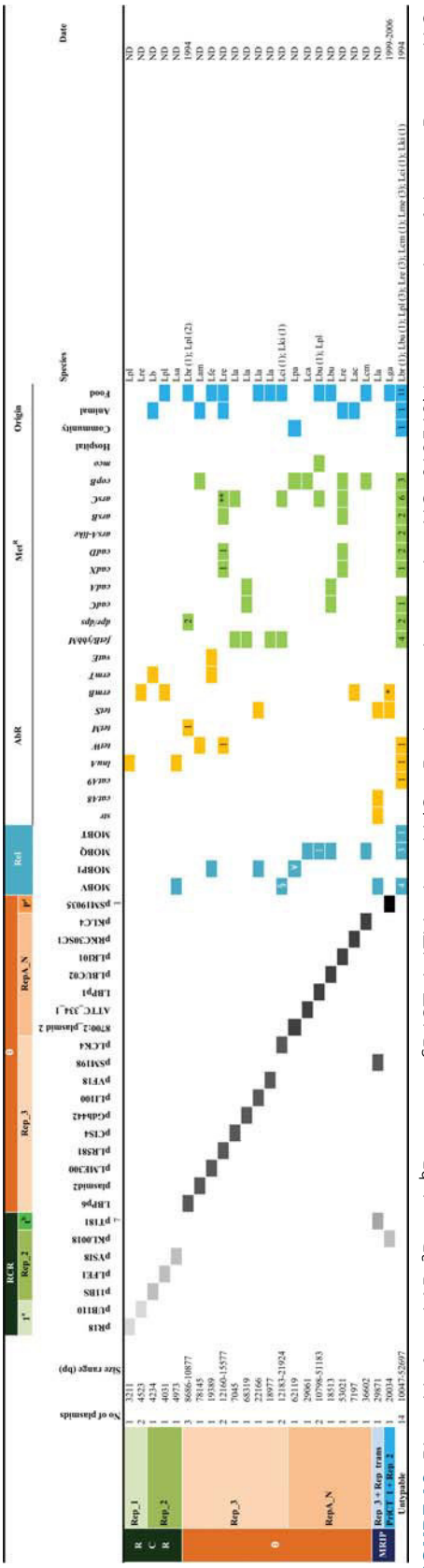
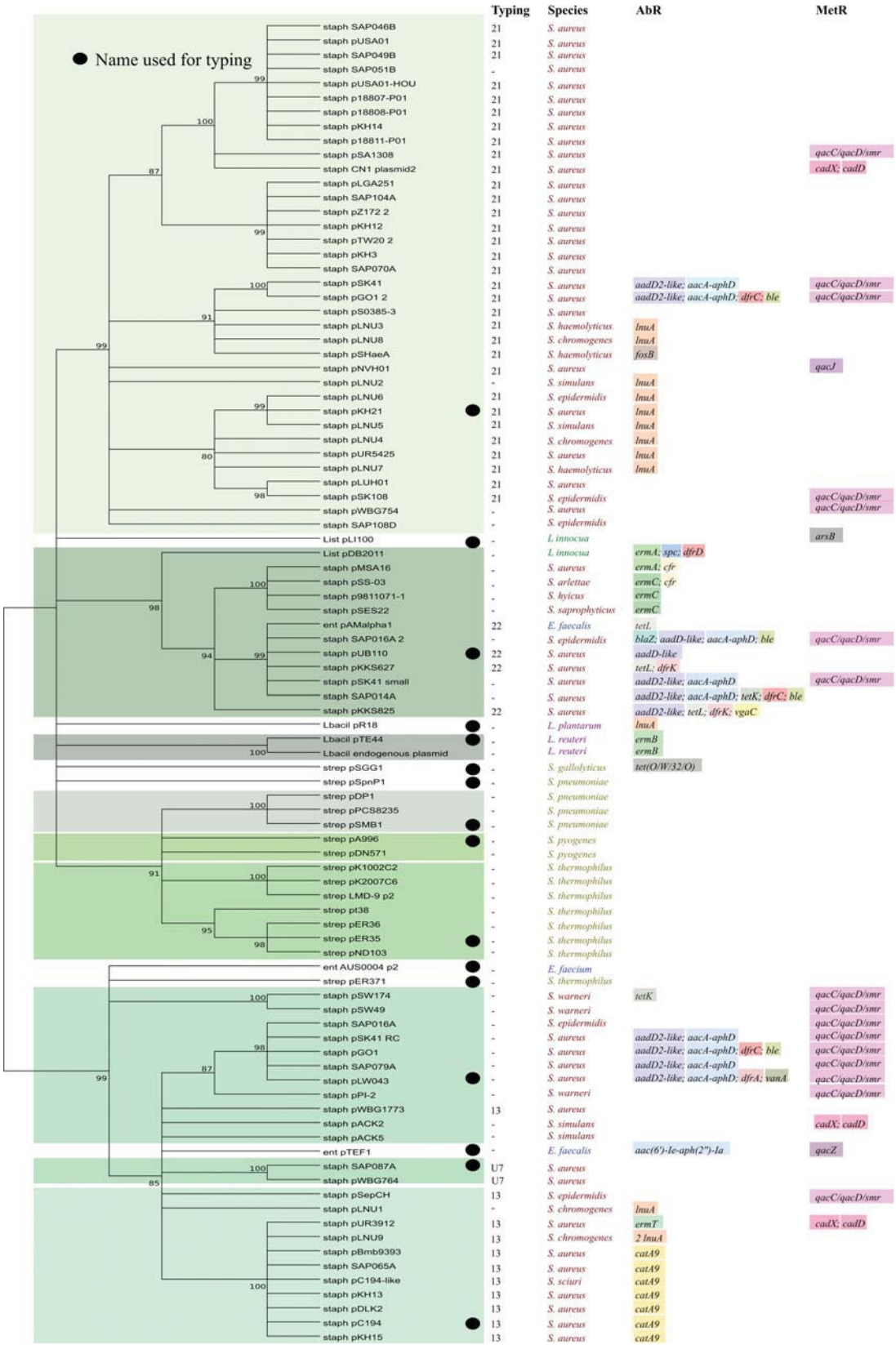


FIGURE 10 Plasmids from LAB. [a]Rep_1. [b]Rep_trans. [c]PriCT_1. *This plasmid (GenBank accession number NC_010540) has two copies of the *ermB* gene. **One of these plasmids (GenBank accession number NC_010603) has a truncated copy of the *arsC* gene. [§]One of these plasmids (GenBank accession number NC_014133) appears to have three copies of the MOB_V gene. [¥]One of these plasmids (GenBank accession number NC_022123) appears to have two copies of the MOB_{P1} gene. Abbreviations: MRip, multi-RIP; Lb, *Lactobacillus* spp; Lca, *Lactobacillus acidophilus*; Lam, *Lactobacillus amylovorus*; Lbr, *Lactobacillus brevis*; Lbu, *Lactobacillus bucheneri*; Lca, *Lactobacillus casei*; Lfe *Lactobacillus fermentum*; Lpa, *Lactobacillus paracasei*; Lpl, *Lactobacillus plantarum*; Lre, *Lactobacillus reuteri*; Lsa, *Lactobacillus sakei*; Lga, *Lactococcus garvieae*; Lla, *Lactococcus lactis*; Lcm, *Leuconostoc carnosum*; Lci, *Leuconostoc citreum*; Lki, *Leuconostoc kimchii*; Lme, *Leuconostoc mesenteroides*. doi:10.1128/microbiolspec.PLAS-0039-2014.f10

homologs identified are phylogenetically congruent with their host background (Fig. 17), suggesting that the replicons have evolved along with their current hosts and that intergenus movement of RepA_N plasmids does not often occur. Such RepA_N clusters correspond to plasmids from *Staphylococcus* (Met^R/*bla* pSK1 and pSK41 MDR plasmids), plasmids from *Enterococcus* (*E. faecalis* pheromone-responsive plasmids and *E. faecium* non-pheromone-responsive plasmids related to pRUM, pLG1, or untypeable megaplasmids), plasmids from *Lactobacillus* and *Lactococcus*, phage homologs from *Streptococcus* (*S. pneumoniae, S. thermophilus)*, and plasmids from *B. subtilis* (e.g., pLS32). Staphylococcal and enterococcal RepA_N plasmids have greatly contributed to the spread of AbR genes among humans and, eventually, animals and will be further described below. They also facilitate the movement of other nonconjugative plasmids and large genomic regions (36, 125, 126).

RepA_N staphylococcal plasmids (Fig. 5)

Large staphylococcal MDR plasmids use evolutionarily related theta-mode replication, although they can be further divided into three types: the Met^R/beta-lactamase-producing plasmids, the pSK1 family, and pSK41-like conjugative elements. All these are compatible and can be identified as the rep_{19}, rep_{20}, and rep_{15} families, respectively, according to Jensen's plasmid typing system (59, 127, 128). The pSK41 family (rep_{15}) is the largest group of conjugative plasmids in staphylococci, traditionally represented by pSK41, pG01, and pJE1, which emerged in the early 1980s associated with resistance to gentamicin due to the presence of Tn*4001* (84, 129). They often confer resistance to other antibiotics such as neomycin, tobramycin and kanamycin (due to the integration of pUB110 plasmids that harbor the *aadD* gene), antiseptics (due to the presence of *qac* genes) (130), and eventually trimethoprim (mediated by Tn*4001*), penicillins (due to the presence of Tn*552*::*blaZ*), and others. Plasmids of this group may also confer resistance to mupirocin (131–133) and vancomycin (134, 135), represented by pUSA03 (which harbors *ileS* and *tetK*) and pWL1043 (which contains Tn*1546*, Tn*4001*, Tn*4002*, Tn*552*, and *qac*C). The pSK41-like plasmids are able to mobilize other plasmids present in the same bacterial cell (133, 136, 137). The pSK1 and Met^R/beta-lactamase plasmids belong to the same incompatibility groups and are also compatible with pSK41 plasmids. Despite their inability to self-transfer, these groups of plasmids have been detected in many staphylococcal species.



RepA_N enterococcal plasmids

This cluster groups pheromone-responsive plasmids of *E. faecalis* and pRUM- and pLG1-like plasmids of *E. faecium* (7) (Fig. 6 and 7).

Pheromone-responsive plasmids. Pheromone-responsive plasmids represent a paradigm of elements in the biology of MGEs and are, together with Inc18 plasmids, the best-known plasmids described to date. For details about the mechanism of replication, conjugation, and evolvability of this plasmid group see references 7, 49, 65, 92, and 138. Plasmids that respond to pheromones are present in most contemporary *E. faecalis* isolates from humans and birds but are only occasionally found among *E. faecium*. Synthesis of pheromones is confined to *E. faecalis*, although *Enterococcus hirae*, *S. aureus*, and *Streptococcus gordonii* may secrete cAM373-like peptides that facilitate the conjugation of pAM373 from *E. faecalis* (139). The description of cAM373-responsive plasmids coding for resistance to glycopeptides (Tn*1546*-*vanA*) highlights the potential risk of the spread of glycopeptide resistance in staphylococci in institutions where VRE are endemic (134, 140). Although pheromone plasmids are unable to replicate in *S. aureus*, their transference and establishment in this host might occur by co-integration with other plasmids able to replicate in this species. In addition, some pAD1 relatives enhance the rate of mobilization of plasmids, conjugative transposons, and PAIs (125).

Plasmids of this family can be classified on the basis of responses to pheromones in different incompatibility groups (139) or according to RIP diversity (59, 68) within rep_8 (pAM373) and rep_9 (further split into subgroups $rep_{9a(pAD1)}$ and $rep_{9b(pTEF2)}$) families (59, 68). Transfer systems of MOBC or MOBP families have been detected in plasmids of this family.

Pheromone-responsive plasmids may encode putative virulence traits (aggregation substance, hemolysin/bacteriocin) and a diversity of AbR elements located on transposable elements such as Tn*916*-like (*tetM*), Tn*4001* (*aac-aph*), Tn*1546* (*vanA*), Tn*1549* (*vanB*), and a composite transposon containing a β-lactamase gene flanked by two IS*4* copies (7). The *par* locus encodes a unique antisense-regulated toxin-antitoxin system present in the plasmid pAD1, but *par* homologs have been detected on other plasmids and chromosomes of *E. faecalis* and *Staphylococcus, Clostridium, Listeria*, and *Lactobacillus* species (141). Toxin-antitoxin systems associated with other plasmid families such as ες and *relBE* have been detected on members of this plasmid group, reflecting rearrangements with representatives of other plasmid families (7). Even though to date, only a few members of pheromone-responsive plasmids have been fully sequenced, typing surveys reveal a wide diversity of plasmids among populations, often containing RIPs, RELs, or regions from plasmids of different origins (68, 71).

pRUM-like plasmids. pRUM-like plasmids (represented by pRUM, p5373c, pS177, and pDO2) are mosaic plasmids of variable size (>30 kb) that comprise diverse genetic elements of different origins (transposons, insertion sequences, small theta-replicating plasmids, bacteriocin clusters). They can be identified as the rep_{17} family according to PCR-based typing systems (59) but differ in the RIP sequence, the MOB system, and the presence of the toxin-antitoxin Axe-Txe locus (71, 142, 143). Both Inc18 and pRUM plasmids are driving the spread of glycopeptide resistance among contemporary isolates of *E. faecium* by carrying Tn*1546* (*vanA*) or Tn*1549* (*vanB*). Two types of pRUM plasmids are currently widespread among VRE and vancomycin-susceptible *E. faecium* isolates from different hosts. One contains RepA and Axe-Txe from pRUM and, eventually, the mobilization system of pC223 from *S. aureus* (70, 71, 142–144). The other type is characterized by a RepA protein that is 95% identical to RepA-pRUM, lacking postsegregational killing Axe-Txe and the presence of a MOB_{P7} relaxase originally detected in pEF1, a plasmid with an environmental origin. Tn*1546* is frequently located on both types of pRUM plasmids, frequently containing replicons of other plasmid families (author's unpublished results).

FIGURE 11 Similarity of *rep*-like sequences encoding RIPs of the Rep_1 family. A neighbor-joining tree of gene sequences coding for RIPs of the Rep_1 family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different backgrounds colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. doi:10.1128/microbiolspec.PLAS-0039-2014.f11

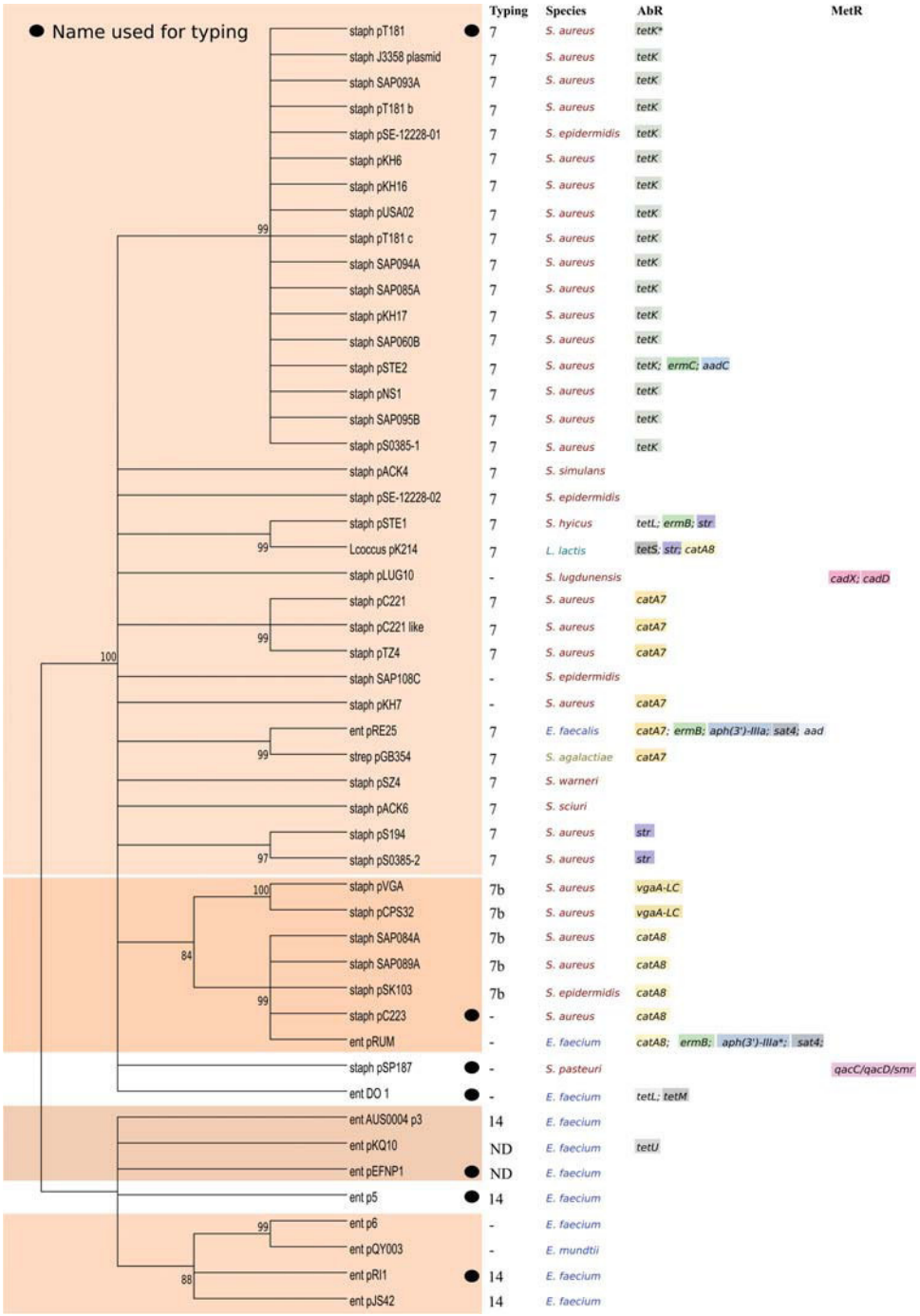


FIGURE 12 Similarity of *rep*-like sequences encoding RIPs of the Rep_trans family. A neighbor-joining tree of gene sequences coding for RIPs of the Rep_trans family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different backgrounds colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. *Truncated gene. **Similar to *E. faecalis ant6-Ia* and *aadE*. Abbreviations: ND, not determined. doi:10.1128/microbiolspec.PLAS-0039-2014.f12

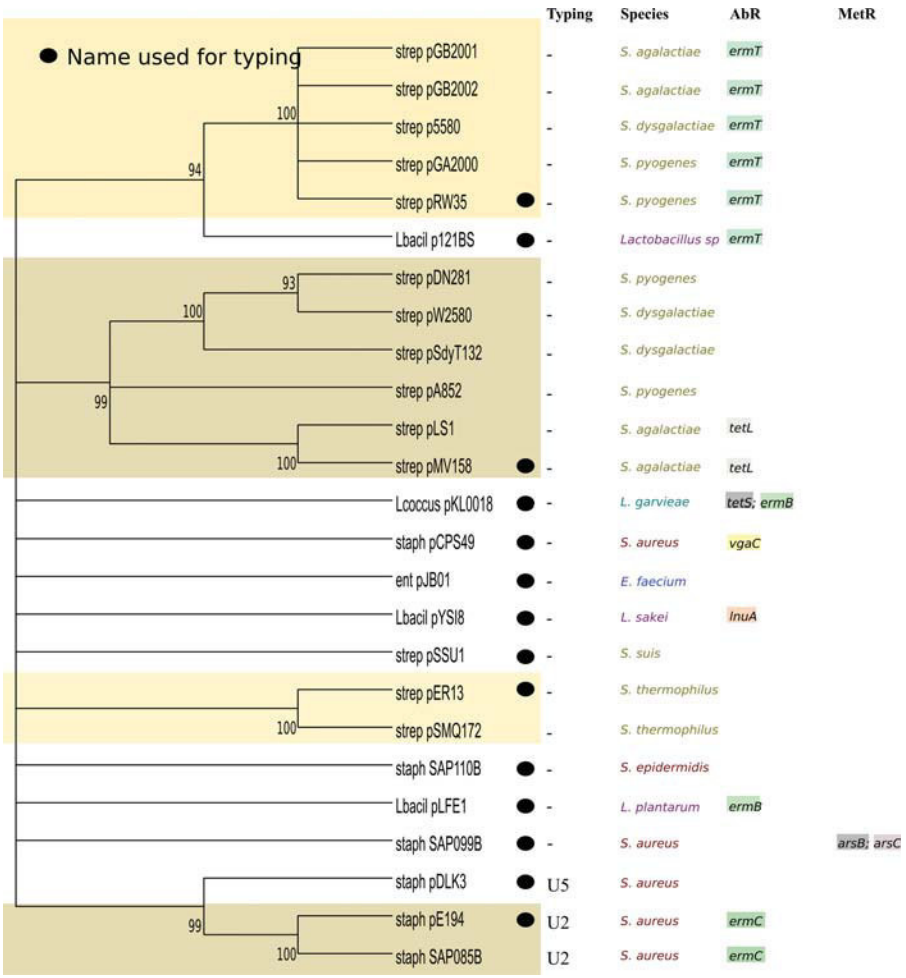


FIGURE 13 Similarity of *rep*-like sequences encoding RIPs of the Rep_2 family. A neighbor-joining tree of gene sequences coding for RIPs of the Rep_2 family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different background colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. doi:10.1128/microbiolspec.PLAS-0039-2014.f13

Large plasmids. Plasmids larger than 150 kb are widely distributed among *E. faecium*, *Enterococcus durans*, and *E. hirae* from different origins, but they have not been detected among *E. faecalis* (71, 144–150). To date, only a handful of *E. faecium* megaplasmids have been fully sequenced (AUS0085_p1 [NC_021987], pNB2354_1 [NC_020208], DO_3 [NC_017963], and pLG1, although this last one has not been closed [148]). All of them contain a RIP similar to $RepA_{pAD1}$, making them part of the RepA_N family (Fig. 7 and 17, Supplementary Table S1) (59, 71, 148). A similar RIP has also been found in a 130-kb plasmid (NC_021987) from a VRE ST203 *E. faecium* strain isolated in 2009 in Australia (151). Although RIP sequences of pLG1 plasmids are often identified among enterococcal megaplasmids, most of them do not hybridize with known RIP genes included in published schemes (71, 148, 152). Enterococcal megaplasmids carry genes involved in sugar metabolism (mannitol, glycerol, sorbitol, raffinose, complex carbohydrates), AbR (macrolides, glycopeptides, aminoglycosides), Met^R (copper-*tcrYAZB*), and enhanced adhesion (71, 126, 144, 147–149, 152–154). They are frequently involved in the acquisition or persistence of AbR among *E. faecium* isolates from food animals (144, 150).

GENE AND PLASMID FLOW SHAPES THE EVOLUTIONARY ECOLOGY OF *FIRMICUTES*

As described in previous sections, the acquisition of novel traits encoding adaptive resistance to antimicrobials in *Firmicutes* is mainly due to genes located on plasmids and transposable elements. This acquisition is, certainly, regulated by interactions at genetic and ecological (social) levels. Interplay between genes, mobile genetic elements, and microbial populations and their relation with the host population and local or global environments shapes the plasmid flow. Such flow can be modified by "external" (supra-cellular) changes, including variations in the host population structure and

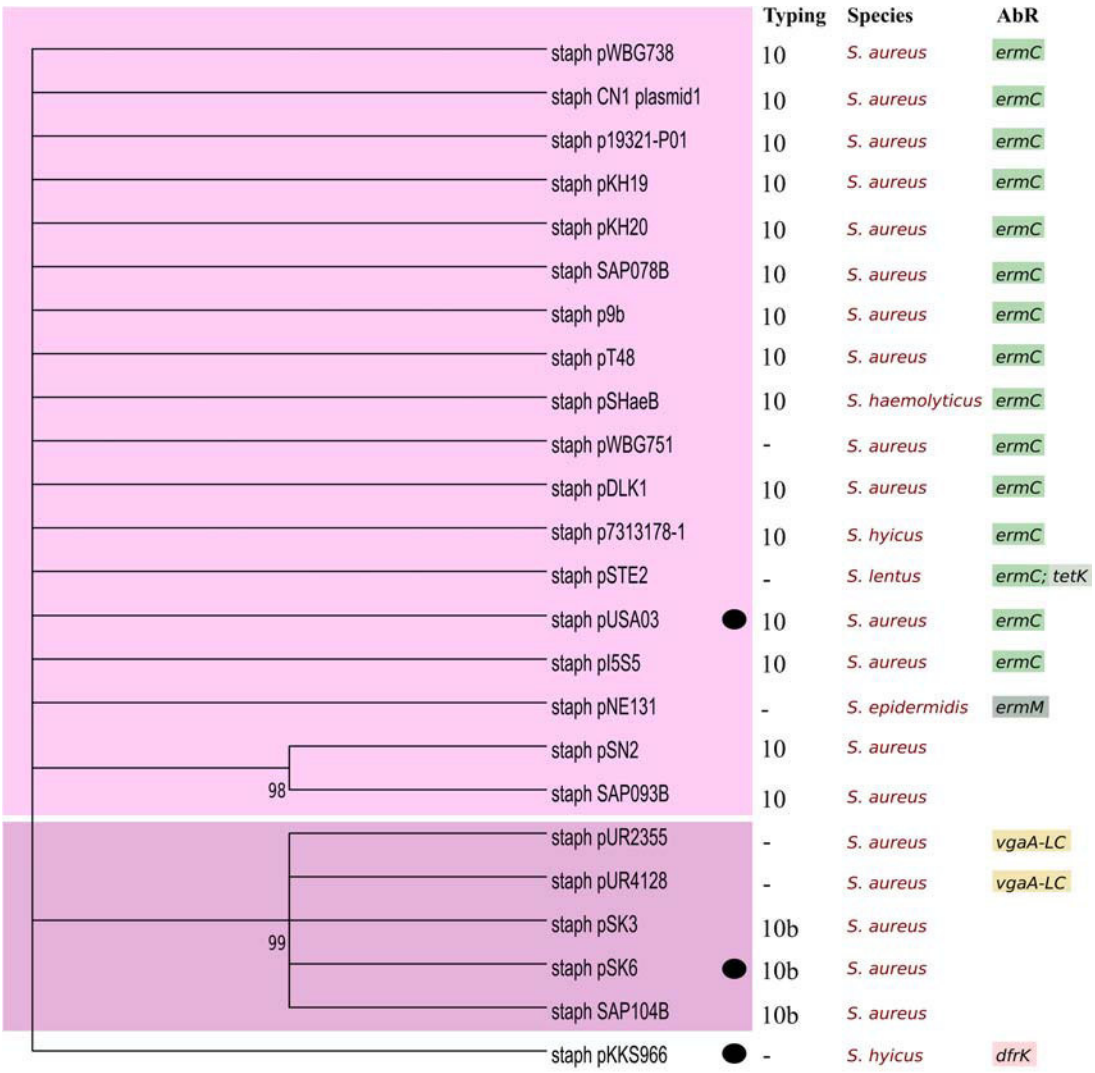


FIGURE 14 Similarity of *rep*-like sequences encoding RIPs of the Rep_L family. A neighbor-joining tree of gene sequences coding for RIPs of the Rep_L family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different background colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. doi:10.1128/microbiolspec.PLAS-0039-2014.f14

size (e.g., mass rearing, crowding) and their associated chemical or behavioral landscape (e.g., use of different antimicrobials, immunization, global food supply, international travel). These changes ultimately determine the density and diversity of particular bacterial populations in particular habitats, leading to ecological specialization, clonalization, and gradual emergence of gene flow barriers (23, 155, 156), a process that mimics the general dynamics of speciation, as bacterial clones and species constitute ecological units of microbial biodiversity.

The challenge to define "units of biodiversity" in microbial community ecology has approached the concept of genes as "defining elements of networks and metacommunities" (155). In such a context, extrachromosomal elements greatly influence the HGT interactions between microbial organisms and are the

building forces for the establishment of "gene exchange communities" (155, 157). The selective power of antimicrobials (antibiotics [Ab], heavy metal, biocides) may then shape this multilevel bacterial population biology (158, 159), involving genes, plasmids (MGEs), bacterial clones and species, and gene exchange communities. The evolutionary tradeoff between early and late stages of adaptation to such selective pressures may determine the local evolvability of clonal and plasmid populations by increasing the number of genotypes resulting from chromosomal and plasmid recombination processes that facilitate further ecological differentiation (18). To establish effective public health interventions to fight the AbR problem in its eco-biological dimension, we then need to define the gene exchange communities relevant for the acquisition, evolution, and spread of resistance (160, 161). Below, we will specifically discuss the role of AbR genes and plasmids in the ecological differentiation of bacterial populations of the main *Firmicutes* genera.

Antimicrobial Resistance Genes and Bacterial Population Ecology

The environmental origin of AbR genes has been extensively discussed, but very few AbR genes identified in the environment are found in human or animal pathogens, which indicates severe bottlenecks for their acquisition and transmission (162, 163). However, the gut microbiota is increasingly considered a significant reservoir of AbR genes (3), which is supported by studies that associate widely spread AbR genes of relevance in clinical therapy, such as *ermB*, *ermT, ermC* (encoding resistance to macrolides), *vanB* (coding for resistance to glycopeptides), and *cfr* (coding for resistance to different antimicrobials), with members of the normal microbiota such as species of the *Clostridium* group XIVa now reclassified as family *Lachnospiraceae* (*Clostridium bolteae*, *Clostridium innocuum*–like, *Clostridium lavalense*, *Clostridium symbiosum*) and some lactic bacteria (3, 88, 164–168).

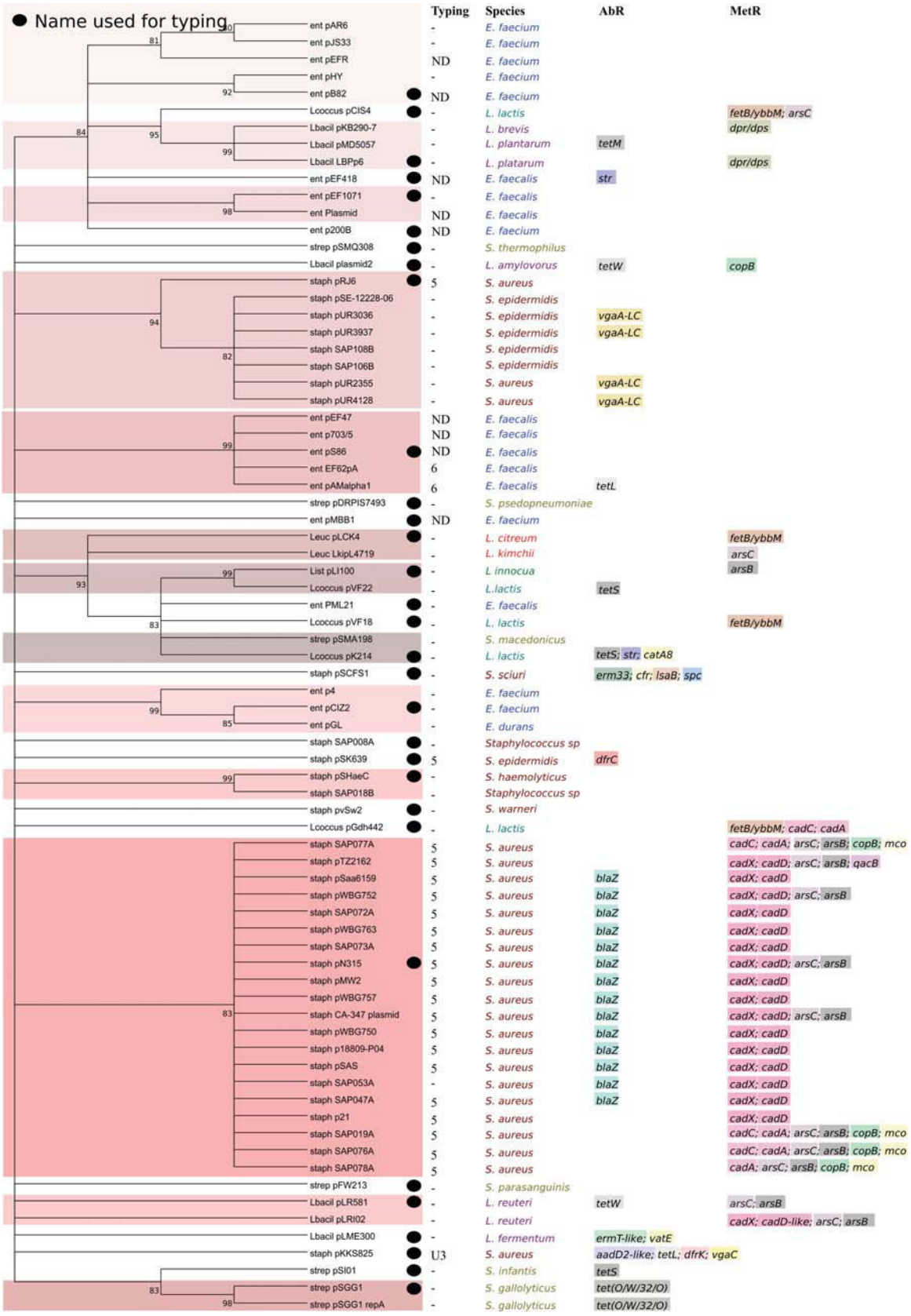
Recent work demonstrates that a given AbR gene (or genetic element such as Tn*1549_vanB*) may be independently acquired by different clonal populations in the intestine of a particular host (165). Once an AbR gene is present in gut commensals (independent of the origin of the gene), members of the normal intestinal flora of humans and animals can exchange such genes among themselves or with bacterial pathogens, which might be present in low numbers or just be passing through the intestine after being transferred from other body sites or with food intake, using different intermediates in the case of distant bacteria (3, 165, 169).

The rapid emergence in *Firmicutes* of genes coding for AbR, Met^R, and Bc^R immediately after their introduction and significant (often massive) use in different settings has been demonstrated for chloramphenicol (*catA*), tetracyclines (*tetL*), macrolides (*ermB)*, neomycin (*aad*), gentamicin (*aac6aph2*), trimethoprim (*dfr*), betalactams (*blaZ*), and antiseptics (*qac*) in hospitals during the 1950s to 1970s, and for tylosin (*ermC*, *ermT*), phenicols (*fex*), pleuromutilins (*cfr*), and zinc-bacitracin in animals during the 1990s, thus supporting the hypothesis of the existence of a previous gastrointestinal reservoir of genes that were selected for the first time as AbR genes (gene exaptation) (84, 88, 91).

Plasmids and Bacterial Population Ecology

The number and types of *Firmicutes* plasmids and integrative-conjugative elements (currently considered as plasmids under the perspective of evolutionary biology [22]) greatly vary with the different bacterial species, certainly as a result of both ecological specialization and selective events resulting from exposure to different anthropogenic activities. Most (if not all) of the contemporary isolates belonging to different species of staphylococci, enterococci, lactobacilli, and others contain plasmids of different families in a consistent pattern (for instance, RCR, small theta, or megaplasmids in *E. faecium*; pheromone plasmids in *E. faecalis*) (7, 68, 71). Such frequent plasmid-bacteria host correspondence indicates a basic coadaptive evolutionary relation between two different types of organisms.

For a long time, plasmids were considered as "organisms," units of a continuous lineage with an individual evolutionary history, and hence producing evolved populations, in line with the Luria and other seminal works in the field (46, 240). However, plasmids are not necessarily discrete units or individuals as classically considered in evolutionary theory (20, 170, 240). Organisms are units of selection, evolutionary units in a sense "evolutionary individuals," defined as any entity that, independently from the number of elements that enters into its composition or from its hierarchical level of complexity, is selected and evolves as a unit (170, 171). The frequent out-of-equilibrium events that characterize the interplay between bacterial hosts, plasmids, and gene populations is explained because selective events might act independently on these different evolutionary individuals, as predicted in the "levels of selection" conceptual frame (20, 172–174). However, it is of note that we should recognize "levels of individuality"; for instance, a substantial number of *Firmicutes* plasmids have a lower-level self-identity than their bacterial hosts (18, 155)



because of the more complex genetic interplay with other mobile genetic elements which in turn are also "leaky individuals," frequently mosaics of individuals with a partial or contingent self-identity, produced under the effect of adaptive challenges when confronting variable environments (155, 175). Even if this problem of "individual constancy" (176) makes it difficult to study the network of plasmids and hosts in *Firmicutes*, and such a network were biased by sampling, we should accept the existence of a certain interactive frame.

Valeria Souza, still following Maynard Smith's ideas about the population structure of bacteria, proposed in 1997 to classify plasmid-bacteria interactions in four patterns, namely, (i) the plasmid-host clonal pattern, where the plasmid phylogeny is mirrored by host phylogeny; (ii) the limited transfer pattern, in which the plasmid flow is limited to closely related (genetically and/or ecologically) strains; (iii) panmictic plasmid spread, in the case of plasmids that circulate among a variety of hosts (the stability of the association being dependent on the benefits and costs of plasmid carriage); and (iv) epidemic plasmid dispersal, in which "successful" plasmids spread in bacterial populations because they provide a clear advantage in high-potency selective landscapes (49, 170). Although illustrative and useful for epidemiological purposes, this single centric view should not replace the complex interplay between different elements that may result in the emergence of different chimeric configurations (49, 177). Therefore, these "patterns" should be currently understood as possible interactive states, even though some of them could be more ephemeral than others, depending on the coevolutionary history, the adaptive demands of the plasmids, and the bacterial populations and communities.

Plasmids and Population Biology of Firmicutes

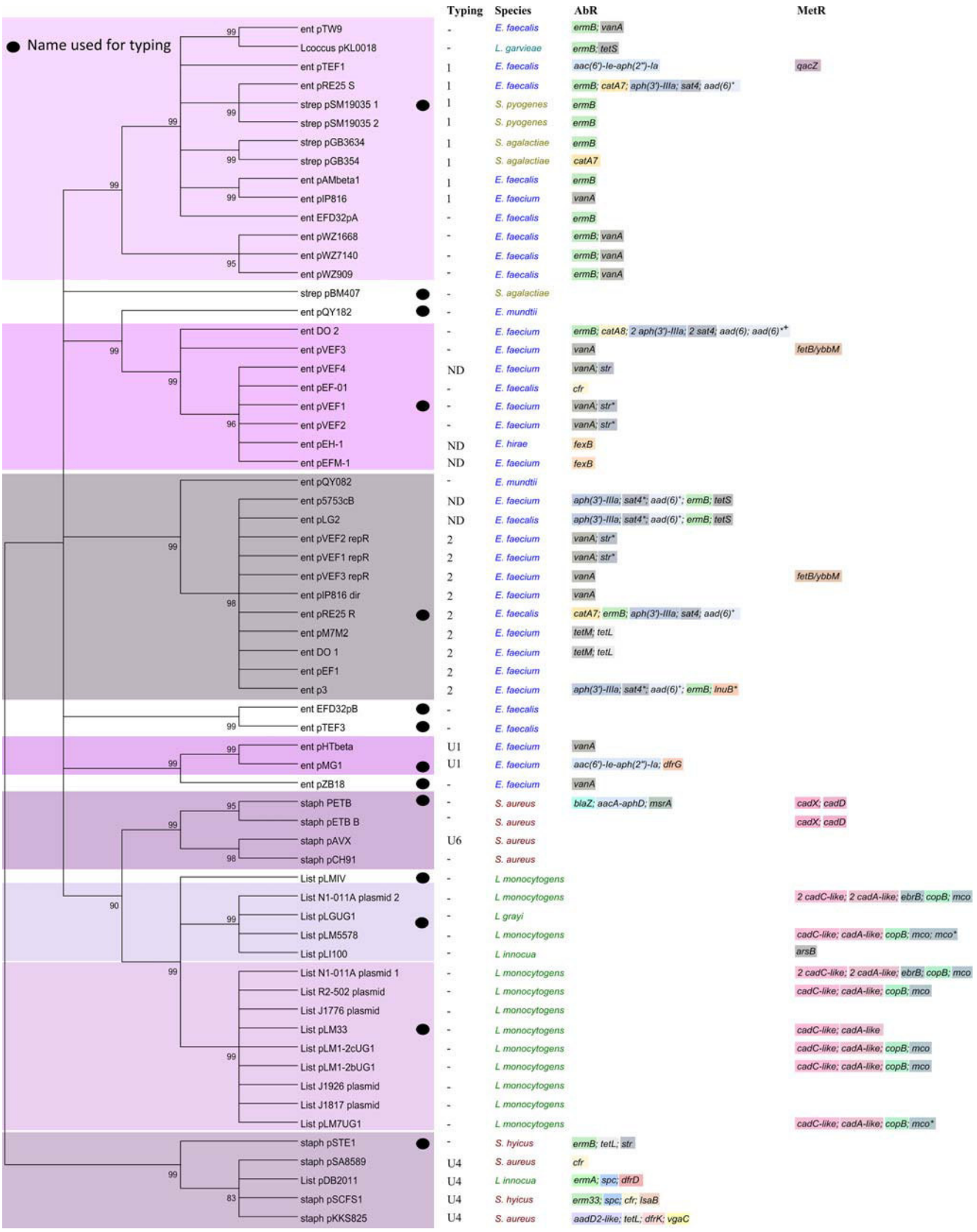
This section will focus on the genera of *Firmicutes* that are relevant to the problem of AbR (1).

Streptococcus

The genus *Streptococcus*, a main hub in gene networks in this and other studies (11, 12), is one of the most heterogeneous groups within the phyla *Firmicutes*. Remarkably, the 138 known species of streptococci found as opportunistic pathogens or commensals (many of them zoonotic pathogens) in humans, horses, pigs, cows, and fish have recently been divided into seven species groups on the basis of 16S rDNA gene sequencing, chemotaxonomic approaches, and DNA hybridization, namely the bovis, pyogenic, mitis, mutans, salivarius, anginosus, and unknown groups (178–180). HGT seems to play a relevant role in the adaptation and cohesiveness of the groups (179). Available information about streptococcal plasmids is scarce, with only a few plasmids being fully sequenced, representing an unbalanced sample of species and ecological groups (Supplementary Table S1). Figure 10 illustrates the 20 AbR plasmids currently found in streptococci.

The streptococcal groups bovis and mutans rarely harbor plasmids, although they can be relevant in the adaptation of particular species. *S. thermophilus*, a nonpathogenic species in the bovis group that is used in the dairy industry (181), contains a set of plasmids harboring heat shock proteins; *Streptococcus mutans*, a member of the human indigenous flora that is transmitted mostly from mother to child, often carries 5- to 6-kb cryptic plasmids that parallel the evolution of lineages associated with racial cohorts and geographical locations (182). Megaplasmids in the group salivarius coding for different lantibiotics favor their persistence in the oral cavity (183). Conversely, the pyogenic group, which is represented by species of clinical interest such as *S. agalactiae* and *S. pyogenes* (also called GAS and GBS, respectively), frequently carry plasmids that code for AbR genes aside from bacteriocins. Inc18 plasmids are widely spread among streptococci and seem to have determined the selection of certain populations resistant to chloramphenicol, aminoglycosides, and macrolides since the late 1970s in different groups of streptococci and enterococci (105, 110, 184). Rep_2 plasmids carrying *erm(T)* seem to have recently spread among GAS and GBS clinical isolates of different countries, having contributed to the increase of macrolide resistance rates in these species since the mid-1990s, either by clonal

FIGURE 15 Similarity of *rep*-like sequences encoding RIPs of the Rep_3 family. A neighbor-joining tree of gene sequences coding for RIPs of the Rep_3 family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different background colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. Abbreviations: ND, not determined. doi:10.1128/microbiolspec.PLAS-0039-2014.f15



expansion, in the case of GAS, or by plasmid transference among unrelated clonal backgrounds, in the case of GBS (8, 185).

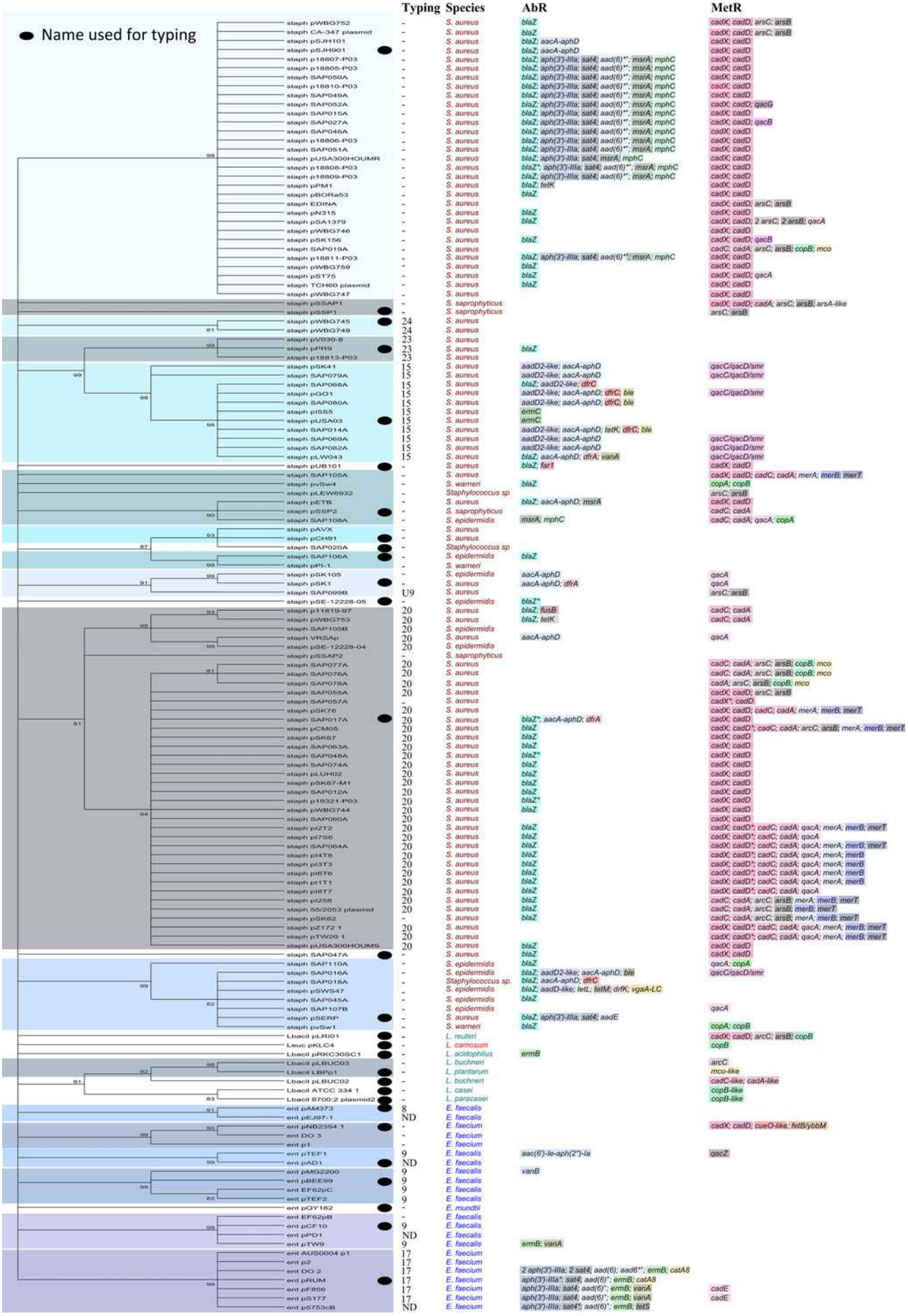
These *erm(T)*-containing plasmids are also spread among other non-streptococcal species, such as *Enterococcus*, *Staphylococcus,* and *Lactobacillus* (89, 186, 187). Often, streptococcal plasmids are mobilized by coresident integrative-conjugative elements belonging to the ICESa2603 family (188). Resistance to macrolides (*ermB*, *mefA)*, tetracyclines (*tetM*, *tetS*, and other mosaic *tet* genes), aminoglycosides (*aph3, aadA6*, Tn*4001*), or vancomycin (*vanA*, *vanB*) is commonly detected among isolates of this group, but the location of determinants seems to be linked to transposable elements often involving insertion sequences (reviewed in reference 181). *Streptococcus suis*, a particularly virulent emerging zoonotic pathogen that remains an outlier to the mitis, sanguinis, and anginosus groups is known to carry plasmids, although they have been scarcely characterized (189, 190). Relevant AbR genes coding for chloramphenicol (*cfr* and *fexA*) and lincosamides (*lnu*) embedded in composite regions similar to those present in plasmids of *E. faecalis* have been located in streptococcal plasmids of approximately 100 kb (191). Smaller plasmids carrying *tetB* associated with Gram-negative species have been described (192).

Enterococcus

The genus *Enterococcus* comprises different species, members of the intestinal flora of animals and humans able to cause disease in their hosts (193). Although seminal works in the field of plasmid biology focus on particular enterococcal plasmids and transposons, such as pheromone-responsive plasmids or Tn*916*, which became paradigms of different mechanisms of conjugation, the plasmidome of enterococcal species has scarcely been analyzed (7). Recent studies revealed that most strains of *E. faecium* and *E. faecalis*, the two main species detected in humans and animals, carry a number of plasmids of different families that include species-specific plasmids (e.g., narrow host range RCRs and RepA_N plasmids such as megaplasmids in *E. faecium* and pheromone-responsive plasmids in *E. faecalis*) and broad host range plasmids (e.g., Inc18), plasmid chimeras being abundant and still difficult to classify (Fig. 6 and 7; see previous section and comprehensive reviews in references 7, 141). Megaplasmids of *E. faecium* or pheromone-responsive *E. faecalis* plasmids enhance the ability to colonize, invade, and form biofilms (65, 126, 154). Conjugative plasmids may influence the mobilization of nonconjugative elements and chromosomal regions and facilitate the acquisition of different adaptive traits and genome evolvability (71, 125, 126). Most enterococcal plasmids are able to acquire and disseminate AbR genes by different mechanisms of genetic exchange. However, the role of plasmids in the population structure and evolvability of these enterococcal species has been poorly addressed (194–197) due to the overrepresentation of recent clinical and animal isolates of specific lineages commonly associated with AbR included in most studies (7, 141) and due to the lack of available plasmid sequences. Similar plasmids have been found in *E. faecium* and other enterococcal species that may play equivalent functional roles in the gastrointestinal tract such as *Enterococcus avium, Enterococcus raffinosus, E. durans*, and *E. hirae* (195, 198).

AbR genes are located on plasmids that often contain different replicons associated with different narrow (RCR, RepA_N) and broad host range (Inc18) plasmids. Inc18 streptococcal plasmids greatly influenced the worldwide increase of aminoglycoside-macrolide resistance among *E. faecalis* isolates from humans and animals during the 1970s (199). They also contributed to the spread of vancomycin resistance among *E. faecium* of animal origin in Europe and *E. faecalis* from hospitalized patients in the United States (7, 70, 71). Diverse narrow host range plasmids have been involved in local expansions of enterococci conferring resistance to first-line antibiotics such as gentamicin (Tn*4001*) or beta-lactams (Δ*Tn552 blaZ*) (152) and beta-lactamase-producing *E. faecalis* and *E. faecium* (152) (200–202), which highlights the role of endogenous plasmids and recombination in the adaptation of particular lineages

FIGURE 16 Similarity of *rep*-like sequences encoding RIPs with the PriCT_1 domain. A neighbor-joining tree of gene sequences coding for RIPs with PriCT_1 domains was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different background colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. Abbreviations: ND, not determined. doi:10.1128/microbiolspec.PLAS-0039-2014.f16



(*E. faecium* ST17, ST18, ST78 and *E. faecalis* ST6 and ST16) (7, 67, 144, 203).

Analysis of the same AbR genes in different species (*cfr*, *bac*, lincosamide resistance genes) reflects the impact of recombination events between genes, MGEs, and different populations of *Firmicutes* (*Staphylococcus*, *Clostridium*, *Lactobacillus*, *Lactococcus*, and *Enterococcus*) and other Gram-negative organisms (200, 204) in the gastrointestinal tract of animals and humans (120, 144, 205, 206).

Staphylococci

These organisms are opportunistic pathogens and members of the commensal flora of skin and mucous membranes of humans and animals (207–209) and, thus, are part of a microbial community with limited contact with members of other main genera of *Firmicutes* that inhabit distinct body sites (210). Figures 1 and 2 show the limited plasmid connectivity of staphylococci with other genera. However, HGT and the acquisition of AbR and Met^R is relevant in the evolvability of this genus, mainly due to genetic exchange events between closely related species (Fig. 5) (9, 211–213). Comprehensive reviews address the essentially clonal population structure of *S. aureus* (214–216) and other staphylococcal species (207) and also the impact of HGT in the evolutionary history of staphylococcal populations (9, 217–219), with emphasis on the description of the plasmids associated with AbR genetic elements (9, 51, 84, 220) and their influence on the variability of lineages (217, 219, 221–224).

Plasmids, transposons, and staphylococcal chromosomal cassettes (SCC*mec*) are infrequently transferred among isolates of a different origin. A close association of MGE and particular staphylococcal lineages has been suggested (31, 225), with country-specific variations (208, 226). This highlights the relevance of local conditions and the emergence of gene flow barriers in the ecological differentiation of staphylococcal lineages such as in the case of *S. aureus* CC30 (219, 227). The origin, rapid spread, and evolution of staphylococcal populations resistant to beta-lactams was mainly influenced by the interplay of genetic elements including plasmids (84, 177).

Clostridium

Clostridium is a large and extremely heterogeneous genus that has traditionally grouped more than 100 species widely distributed in the gut microbiota of mammals, amphibians, and insects and in soils. An extensive update of clostridial classification is included in the latest edition of *Bergey's Manual*, although many unrelated species still retain the *Clostridium* name, causing major confusion in the clostridial taxonomy (228). To date, only 60 plasmids have been fully sequenced, mainly corresponding to *C. perfringens*, *Clostridium botulinum*, and other group I clostridia species (1 *Clostridium butyricum*, 2 *Clostridium kluyveri*, 3 *Clostridium acetobutylicum*). Some species in which plasmids were analyzed have been moved to other genera such as *Clostridium aciduricidi* (now *Anaerococcus prevotii* type XII) and *Clostridium thermocellum* (now belonging to the family *Ruminococcaceae*). Several sequenced plasmids correspond to the same strain and are mostly from contemporary isolates, thus limiting the possible knowledge about the role of plasmids in the evolution of these species (Supplementary Table S1). Only narrow host range conjugative plasmids of *C. perfringens* (CpCP) or linear megaplasmids from *C. butyricum* have been associated with AbR.

CpCP plasmids belong to the pCW3 family and are widely spread among isolates of *C. perfringens*, carrying genes encoding AbR (tetracycline [*tetAB(P)*], chloramphenicol [*catP*-Tn*4451*], lincomycin [*lnuP*-tISCpe8]) and/or enterotoxins, ε-toxin, or iota-toxin production that determine different toxinotypes (56, 229–231). All pCW3-like plasmids have a conjugative transfer locus of 11 open reading frames (*orf*s) (*tcp* [transfer *C. perfringens*]) that includes an integrase and a T4CP protein but lacks relaxase (73, 231). A transposable origin similar to that of Tn*916* has been suggested for the *tcp* module of pCW3-like plasmids, which would have acquired a replication machinery specific to this species. Often,

FIGURE 17 Similarity of *rep*-like sequences encoding RIPs of the RepA_N family. A neighbor-joining tree of gene sequences coding for replication initiator proteins of the RepA_N family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different background colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. *Truncated gene. $^+$Similar to *E. faecalis ant6-Ia* and *aadE*. Abbreviations: ND, not determined. doi:10.1128/microbiolspec.PLAS-0039-2014.f17

C. perfringens isolates harbor more than one pCW3 plasmid, which carry different adaptative traits and partition machineries. The presence of different partition systems explains the coexistence of different plasmids with the same type of RIP in the same cell (56, 231–233).

These plasmids can be transferred (and eventually serve as donors of AbR genes) but cannot replicate in other species such as *P. difficile*, *Clostridium sordelli*, or *Clostridium septicum*, which could explain the confinement of some AbR genes in these populations (234). An evolutionary scenario for CpCP has been reported, with pCW3 (*tetAB-P*) and pIP401 (*tetAB-P* and Tn*4451*) being suggested as the precursors of this family, which would have acquired different toxins by homologous recombination involving composite transposons flanked by insertion sequences (56). Large linear plasmids containing AbR have recently been described in neurotoxigenic *C. butyricum*, one of the six phylogroups able to produce the botulinum toxin (34, 235). These plasmids contain four beta-lactamase genes, transcriptional regulators and two-component regulatory systems, involved in the regulation of expression of the *bont/A* gene and a region with a functional CRISPR-cas locus that provides a defense against invading genetic elements present in the intestinal environment.

Acquired resistance to tetracyclines (*tetM*, *tetL*, *tetK*, *tetO*, *tetW*), chloramphenicol, macrolides (*ermB*, *lnu*), and bacitracin (a bacitracin efflux pump and an overproduced undecaprenol kinase gene located on a genetic island flanked by copies of IS*1216*) has been reported in human and animal clostridium species including *C. perfringens*, often associated with conjugative transposons and plasmids widespread in other species (234, 236, 237). A detailed analysis of AbR networks suggests further ecological connections with mobile genetic elements of other prokaryotic groups (Fig. 1 and 2).

CONCLUSION

This work offers for the first time an integrated and comprehensive analysis of the dynamics of AbR genes in Gram-positive bacteria and highlights the need for a population view to analyze the problem of antibiotic resistance. The article analyzed the relevance of the plasmidome in the emergence, spread, and maintenance of genes encoding resistance to antimicrobials (antibiotics, heavy metals, and biocides) and their influence on the structure of bacterial populations in the light of evolutionary ecology. A critical revision of plasmid typing systems highlights the limitation of available knowledge about plasmid diversity in this group of bacteria.

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REFERENCES

1. Centers for Disease Control and Prevention. 2013. Threat Report 2013. Antimicrobial Resistance. http://www.cdc.gov/drugresistance/threat-report-2013/

2. Murphree CA, Heist EP, Moe LA. 2014. Antibiotic resistance among cultured bacterial isolates from bioethanol fermentation facilities across the United States. *Curr Microbiol* **69:**277–285.

3. Salyers AA, Gupta A, Wang Y. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends Microbiol* **12:**412–416.

4. European Food Safety Authority. 2012. Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in *Salmonella*, *Campylobacter* and indicator *Escherichia coli* and *Enterococcus* spp. bacteria transmitted through food. *EFSA J* **10:**2742.

5. European Food Safety Authority. 2012. Technical specifications for the analysis and reporting of data on antimicrobial resistance (AMR) in the European Union Summary Report. *EFSA J* **10:**2587.

6. Schwarz S, Shen J, Wendlandt S, Feßler AT, Wang Y, Kadlec K, Wu C. 2015. Plasmid-mediated antimicrobial resistance in staphylococci and other *Firmicutes*. *In* Tolmasky ME, Alonso JC (ed), *Plasmids: Biology and Impact in Biotechnolgy and Discovery*. ASM Press, Washington, DC, in press.

7. Clewell DB, Weaver KE, Dunny GM, Coque TM, Francia MV, Hayes F. 2014. Extrachromosomal and mobile elements in enterococci: transmission, maintenance, and epidemiology. *In* Gilmore MS, Clewell DB, Ike Y, Shankar N (ed), *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary, Boston, MA.

8. DiPersio LP, DiPersio JR, Beach JA, Loudon AM, Fuchs AM. 2011. Identification and characterization of plasmid-borne erm(T) macrolide resistance in group B and group A *Streptococcus*. *Diagn Microbiol Infect Dis* **71:**217–223.

9. Lindsay JA. 2014. *Staphylococcus aureus* genomics and the impact of horizontal gene transfer. *Int J Med Microbiol* **304:**103–109.

10. Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems RJL, Earl AM, Gilmore MS. 2013. Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. MBio **4**(4):e00534-13. doi:10.1128/mBio.00534-13.

11. Beiko RG, Harlow TJ, Ragan MA. 2005. Highways of gene sharing in prokaryotes. *Proc Natl Acad Sci USA* **102:**14332–14337.

12. Halary S, Leigh JW, Cheaib B, Lopez P, Bapteste E. 2010. Network analyses structure genetic diversity in independent genetic worlds. *Proc Natl Acad Sci USA* **107:**127–132.

13. Popa O, Dagan T. 2011. Trends and barriers to lateral gene transfer in prokaryotes. *Curr Opin Microbiol* **14:**615–623.

14. Popa O, Hazkani-Covo E, Landan G, Martin W, Dagan T. 2011. Directed networks reveal genomic barriers and DNA repair bypasses to lateral gene transfer among prokaryotes. *Genome Res* **21:**599–609.

15. Tamminen M, Virta M, Fani R, Fondi M. 2012. Large-scale analysis of plasmid relationships through gene-sharing networks. *Mol Biol Evol* **29:**1225–1240.

16. Nogueira T, Rankin DJ, Touchon M, Taddei F, Brown SP, Rocha EPC. 2009. Horizontal gene transfer of the secretome drives the evolution of bacterial cooperation and virulence. *Curr Biol* **19:**1683–1691.

17. McGinty SE, Rankin DJ, Brown SP. 2011. Horizontal gene transfer and the evolution of bacterial cooperation. *Evolution* **65:**21–32.

18. Friedman J, Alm EJ, Shapiro BJ. 2013. Sympatric speciation: when is it possible in bacteria? *PLoS One* **8:**e53539. doi:10.1371/journal.pone.0053539.

19. Hobman JL. 2015. *Molecular Life Sciences*. Springer, New York, NY.

20. Eberhard WG. 1990. Evolution in bacterial plasmids and levels of selection. *Q Rev Biol* **65:**3–22.

21. Kloesges T, Popa O, Martin W, Dagan T. 2011. Networks of gene sharing among 329 proteobacterial genomes reveal differences in lateral gene transfer frequency at different phylogenetic depths. *Mol Biol Evol* **28:**1057–1074.

22. Guglielmini J, Quintais L, Garcillán-Barcia MP, de la Cruz F, Rocha EPC. 2011. The repertoire of ICE in prokaryotes underscores the unity, diversity, and ubiquity of conjugation. *PLoS Genet* **7:**e1002222. doi:10.1371/journal.pgen.1002222.

23. Shapiro BJ, Friedman J, Cordero OX, Preheim SP, Timberlake SC, Szabó G, Polz MF, Alm EJ. 2012. Population genomics of early events in the ecological differentiation of bacteria. *Science* **336:**48–51.

24. DeMaere MZ, Williams TJ, Allen MA, Brown MV, Gibson JAE, Rich J, Lauro FM, Dyall-Smith M, Davenport KW, Woyke T, Kyrpides NC, Tringe SG, Cavicchioli R. 2013. High level of intergenera gene exchange shapes the evolution of haloarchaea in an isolated Antarctic lake. *Proc Natl Acad Sci USA* **110:**16939–16944.

25. Toussaint A, Chandler M. 2012. Prokaryote genome fluidity: toward a system approach of the mobilome. *Methods Mol Biol* **804:**57–80.

26. Shen J, Wang Y, Schwarz S. 2013. Presence and dissemination of the multiresistance gene *cfr* in Gram-positive and Gram-negative bacteria. *J Antimicrob Chemother* **68:**1697–1706.

27. Bonafede ME, Carias LL, Rice LB. 1997. Enterococcal transposon Tn5384: evolution of a composite transposon through cointegration of enterococcal and staphylococcal plasmids. *Antimicrob Agents Chemother* **41:**1854–1858.

28. Hung W-C, Takano T, Higuchi W, Iwao Y, Khokhlova O, Teng L-J, Yamamoto T. 2012. Comparative genomics of community-acquired ST59 methicillin-resistant *Staphylococcus aureus* in Taiwan: novel mobile resistance structures with IS1216V. *PLoS One* **7:**e46987. doi:10.1371/journal.pone.0046987.

29. Marraffini LA, Sontheimer EJ. 2010. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat Rev Genet* **11:**181–190.

30. Johnston C, Martin B, Polard P, Claverys J-P. 2013. Postreplication targeting of transformants by bacterial immune systems? *Trends Microbiol* **21:**516–521.

31. McCarthy AJ, Lindsay JA. 2012. The distribution of plasmids that carry virulence and resistance genes in *Staphylococcus aureus* is lineage associated. *BMC Microbiol* **12:**104.

32. Roberts GA, Houston PJ, White JH, Chen K, Stephanou AS, Cooper LP, Dryden DTF, Lindsay JA. 2013. Impact of target site distribution for type I restriction enzymes on the evolution of methicillin-resistant *Staphylococcus aureus* (MRSA) populations. *Nucleic Acids Res* **41:**7472–7484.

33. Attaiech L, Olivier A, Mortier-Barrière I, Soulet A-L, Granadel C, Martin B, Polard P, Claverys J-P. 2011. Role of the single-stranded DNA-binding protein SsbB in pneumococcal transformation: maintenance of a reservoir for genetic plasticity. *PLoS Genet* **7:**e1002156. doi:10.1371/journal.pgen.1002156.

34. Iacobino A, Scalfaro C, Franciosa G. 2013. Structure and genetic content of the megaplasmids of neurotoxigenic clostridium butyricum type E strains from Italy. *PLoS One* **8:**e71324. doi:10.1371/journal.pone.0071324.

35. Hatoum-Aslan A, Maniv I, Samai P, Marraffini LA. 2014. Genetic characterization of antiplasmid immunity through a type III-A CRISPR-Cas system. *J Bacteriol* **196:**310–317.

36. Palmer KL, Gilmore MS. 2010. Multidrug-resistant enterococci lack CRISPR-cas. *MBio* **1**(4)**:**e00227-10. doi:10.1128/mBio.00227-10.

37. Serbanescu MA, Cordova M, Krastel K, Flick R, Beloglazova N, Latos A, Yakunin AF, Senadheera DB, Cvitkovitch DG. 2014. Role of the *Streptococcus mutans* CRISPR/Cas systems in immunity and cell physiology. *J Bacteriol.* [Epub ahead of print.] doi:10.1128/JB.02333-14.

38. Hermsen R, Deris JB, Hwa T. 2012. On the rapidity of antibiotic resistance evolution facilitated by a concentration gradient. *Proc Natl Acad Sci USA* **109:**10775–10780.

39. Shu C-C, Chatterjee A, Hu W-S, Ramkrishna D. 2013. Role of intracellular stochasticity in biofilm growth. Insights from population balance modeling. *PLoS One* **8:**e79196. doi:10.1371/journal.pone.0079196.

40. Król JE, Wojtowicz AJ, Rogers LM, Heuer H, Smalla K, Krone SM, Top EM. 2013. Invasion of *E. coli* biofilms by antibiotic resistance plasmids. *Plasmid* **70:**110–119.

41. Hermsen R, Hwa T. 2010. Sources and sinks: a stochastic model of evolution in heterogeneous environments. *Phys Rev Lett* **105:**248104.

42. Nielsen KM, Bøhn T, Townsend JP. 2014. Detecting rare gene transfer events in bacterial populations. *Front Microbiol* **4:**415.

43. McCarthy AJ, Loeffler A, Witney AA, Gould KA, Lloyd DH, Lindsay JA. 2014. Extensive horizontal gene transfer during *Staphylococcus aureus* co-colonization *in vivo*. *Genome Biol Evol* **6:**2697–2708.

44. Gullberg E, Albrecht LM, Karlsson C, Sandegren L, Andersson DI. 2014. Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. *MBio* **5:**e01918-14. doi:10.1128/mBio.01918-14.

45. Baquero F, Coque TM. 2014. Widening the spaces of selection: evolution along sublethal antimicrobial gradients. *MBio* **5:**e02270-14. doi:10.1128/mBio.02270-14

46. Datta N. 1985. Plasmids in bacteria, p 3–16. *In* Helinski DR, Cohen SN, Clewell DB, Jackson DA, Hollaender A (ed), *Plasmids in Bacteria*. Basic Life Sciences, Vol. 30. Springer US, New York, New York.

47. Eberhard WG. 1989. Why do bacterial plasmids carry some genes and not others? *Plasmid* **21:**167–174.

48. Souza V, Eguiarte LE. 1997. Bacteria gone native vs. bacteria gone awry?: plasmidic transfer and bacterial evolution. *Proc Natl Acad Sci USA* **94:**5501–5503.

49. Kozlowicz BK, Dworkin M, Dunny GM. 2009. Pheromone-inducible conjugation in *Enterococcus faecalis*: a model for the evolution of biological complexity? *Int J Med Microbiol* **296:**141–147.

50. Taylor DE, Gibreel A, Lawley TD, Tracz DM. 2004. Antibiotic resistance plasmids, p 473–491. *In* Funnell B, Phillips G (ed), *Plasmid Biology*. ASM Press, Washington, DC.

51. Shearer JES, Wireman J, Hostetler J, Forberger H, Borman J, Gill J, Sanchez S, Mankin A, Lamarre J, Lindsay JA, Bayles K, Nicholson A, O'Brien F, Jensen SO, Firth N, Skurray RA, Summers AO. 2011. Major families of multiresistant plasmids from geographically and epidemiologically diverse staphylococci. *G3* **1:**581–591.

52. Lacey RW. 1975. Antibiotic resistance plasmids of *Staphylococcus aureus* and their clinical importance. *Bacteriol Rev* **39:**1–32.

53. Novick RP. 1989. Staphylococcal plasmids and their replication. *Annu Rev Microbiol* **43:**537–565.

54. Mills S, McAuliffe OE, Coffey A, Fitzgerald GF, Ross RP. 2006. Plasmids of lactococci: genetic accessories or genetic necessities? *FEMS Microbiol Rev* **30:**243–273.

55. Wang TT, Lee BH. 1997. Plasmids in *Lactobacillus*. *Crit Rev. Biotechnol* **17:**227–272.

56. Li J, Adams V, Bannam TL, Miyamoto K, Garcia JP, Uzal FA, Rood JI, McClane BA. 2013. Toxin plasmids of *Clostridium perfringens*. *Microbiol Mol Biol Rev* **77:**208–233.

57. Dib JR, Liebl W, Wagenknecht M, Farías ME, Meinhardt F. 2013. Extrachromosomal genetic elements in *Micrococcus*. *Appl Microbiol Biotechnol* **97:**63–75.

58. Guglielmetti S, Mayo B, Álvarez-Martín P. 2013. Mobilome and genetic modification of bifidobacteria. *Benef Microbes* **4:**143–166.

59. Jensen LB, Garcia-Migura L, Valenzuela AJ, Løhr M, Hasman H, Aarestrup FM. 2010. A classification system for plasmids from enterococci and other Gram-positive bacteria. *J Microbiol Methods* **80:** 25–43.

60. Iordanescu S, Surdeanu M, Della Latta P, Novick R. 1978. Incompatibility and molecular relationships between small staphylococcal plasmids carrying the same resistance marker. *Plasmid* **1:**468–479.

61. Macrina FL, Archer GL. 1993. *Bacterial Conjugation*. Plenum, New York, NY.

62. Novick RP. 1987. Plasmid incompatibility. *Microbiol Rev* **51:**381–395.

63. Projan SJ, Novick R. 1988. Comparative analysis of five related staphylococcal plasmids. *Plasmid* **19:**203–221.

64. Brantl S, Behnke D, Alonso JC. 1990. Molecular analysis of the replication region of the conjugative *Streptococcus agalactiae* plasmid pIP501 in *Bacillus subtilis*. Comparison with plasmids pAM beta 1 and pSM19035. *Nucleic Acids Res* **18:**4783–4790.

65. Dunny GM. 2013. Enterococcal sex pheromones: signaling, social behavior, and evolution. *Annu Rev Genet* **47:**457–482.

66. Lozano C, García-Migura L, Aspiroz C, Zarazaga M, Torres C, Aarestrup FM. 2012. Expansion of a plasmid classification system for Gram-positive bacteria and determination of the diversity of plasmids in *Staphylococcus aureus* strains of human, animal, and food origins. *Appl Environ Microbiol* **78:**5948–5955.

67. Sadowy E, Luczkiewicz A. 2014. Drug-resistant and hospital-associated *Enterococcus faecium* from wastewater, riverine estuary and anthropogenically impacted marine catchment basin. *BMC Microbiol* **14:**66.

68. Wardal E, Gawryszewska I, Hryniewicz W, Sadowy E. 2013. Abundance and diversity of plasmid-associated genes among clinical isolates of *Enterococcus faecalis*. *Plasmid* **70:**329–342.

69. Wardal E, Markowska K, Zabicka D, Wróblewska M, Giemza M, Mik E, Połowniak-Pracka H, Woźniak A, Hryniewicz W, Sadowy E. 2014. Molecular analysis of vanA outbreak of *Enterococcus faecium* in two Warsaw hospitals: the importance of mobile genetic elements. *Biomed Res Int* **2014:**575367.

70. Rosvoll TCS, Pedersen T, Sletvold H, Johnsen PJ, Sollid JE, Simonsen GS, Jensen LB, Nielsen KM, Sundsfjord A. 2010. PCR-based plasmid typing in *Enterococcus faecium* strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHTbeta-related replicons associated with glycopeptide resistance and stabilizing toxin-antitoxin systems. *FEMS Immunol Med Microbiol* **58:**254–268.

71. Freitas AR, Novais C, Tedim AP, Francia MV, Baquero F, Peixe L, Coque TM. 2013. Microevolutionary events involving narrow host plasmids influences local fixation of vancomycin-resistance in *Enterococcus* populations. *PLoS One* **8:**e60589. doi:10.1371/journal.pone.0060589.

72. Francia MV, Varsaki A, Garcillán-Barcia MP, Latorre A, Drainas C, de la Cruz F. 2004. A classification scheme for mobilization regions of bacterial plasmids. *FEMS Microbiol Rev* **28:**79–100.

73. Garcillan-Barcia MP, Francia MV, de la Cruz F. 2009. The diversity of conjugative relaxases and its application in plasmid classification. *FEMS Microbiol Rev* **33:**657–687.

74. Smillie C, Garcillán-Barcia MP, Francia MV, Rocha EPC, de la Cruz F. 2010. Mobility of plasmids. *Microbiol Mol Biol Rev* **74:**434–452.

75. Lorenzo-Díaz F, Fernández-López C, Garcillán-Barcia MP, Espinosa M. 2014. Bringing them together: plasmid pMV158 rolling circle replication and conjugation under an evolutionary perspective. *Plasmid* **74:**15–31.

76. Weaver KE, Kwong SM, Firth N, Francia MV. 2009. The RepA_N replicons of Gram-positive bacteria: a family of broadly distributed but narrow host range plasmids. *Plasmid* **61:**94–109.

77. Gruss A, Ehrlich SD. 1989. The Family of highly interrelated single-stranded deoxyribonucleic acid plasmids. *Microbiol Rev* **53:**231–241.

78. del Solar G, Moscoso M, Espinosa M. 1993. Rolling circle-replicating plasmids from Gram-positive and Gram-negative bacteria: a wall falls. *Mol Microbiol* **8:**789–796.

79. Khan SA. 1997. Rolling-circle replication of bacterial plasmids. *Microbiol Mol Biol Rev* **61:**442–455.

80. Widdowson CA, Adrian PV, Klugman KP. 2000. Acquisition of Chloramphenicol resistance by the linearization and integration of the entire staphylococcal plasmid pC194 into the chromosome of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* **44:**393–395.

81. Del Solar G, Moscoso M, Espinosa M. 1993. Rolling circle-replicating plasmids from Gram-positive and Gram-negative bacteria: a wall falls. *Mol Microbiol* **8:**789–796.

82. Heng NCK, Burtenshaw GA, Jack RW, Tagg JR. 2004. Sequence analysis of pDN571, a plasmid encoding novel bacteriocin production in M-type 57 *Streptococcus pyogenes*. *Plasmid* **52:**225–229.

83. Romero P, Llull D, García E, Mitchell TJ, López R, Moscoso M. 2007. Isolation and characterization of a new plasmid pSpnP1 from a multidrug-resistant clone of *Streptococcus pneumoniae*. *Plasmid* **58:**51–60.

84. Gillespie MT, May JW, Skurray RA. 1985. Antibiotic resistance in *Staphylococcus aureus* isolated at an Australian hospital between 1946 and 1981. *J Med Microbiol* **19:**137–147.

85. Schiwon K, Arends K, Rogowski KM, Fürch S, Prescha K, Sakinc T, Van Houdt R, Werner G, Grohmann E. 2013. Comparison of antibiotic resistance, biofilm formation and conjugative transfer of *Staphylococcus* and *Enterococcus* isolates from International Space Station and Antarctic Research Station Concordia. *Microb Ecol* **65:**638–651.

86. Shiwa Y, Yanase H, Hirose Y, Satomi S, Araya-Kojima T, Watanabe S, Zendo T, Chibazakura T, Shimizu-Kadota M, Yoshikawa H, Sonomoto K. 2014. Complete genome sequence of *Enterococcus mundtii* QU 25, an efficient L-(+)-lactic acid-producing bacterium. *DNA Res* **21:**369–377.

87. Garcia-Migura L, Hasman H, Jensen LB. 2009. Presence of pRI1: a small cryptic mobilizable plasmid isolated from *Enterococcus faecium* of human and animal origin. *Curr Microbiol* **58:**95–100.

88. De Vries LE, Christensen H, Agersø Y. 2012. The diversity of inducible and constitutively expressed erm(C) genes and association to different replicon types in staphylococci plasmids. *Mob Genet Elements* **2:**72–80.

89. Gómez-Sanz E, Kadlec K, Feßler AT, Zarazaga M, Torres C, Schwarz S. 2013. Novel erm(T)-carrying multiresistance plasmids from porcine and human isolates of methicillin-resistant *Staphylococcus aureus* ST398 that also harbor cadmium and copper resistance determinants. *Antimicrob Agents Chemother* **57:**3275–3282.

90. Firth N, Skurray RA. 1998. Mobile elements in the evolution and spread of multiple-drug resistance in staphylococci. *Drug Resist Updat* **1:**49–58.

91. Charlebois A, Jalbert L-A, Harel J, Masson L, Archambault M. 2012. Characterization of genes encoding for acquired bacitracin resistance in *Clostridium perfringens*. *PLoS One* **7:**e44449. doi:10.1371/journal.pone.0044449.

92. Palmer KL, Kos VN, Gilmore MS. 2010. Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. *Curr Opin Microbiol* **13:**632–639.

93. Montilla A, Zavala A, Cáceres Cáceres R, Cittadini R, Vay C, Gutkind G, Famiglietti A, Bonofiglio L, Mollerach M. 2014. Genetic environment of the lnu(B) gene in a *Streptococcus agalactiae* clinical isolate. *Antimicrob Agents Chemother* **58:**5636–5637.

94. Zong Z. 2013. Characterization of a complex context containing mecA but lacking genes encoding cassette chromosome recombinases in *Staphylococcus haemolyticus*. *BMC Microbiol* **13:**64.

95. Locke JB, Zuill DE, Scharn CR, Deane J, Sahm DF, Denys GA, Goering RV, Shaw KJ. 2014. Linezolid-resistant *Staphylococcus aureus* strain 1128105, the first known clinical isolate possessing the cfr multidrug resistance gene. *Antimicrob Agents Chemother* **58:**6592–6598.

96. Goessweiner-Mohr N, Arends K, Keller W, Grohmann E. 2013. Conjugative type IV secretion systems in Gram-positive bacteria. *Plasmid* **70:**289–302.

97. Li J, Li B, Wendlandt S, Schwarz S, Wang Y, Wu C, Ma Z, Shen J. 2014. Identification of a novel vga(E) gene variant that confers resistance to pleuromutilins, lincosamides and streptogramin A antibiotics in staphylococci of porcine origin. *J Antimicrob Chemother* **69:**919–923.

98. Lozano C, Aspiroz C, Rezusta A, Gómez-sanz E, Simon C, Gómez P, Ortega C, José M, Zarazaga M, Torres C. 2012. Identification of novel vga(A)-carrying plasmids and a Tn 5406-like transposon in meticillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* of human and animal origin. *Int J Antimicrob Agents* **40:**306–312.

99. Brantl S. 2014. Antisense-RNA mediated control of plasmid replication: pIP501 revisited. *Plasmid*. [Epub ahead of print.] doi:10.1016/j.plasmid.2014.07.004.

100. Grohmann E, Muth G, Espinosa M. 2003. Conjugative plasmid transfer in Gram-positive bacteria. *Microbiol Mol Biol Rev* **67:**277–301.

101. Behnke D, Malke H, Hartmann M, Walter F. 1979. Post-transformational rearrangement of an *in vitro* reconstructed group-A streptococcal erythromycin resistance plasmid. *Plasmid* **2:**605–616.

102. Clewell DB, Yagi Y, Dunny GM, Schultz SK. 1974. Characterization of three plasmid deoxyribonucleic acid molecules in a strain of *Streptococcus faecalis*: identification of a plasmid determining erythromycin resistance. *J Bacteriol* **117:**283–289.

103. Horodniceanu T, Bouanchaud DH, Bieth G, Chabbert YA. 1976. R plasmids in *Streptococcus agalactiae* (group B). *Antimicrob Agents Chemother* **10:**795–801.

104. Thompson JK, Collins MA. 2003. Completed sequence of plasmid pIP501 and origin of spontaneous deletion derivatives. *Plasmid* **50:**28–35.

105. Horaud T, Le Bouguenec C, Pepper K. 1985. Molecular genetics of resistance to macrolides, lincosamides and streptogramin B (MLS) in streptococci. *J Antimicrob Chemother* **16:**111–135.

106. Derome A, Hoischen C, Bussiek M, Grady R, Adamczyk M, Kedzierska B, Diekmann S, Barillà D, Hayes F. 2008. Centromere anatomy in the multidrug-resistant pathogen *Enterococcus faecium*. *Proc Natl Acad Sci USA* **105:**2151–2156.

107. Li X, Alvarez V, Harper WJ, Wang HH. 2011. Persistent, toxin-antitoxin system-independent, tetracycline resistance-encoding plasmid from a dairy *Enterococcus faecium* isolate. *Appl Environ Microbiol* **77:**7096–7103.

108. Abajy MY, Kopeć J, Schiwon K, Burzynski M, Döring M, Bohn C, Grohmann E. 2007. A type IV-secretion-like system is required for conjugative DNA transport of broad-host-range plasmid pIP501 in Gram-positive bacteria. *J Bacteriol* **189:**2487–2496.

109. Krah ER, Macrina FL. 1991. Identification of a region that influences host range of the streptococcal conjugative plasmid pIP501. *Plasmid* **25:**64–69.

110. Schaberg DR, Zervos MJ. 1986. Intergeneric and interspecies gene exchange in Gram-positive cocci. *Antimicrob Agents Chemother* **30:**817–822.

111. Teuber M, Meile L, Schwarz F. 1999. Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie Van Leeuwenhoek* **76:**115–137.

112. El-Solh N, Bouanchaud DH, Horodniceanu T, Roussel A, Chabbert YA. 1978. Molecular studies and possible relatedness between R plasmids from groups B and D streptococci. *Antimicrob Agents Chemother* **14:**19–23.

113. Engel HW, Soedirman N, Rost JA, van Leeuwen WJ, van Embden JD. 1980. Transferability of macrolide, lincomycin, and streptogramin resistances between group A, B, and D streptococci, *Streptococcus pneumoniae*, and *Staphylococcus aureus*. *J Bacteriol* **142:**407–413.

114. Clewell DB. 1981. Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. *Microbiol Rev* **45:**409–436.

115. Sletvold H, Johnsen PJ, Simonsen GS, Aasnaes B, Sundsfjord A, Nielsen KM. 2007. Comparative DNA analysis of two vanA plasmids from *Enterococcus faecium* strains isolated from poultry and a poultry farmer in Norway. *Antimicrob Agents Chemother* **51:**736–739.

116. Sletvold H, Johnsen PJ, Wikmark O-G, Simonsen GS, Sundsfjord A, Nielsen KM. 2010. Tn1546 is part of a larger plasmid-encoded genetic unit horizontally disseminated among clonal *Enterococcus faecium* lineages. *J Antimicrob Chemother* **65:**1894–1906.

117. Zhu W, Clark NC, McDougal LK, Hageman J, McDonald LC, Patel JB. 2008. Vancomycin-resistant *Staphylococcus aureus* isolates associated with Inc18-like vanA plasmids in Michigan. *Antimicrob Agents Chemother* **52:**452–457.

118. Zhu W, Murray PR, Huskins WC, Jernigan JA, McDonald LC, Clark NC, Anderson KF, McDougal LK, Hageman JC, Olsen-Rasmussen M, Frace M, Alangaden GJ, Chenoweth C, Zervos MJ, Robinson-Dunn B, Schreckenberger PC, Reller LB, Rudrik JT, Patel JB. 2010. Dissemination of an *Enterococcus* Inc18-Like vanA plasmid associated with vancomycin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* **54:**4314–4320.

119. Teuber M, Schwarz F, Perreten V. 2003. Molecular structure and evolution of the conjugative multiresistance plasmid pRE25 of *Enterococcus faecalis* isolated from a raw-fermented sausage. *Int J Food Microbiol* **88:**325–329.

120. Maki T, Santos MD, Kondo H, Hirono I, Aoki T. 2009. A transferable 20-kilobase multiple drug resistance-conferring R plasmid (pKL0018) from a fish pathogen (*Lactococcus garvieae*) is highly homologous to a conjugative multiple drug resistance-conferring enterococcal plasmid. *Appl Environ Microbiol* **75:**3370–3372.

121. Lim S-K, Tanimoto K, Tomita H, Ike Y. 2006. Pheromone-responsive conjugative vancomycin resistance plasmids in *Enterococcus faecalis* isolates from humans and chicken feces. *Appl Environ Microbiol* **72:**6544–6553.

122. Tanimoto K, Ike Y. 2008. Complete nucleotide sequencing and analysis of the 65-kb highly conjugative *Enterococcus faecium* plasmid pMG1: identification of the transfer-related region and the minimum region required for replication. *FEMS Microbiol Lett* **288:**186–195.

123. Tomita H, Pierson C, Lim SK, Clewell DB, Ike Y. 2002. Possible connection between a widely disseminated conjugative gentamicin resistance (pMG1-like) plasmid and the emergence of vancomycin resistance in *Enterococcus faecium*. *J Clin Microbiol* **40:**3326–3333.

124. Tomita H, Tanimoto K, Hayakawa S, Morinaga K, Ezaki K, Oshima H, Ike Y. 2003. Highly conjugative pMG1-like plasmids carrying Tn1546-like transposons that encode vancomycin resistance in *Enterococcus faecium*. *J Bacteriol* **185:**7024–7028.

125. Manson JM, Hancock LE, Gilmore MS. 2010. Mechanism of chromosomal transfer of *Enterococcus faecalis* pathogenicity island,

capsule, antimicrobial resistance, and other traits. *Proc Natl Acad Sci USA* **107:**12269–12274.

126. Arias CA, Panesso D, Singh KV, Rice LB, Murray BE. 2009. Cotransfer of antibiotic resistance genes and a hylEfm-containing virulence plasmid in *Enterococcus faecium*. *Antimicrob Agents Chemother* **53:**4240–4246.

127. Firth N, Apisiridej S, Berg T, Rourke BAO, Curnock S, Dyke KGH, Skurray RA. 2006. Replication of staphylococcal multiresistance plasmids. *J Bacteriol* **182:**2170–2178.

128. Kwong SM, Lim R, Lebard RJ, Skurray RA, Firth N. 2008. Analysis of the pSK1 replicon, a prototype from the staphylococcal multiresistance plasmid family. *Microbiology* **154:**3084–3094.

129. Berg T, Firth N, Apisiridej S, Leelaporn A, Skurray RA, Hettiaratchi A. 2006. Complete nucleotide sequence of pSK41: evolution of staphylococcal conjugative multiresistance plasmids. *J Bacteriol* **180:**4350–4359.

130. Littlejohn TG, DiBerardino D, Messerotti LJ, Spiers SJ, Skurray RA. 1991. Structure and evolution of a family of genes encoding antiseptic and disinfectant resistance in *Staphylococcus aureus*. *Gene* **101:**59–66.

131. Pérez-Roth E, Kwong SM, Alcoba-Florez J, Firth N, Méndez-Alvarez S. 2010. Complete nucleotide sequence and comparative analysis of pPR9, a 41.7-kilobase conjugative staphylococcal multiresistance plasmid conferring high-level mupirocin resistance. *Antimicrob Agents Chemother* **54:**2252–2257.

132. Pérez-Roth E, Potel-Alvarellos C, Espartero X, Constela-Caramés L, Méndez-Álvarez S, Alvarez-Fernández M. 2013. Molecular epidemiology of plasmid-mediated high-level mupirocin resistance in methicillin-resistant *Staphylococcus aureus* in four Spanish health care settings. *Int J Med Microbiol* **303:**201–204.

133. Udo EE, Jacob LE. 1998. Conjugative transfer of high-level mupirocin resistance and the mobilization of non-conjugative plasmids in *Staphylococcus*. *Microb Drug Resist* **4:**185–193.

134. Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, Flannagan SE, Kolonay JF, Shetty J, Killgore GE, Tenover FC. 2003. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. *Science* **302:**1569–1571.

135. Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, Lin F, Lin J, Carleton HA, Mongodin EF, Sensabaugh GF, Perdreau-Remington F. 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. *Lancet* **367:**731–739.

136. Thomas WD, Archer GL. 1992. Mobilization of recombinant plasmids from *Staphylococcus aureus* into coagulase negative *Staphylococcus* species. *Plasmid* **27:**164–168.

137. Projan SJ, Archer GL. 1989. Mobilization of the relaxable *Staphylococcus aureus* plasmid pC221 by the conjugative plasmid pGO1 involves three pC221 loci. *J Bacteriol* **171:**1841–1845.

138. Wardal E, Sadowy E, Hryniewicz W. 2010. Complex nature of enterococcal pheromone-responsive plasmids. *Pol J Microbiol* **59:**79–87.

139. Clewell DB, Francia MV. 2004. Conjugation in Gram-positive bacteria. *In* Funnell B, Phillips JG (ed), *Plasmid Biology*. ASM Press, Washington, DC.

140. Clewell DB, Francia MV, Flannagan SE, An FY. 2002. Enterococcal plasmid transfer: sex pheromones, transfer origins, relaxases, and the *Staphylococcus aureus* issue. *Plasmid* **48:**193–201.

141. Weaver KE. 2014. The type I toxin-antitoxin par locus from *Enterococcus faecalis* plasmid pAD1: RNA regulation by both cis- and trans-acting elements. *Plasmid*. [Epub ahead of print.] doi:10.1016/j.plasmid.2014.10.001.

142. Grady R, Hayes F. 2003. Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multidrug-resistant, clinical isolate of *Enterococcus faecium*. *Mol Microbiol* **47:**1419–1432.

143. Halvorsen EM, Williams JJ, Bhimani AJ, Billings EA, Hergenrother PJ. 2011. Txe, an endoribonuclease of the enterococcal Axe-Txe toxin-antitoxin system, cleaves mRNA and inhibits protein synthesis. *Microbiology* **157:**387–397.

144. Freitas AR, Coque TM, Novais C, Hammerum AM, Lester CH, Zervos MJ, Donabedian S, Jensen LB, Francia MV, Baquero F, Peixe L. 2011. Human and swine hosts share vancomycin-resistant *Enterococcus faecium* CC17 and CC5 and *Enterococcus faecalis* CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids. *J Clin Microbiol* **49:**925–931.

145. Werner G, Klare I, Witte W. 1999. Large conjugative vanA plasmids in vancomycin-resistant *Enterococcus faecium*. *J Clin Microbiol* **37:**2383–2384.

146. Hasman H, Villadsen AG, Aarestrup FM. 2005. Diversity and stability of plasmids from glycopeptide-resistant *Enterococcus faecium* (GRE) isolated from pigs in Denmark. *Microb Drug Resist* **11:**178–184.

147. Biavasco F, Foglia G, Paoletti C, Zandri G, Magi G, Guaglianone E, Sundsfjord A, Pruzzo C, Donelli G, Facinelli B. 2007. VanA-type enterococci from humans, animals, and food: species distribution, population structure, Tn1546 typing and location, and virulence determinants. *Appl Environ Microbiol* **73:**3307–3319.

148. Laverde Gomez JA, van Schaik W, Freitas AR, Coque TM, Weaver KE, Francia MV, Witte W, Werner G. 2011. A multiresistance megaplasmid pLG1 bearing a hylEfm genomic island in hospital *Enterococcus faecium* isolates. *Int J Med Microbiol* **301:**165–175.

149. Zhang X, Vrijenhoek JEP, Bonten MJM, Willems RJL, van Schaik W. 2011. A genetic element present on megaplasmids allows *Enterococcus faecium* to use raffinose as carbon source. *Environ Microbiol* **13:**518–528.

150. Gordoncillo MJN, Donabedian S, Bartlett PC, Perri M, Zervos M, Kirkwood R, Febvay C. 2013. Isolation and molecular characterization of vancomycin-resistant *Enterococcus faecium* from swine in Michigan, USA. *Zoonoses Public Health* **60:**319–326.

151. Johnson PDR, Ballard SA, Grabsch EA, Stinear TP, Seemann T, Young HL, Grayson ML, Howden BP. 2010. A sustained hospital outbreak of vancomycin-resistant *Enterococcus faecium* bacteremia due to emergence of vanB *E. faecium* sequence type 203. *J Infect Dis* **202:**1278–1286.

152. Rosvoll TCS, Lindstad BL, Lunde TM, Hegstad K, Aasnaes B, Hammerum AM, Lester CH, Simonsen GS, Sundsfjord A, Pedersen T. 2012. Increased high-level gentamicin resistance in invasive *Enterococcus faecium* is associated with aac(6′)Ie-aph(2″)Ia-encoding transferable megaplasmids hosted by major hospital-adapted lineages. *FEMS Immunol Med Microbiol* **66:**166–176.

153. Panesso D, Montealegre MC, Rincón S, Mojica MF, Rice LB, Singh KV, Murray BE, Arias CA. 2011. The hylEfm gene in pHylEfm of *Enterococcus faecium* is not required in pathogenesis of murine peritonitis. *BMC Microbiol* **11:**20.

154. Kim DS, Singh KV, Nallapareddy SR, Qin X, Panesso D, Arias CA, Murray BE. 2010. The fms21 (pilA)-fms20 locus encoding one of four distinct pili of *Enterococcus faecium* is harboured on a large transferable plasmid associated with gut colonization and virulence. *J Med Microbiol* **59:**505–507.

155. Boon E, Meehan CJ, Whidden C, Wong DH-J, Langille MGI, Beiko RG. 2014. Interactions in the microbiome: communities of organisms and communities of genes. *FEMS Microbiol Rev* **38:**90–118.

156. Smillie CS, Smith MB, Friedman J, Cordero OX, David LA, Alm EJ. 2011. Ecology drives a global network of gene exchange connecting the human microbiome. *Nature* **480:**241–244.

157. Skippington E, Ragan MA. 2011. Within-species lateral genetic transfer and the evolution of transcriptional regulation in *Escherichia coli* and *Shigella*. *BMC Genomics* **12:**532.

158. Baquero F, Coque TM, Canto R. 2003. Antibiotics, complexity, and evolution. *ASM News* **69:**547–552.

159. Baquero F, Tedim AP, Coque TM. 2013. Antibiotic resistance shaping multilevel population biology of bacteria. *Front Microbiol* **4:**1–15.

160. Baquero F, Lanza VF, Canton R, Coque TM. 2014. Public health evolutionary biology of antimicrobial resistance: priorities for intervention. *Evol Appl.* [Epub ahead of print.] doi:10.1111/eva.12235.

161. Baquero F, Coque TM, Cantón R. 2014. Counteracting antibiotic resistance: breaking barriers among antibacterial strategies. *Expert Opin Ther Targets* **18:**1–11.

162. Martínez JL. 2008. Antibiotics and antibiotic resistance genes in natural environments. *Science* **321:**365–367.

163. Martínez JL. 2011. Bottlenecks in the transferability of antibiotic resistance from natural ecosystems to human bacterial pathogens. *Front Microbiol* **2:**265.

164. Berryman DI, Rood JI. 1995. The closely related ermB-ermAM genes from *Clostridium perfringens*, *Enterococcus faecalis* (pAM beta 1), and *Streptococcus agalactiae* (pIP501) are flanked by variants of a directly repeated sequence. *Antimicrob Agents Chemother* **39:**1830–1834.

165. Howden BP, Holt KE, Lam MMC, Seemann T, Ballard S, Coombs GW, Tong SYC, Grayson ML, Johnson PDR, Stinear TP. 2013. Genomic insights to control the emergence of vancomycin-resistant enterococci. *MBio* **4**. doi:10.1128/mBio.00412-13.

166. Marvaud J-C, Mory F, Lambert T. 2011. Clostridium clostridioforme and *Atopobium minutum* clinical isolates with vanB-type resistance in France. *J Clin Microbiol* **49:**3436–3438.

167. Ballard SA, Grabsch EA, Johnson PDR, Grayson ML. 2005. Comparison of three PCR primer sets for identification of vanB gene carriage in feces and correlation with carriage of vancomycin-resistant enterococci: interference by vanB-containing anaerobic bacilli. *Antimicrob Agents Chemother* **49:**77–81.

168. Domingo M-C, Huletsky A, Boissinot M, Hélie M-C, Bernal A, Bernard KA, Grayson ML, Picard FJ, Bergeron MG. 2009. *Clostridium lavalense* sp. nov., a glycopeptide-resistant species isolated from human faeces. *Int J Syst Evol Microbiol* **59:**498–503.

169. Marshall BM, Ochieng DJ, Levy SB. 2009. Commensals: underappreciated reservoir of antibiotic resistance. *Microbe* **4:**231–238.

170. Maynard-Smith J. 1982. The century since Darwin. *Nature* **296:**599–601.

171. Baquero F. 2014. Genetic hyper-codes and multidimensional Darwinism: replication modes and codes in evolutionary individuals of the bacterial world. *In* Trueba G (ed), *Why does Evolution Matter?* Cambridge Scholars Publishing, Newcastle Upon Tyne, UK.

172. Baquero F. 2004. From pieces to patterns: evolutionary engineering in bacterial pathogens. *Nat Rev Microbiol* **2:**510–518.

173. Okasha S. 2006. *Evolution and the Levels of Selection*. Clarendon Press, Oxford, UK.

174. Paulsson J. 2002. Multileveled selection on plasmid replication. *Genetics* **161:**1373–1384.

175. Cordero OX, Polz MF. 2014. Explaining microbial genomic diversity in light of evolutionary ecology. *Nat Rev Microbiol* **12:**263–273.

176. Norton B. 1996. Change, constancy, and creativity: the new ecology and some old problems. *Duke Environ Law Policy Forum* **7:**49–70.

177. Hiramatsu K, Ito T, Tsubakishita S, Sasaki T, Takeuchi F, Morimoto Y, Katayama Y, Matsuo M, Kuwahara-Arai K, Hishinuma T, Baba T. 2013. Genomic basis for methicillin resistance in *Staphylococcus aureus*. *Infect Chemother* **45:**117–136.

178. Haines AN, Gauthier DT, Nebergall EE, Cole SD, Nguyen KM, Rhodes MW, Vogelbein WK. 2013. First report of *Streptococcus parauberis* in wild finfish from North America. *Vet Microbiol* **166:**270–275.

179. Richards VP, Palmer SR, Pavinski Bitar PD, Qin X, Weinstock GM, Highlander SK, Town CD, Burne RA, Stanhope MJ. 2014. Phylogenomics and the dynamic genome evolution of the genus *Streptococcus*. *Genome Biol Evol* **6:**741–753.

180. Gao X-Y, Zhi X-Y, Li H-W, Klenk H-P, Li W-J. 2014. Comparative genomics of the bacterial genus *Streptococcus* illuminates evolutionary implications of species groups. *PLoS One* **9:**e101229. doi:10.1371/journal.pone.0101229.

181. Jans C, Meile L, Lacroix C, Stevens MJA. 2014. Genomics, evolution, and molecular epidemiology of the *Streptococcus bovis/Streptococcus equinus* complex (SBSEC). *Infect Genet Evol.* [Epub ahead of print.] doi:10.1016/j.meegid.2014.09.017.

182. Caufield PW, Saxena D, Fitch D, Li Y. 2007. Population structure of plasmid-containing strains of *Streptococcus mutans*, a member of the human indigenous biota. *J Bacteriol* **189:**1238–1243.

183. Wescombe PA, Burton JP, Cadieux PA, Klesse NA, Hyink O, Heng NCK, Chilcott CN, Reid G, Tagg JR. 2006. Megaplasmids encode differing combinations of lantibiotics in *Streptococcus salivarius*. *Antonie Van Leeuwenhoek* **90:**269–280.

184. Pepper K, Le Bouguénec C, de Cespédès G, Horaud T. 1986. Dispersal of a plasmid-borne chloramphenicol resistance gene in streptococcal and enterococcal plasmids. *Plasmid* **16:**195–203.

185. Woodbury RL, Klammer KA, Xiong Y, Bailiff T, Glennen A, Bartkus JM, Lynfield R, Van Beneden C, Beall BW. 2008. Plasmid-borne erm(T) from invasive, macrolide-resistant *Streptococcus pyogenes* strains. *Antimicrob Agents Chemother* **52:**1140–1143.

186. Whitehead TR, Cotta MA. 2001. Sequence analyses of a broad host-range plasmid containing ermT from a tylosin-resistant *Lactobacillus* sp. isolated from swine feces. *Curr Microbiol* **43:**17–20.

187. DiPersio LP, DiPersio JR. 2006. High rates of erythromycin and clindamycin resistance among OBGYN isolates of group B *Streptococcus*. *Diagn Microbiol Infect Dis* **54:**79–82.

188. Palmieri C, Magi G, Creti R, Baldassarri L, Imperi M, Gherardi G, Facinelli B. 2013. Interspecies mobilization of an ermT-carrying plasmid of *Streptococcus dysgalactiae* subsp. equisimilis by a coresident ICE of the ICESa2603 family. *J Antimicrob Chemother* **68:**23–26.

189. Holden MTG, Hauser H, Sanders M, Ngo TH, Cherevach I, Cronin A, Goodhead I, Mungall K, Quail MA, Price C, Rabbinowitsch E, Sharp S, Croucher NJ, Chieu TB, Mai NTH, Diep TS, Chinh NT, Kehoe M, Leigh JA, Ward PN, Dowson CG, Whatmore AM, Chanter N, Iversen P, Gottschalk M, Slater JD, Smith HE, Spratt BG, Xu J, Ye C, Bentley S, Barrell BG, Schultsz C, Maskell DJ, Parkhill J. 2009. Rapid evolution of virulence and drug resistance in the emerging zoonotic pathogen *Streptococcus suis*. *PLoS One* **4:**e6072. doi:10.1371/journal.pone.0006072.

190. Cantin M, Harel J, Higgins R, Gottschalk M. 1992. Antimicrobial resistance patterns and plasmid profiles of *Streptococcus suis* isolates. *J Vet Diagn Invest* **4:**170–174.

191. Wang Y, Li D, Song L, Liu Y, He T, Liu H, Wu C, Schwarz S, Shen J. 2013. First report of the multiresistance gene *cfr* in *Streptococcus suis*. *Antimicrob Agents Chemother* **57:**4061–4063.

192. Chander Y, Oliveira SR, Goyal SM. 2011. Identification of the tet(B) resistance gene in *Streptococcus suis*. *Vet J* **189:**359–360.

193. Lebreton F, Willems RJL, Gilmore MS. 2014. *Enterococcus* diversity, origins in nature, and gut colonization. *In* Gilmore MS, Clewell DB, Ike Y, Shankar N (ed), *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary, Boston, MA.

194. Willems RJL, Top J, van Schaik W, Leavis H, Bonten M, Sirén J, Hanage WP, Corander J. 2012. Restricted gene flow among hospital subpopulations of *Enterococcus faecium*. *MBio* **3:**e00151-12. doi:10.1128/mBio.00151-12.

195. Tedim AP, Ruiz-Garbajosa P, Corander J, Rodríguez CM, Cantón R, Willems R, Baquero F, Coque TM. 2014. Population biology of *Enterococcus* from intestinal colonization in hospitalized and non-hospitalized individuals in different age groups. *Appl Environ Microbiol* **81.** [Epub ahead of print.] doi:10.1128/AEM.03661-14.

196. McBride SM, Fischetti VA, Leblanc DJ, Moellering RC, Gilmore MS. 2007. Genetic diversity among *Enterococcus faecalis*. *PLoS One* **2:**e582. doi:10.1371/journal.pone.0000582.

197. Kuch A, Willems RJL, Werner G, Coque TM, Hammerum AM, Sundsfjord A, Klare I, Ruiz-Garbajosa P, Simonsen GS, van Luit-Asbroek M, Hryniewicz W, Sadowy E. 2012. Insight into antimicrobial susceptibility and population structure of contemporary human *Enterococcus faecalis* isolates from Europe. *J Antimicrob Chemother* **67:**551–558.

198. López M, Tedim AP, Baquero F, Torres C, Coque TM. 2011. Plasmid diversity among *Enterococcus* spp from humans and animals (1991-2010) Poster presented at 4th Congress of European Microbiologist, FEMS. Geneva, Switzerland.

199. LeBlanc DJ, Inamine JM, Lee LN. 1986. Broad geographical distribution of homologous erythromycin, kanamycin, and streptomycin resistance determinants among group D streptococci of human and animal origin. *Antimicrob Agents Chemother* **29:**549–555.

200. Murray BE, An FY, Clewell DB. 1988. Plasmids and pheromone response of the beta-lactamase producer *Streptococcus* (*Enterococcus*) *faecalis* HH22. *Antimicrob Agents Chemother* **32:**547–551.

201. Murray BE, Singh KV, Markowitz SM, Lopardo HA, Patterson JE, Zervos MJ, Rubeglio E, Eliopoulos GM, Rice LB, Goldstein FW. 1991. Evidence for clonal spread of a single strain of beta-lactamase-producing *Enterococcus* (*Streptococcus*) *faecalis* to six hospitals in five states. *J Infect Dis* **163:**780–785.

202. Patterson JE, Singh KV, Murray BE. 1991. Epidemiology of an endemic strain of beta-lactamase-producing *Enterococcus faecalis*. *J Clin Microbiol* **29:**2513–2516.

203. Werner GDA, Strassmann JE, Ivens ABF, Engelmoer DJP, Verbruggen E, Queller DC, Noë R, Johnson NC, Hammerstein P, Kiers ET. 2014. Evolution of microbial markets. *Proc Natl Acad Sci USA* **111:**1237–1244.

204. Datta N, Hughes VM. Plasmids of the same Inc groups in *Enterobacteria* before and after the medical use of antibiotics. *Nature* **306:**616–617.

205. Wang X-M, Li X-S, Wang Y-B, Wei F-S, Zhang S-M, Shang Y-H, Du X-D. 2015. Characterization of a multidrug resistance plasmid from *Enterococcus faecium* that harbours a mobilized bcrABDR locus. *J Antimicrob Chemother* **70:**609–611.

206. Devirgiliis C, Zinno P, Perozzi G. 2013. Update on antibiotic resistance in foodborne *Lactobacillus* and *Lactococcus* species. *Front Microbiol* **4:**301.

207. Becker K, Heilmann C, Peters G. 2014. Coagulase-negative staphylococci. *Clin Microbiol Rev* **27:**870–926.

208. Lindsay JA, Holden MTG. 2004. *Staphylococcus aureus*: superbug, super genome? *Trends Microbiol* **12:**378–385.

209. Lindsay JA. 2010. Genomic variation and evolution of *Staphylococcus aureus*. *Int J Med Microbiol* **300:**98–103.

210. Dethlefsen L, McFall-Ngai M, Relman DA. 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* **449:**811–818.

211. Lindsay JA, Knight GM, Budd EL, McCarthy AJ. 2012. Shuffling of mobile genetic elements (MGEs) in successful healthcare-associated MRSA (HA-MRSA). *Mob Genet Elements* **2:**239–243.

212. Livermore DM. 2000. Antibiotic resistance in staphylococci. *Int J Antimicrob Agents* **16**(Suppl 1)**:**S3–S10.

213. Sidhu MS, Heir E, Leegaard T, Wiger K, Holck A. 2002. Frequency of disinfectant resistance genes and genetic linkage with beta-lactamase transposon Tn552 among clinical staphylococci. *Antimicrob Agents Chemother* **46:**2797–2803.

214. Willems RJL, Hanage WP, Bessen DE, Feil EJ. 2011. Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance. *FEMS Microbiol Rev* **35:**872–900.

215. Lindsay JA, Holden MTG. 2006. Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. *Funct Integr Genomics* **6:**186–201.

216. Lutz C, Erken M, Noorian P, Sun S, McDougald D. 2013. Environmental reservoirs and mechanisms of persistence of *Vibrio cholerae*. *Front Microbiol* **4:**375.

217. Lindsay JA. 2014. Evolution of *Staphylococcus aureus* and MRSA during outbreaks. *Infect Genet Evol* **21:**548–553.

218. McDougal LK, Fosheim GE, Nicholson A, Bulens SN, Limbago BM, Shearer JES, Summers AO, Patel JB. 2010. Emergence of resistance among USA300 methicillin-resistant *Staphylococcus aureus* isolates causing invasive disease in the United States. *Antimicrob Agents Chemother* **54:** 3804–3811.

219. McCarthy AJ, Witney AA, Gould KA, Moodley A, Guardabassi L, Voss A, Denis O, Broens EM, Hinds J, Lindsay JA. 2011. The distribution of mobile genetic elements (MGEs) in MRSA CC398 is associated with both host and country. *Genome Biol Evol* **3:**1164–1174.

220. Alibayov B, Baba-Moussa L, Sina H, Zdeňková K, Demnerová K. 2014. *Staphylococcus aureus* mobile genetic elements. *Mol Biol Rep* **41:**5005–5018.

221. Uhlemann A-C, Dordel J, Knox JR, Raven KE, Parkhill J, Holden MTG, Peacock SJ, Lowy FD. 2014. Molecular tracing of the emergence, diversification, and transmission of *S. aureus* sequence type 8 in a New York community. *Proc Natl Acad Sci USA* **111:**6738–6743.

222. Rossi F, Diaz L, Wollam A, Panesso D, Zhou Y, Rincon S, Narechania A, Xing G, Di Gioia TSR, Doi A, Tran TT, Reyes J, Munita JM, Carvajal LP, Hernandez-Roldan A, Brandão D, van der Heijden IM, Murray BE, Planet PJ, Weinstock GM, Arias CA. 2014. Transferable vancomycin resistance in a community-associated MRSA lineage. *N Engl J Med* **370:**1524–1531.

223. Strommenger B, Bartels MD, Kurt K, Layer F, Rohde SM, Boye K, Westh H, Witte W, De Lencastre H, Nübel U. 2014. Evolution of methicillin-resistant *Staphylococcus aureus* towards increasing resistance. *J Antimicrob Chemother* **69:**616–622.

224. Locke JB, Zuill DE, Scharn CR, Deane J, Sahm DF, Goering RV, Jenkins SG, Shaw KJ. 2014. Identification and characterization of linezolid-resistant cfr-positive *Staphylococcus aureus* USA300 isolates from a New York City Medical Center. *Antimicrob Agents Chemother* **58:**6949–6952.

225. Sung JM-L, Lloyd DH, Lindsay JA. 2008. *Staphylococcus aureus* host specificity: comparative genomics of human versus animal isolates by multi-strain microarray. *Microbiology* **154:**1949–1959.

226. Holden MTG, Feil EJ, Lindsay JA, Peacock SJ, Day NPJ, Enright MC, Foster TJ, Moore CE, Hurst L, Atkin R, Barron A, Bason N, Bentley SD, Chillingworth C, Chillingworth T, Churcher C, Clark L, Corton C, Cronin A, Doggett J, Dowd L, Feltwell T, Hance Z, Harris B, Hauser H, Holroyd S, Jagels K, James KD, Lennard N, Line A, Mayes R, Moule S, Mungall K, Ormond D, Quail MA, Rabbinowitsch E, Rutherford K, Sanders M, Sharp S, Simmonds M, Stevens K, Whitehead S, Barrell BG, Spratt BG, Parkhill J. 2004. Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc Natl Acad Sci USA* **101:**9786–9791.

227. Robinson DA, Kearns AM, Holmes A, Morrison D, Grundmann H, Edwards G, O'Brien FG, Tenover FC, McDougal LK, Monk AB, Enright MC. 2005. Re-emergence of early pandemic *Staphylococcus aureus* as a community-acquired meticillin-resistant clone. *Lancet* **365:**1256–1258.

228. Yutin N, Galperinr MY. 2014. A genomic update on clostridial phylogeny: Gram-negative spore-formers and other misplaced clostridia. *Environ Microbiol* **15:**2631–2641.

229. Magot M. 1984. Physical characterization of the *Clostridium perfringens* tetracycline-chloramphenicol resistance plasmid pIP401. *Ann Microbiol* **135B:**269–282.

230. Lyras D, Adams V, Ballard SA, Teng WL, Howarth PM, Crellin PK, Bannam TL, Songer JG, Rood JI. 2009. tISCpe8, an IS1595-family lincomycin resistance element located on a conjugative plasmid in *Clostridium perfringens*. *J Bacteriol* **191:**6345–6351.

231. Bannam TL, Teng WL, Bulach D, Lyras D, Rood JI. 2006. Functional identification of conjugation and replication regions of the tetracycline resistance plasmid pCW3 from *Clostridium perfringens*. *J Bacteriol* **188:**4942–4951.

232. Gurjar A, Li J, McClane BS. 2010. Characterization of toxin plasmids in *Clostridium perfringens* type C isolates. *Infect Immun* **78:**4860–4869.

233. Nowell VJ, Kropinski AM, Songer JG, MacInnes JI, Parreira VR, Prescott JF. 2012. Genome sequencing and analysis of a type A *Clostridium perfringens* isolate from a case of bovine clostridial abomasitis. *PLoS One* **7:**e32271. doi:10.1371/journal.pone.0032271.

234. Sasaki Y, Yamamoto K, Tamura Y, Takahashi T. 2001. Tetracycline-resistance genes of *Clostridium perfringens*, *Clostridium septicum* and *Clostridium sordellii* isolated from cattle affected with malignant edema. *Vet Microbiol* **83:**61–69.

235. Franciosa G, Scalfaro C, Di Bonito P, Vitale M, Aureli P. 2011. Identification of novel linear megaplasmids carrying a ß-lactamase gene in neurotoxigenic *Clostridium butyricum* type E strains. *PLoS One* **6:**e21706. doi:10.1371/journal.pone.0021706.

236. Slavić D, Boerlin P, Fabri M, Klotins KC, Zoethout JK, Weir PE, Bateman D. 2011. Antimicrobial susceptibility of *Clostridium perfringens* isolates of bovine, chicken, porcine, and turkey origin from Ontario. *Can J Vet Res* **75:**89–97.

237. Farrow KA, Lyras D, Rood JI. 2000. The macrolide-lincosamide-streptogramin B resistance determinant from *Clostridium difficile* 630 contains two erm(B) genes. *Antimicrob Agents Chemother* **44:**411–413.

238. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25:**3389–3402.

239. Li W, Godzik A. 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* **22:**1658–1659.

240. Lederberg J. 1998. Plasmid (1952–1997). *Plasmid* **39:**1–9.

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Microevolutionary Events Involving Narrow Host Plasmids Influences Local Fixation of Vancomycin-Resistance in *Enterococcus* Populations

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Abstract

Vancomycin-resistance in enterococci (VRE) is associated with isolates within ST18, ST17, ST78 *Enterococcus faecium* (Efm) and ST6 *Enterococcus faecalis* (Efs) human adapted lineages. Despite of its global spread, vancomycin resistance rates in enterococcal populations greatly vary temporally and geographically. Portugal is one of the European countries where Tn*1546* (*vanA*) is consistently found in a variety of environments. A comprehensive multi-hierarchical analysis of VRE isolates (75 Efm and 29 Efs) from Portuguese hospitals and aquatic surroundings (1996–2008) was performed to clarify the local dynamics of VRE. Clonal relatedness was established by PFGE and MLST while plasmid characterization comprised the analysis of known relaxases, rep initiator proteins and toxin-antitoxin systems (TA) by PCR-based typing schemes, RFLP comparison, hybridization and sequencing. Tn*1546* variants were characterized by PCR overlapping/sequencing. Intra- and inter-hospital dissemination of Efm ST18, ST132 and ST280 and Efs ST6 clones, carrying rolling-circle (pEFNP1/pRI1) and theta-replicating (pCIZ2-like, Inc18, pHTβ-like, two pRUM-variants, pLG1-like, and pheromone-responsive) plasmids was documented. Tn*1546* variants, mostly containing IS*Ef1* or IS*1216*, were located on plasmids (30–150 kb) with a high degree of mosaicism and heterogeneous RFLP patterns that seem to have resulted from the interplay between broad host Inc18 plasmids (pIP501, pRE25, pEF1), and narrow host RepA_N plasmids (pRUM, pAD1-like). TAs of Inc18 (ω-ε-ζ) and pRUM (Axe-Txe) plasmids were infrequently detected. Some plasmid chimeras were persistently recovered over years from different clonal lineages. This work represents the first multi-hierarchical analysis of VRE, revealing a frequent recombinatorial diversification of a limited number of interacting clonal backgrounds, plasmids and transposons at local scale. These interactions provide a continuous process of parapatric clonalization driving a full exploration of the local adaptive landscape, which might assure long-term maintenance of resistant clones and eventually fixation of Tn*1546* in particular geographic areas.

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Introduction

Since its first description in the late 80's, vancomycin-resistant enterococci (VRE) have been increasingly reported worldwide, but presenting remarkable geographical and temporal differences in local rates (http://www.cddep.org/ResistanceMap/bug-drug/EFa-VC) [1-3]. Vancomycin-resistant *Enterococcus faecium* (VREfm) became endemic in most North American hospitals since the mid 90's [1,2,4–6] while their overall occurrence in Europe remained low until recently, when VRE nosocomial outbreaks started to be increasingly reported in some European countries (Annual Report of the European Antimicrobial Resistance Surveillance Network, EARS-Net, 2009) [1,3,7,8]. Despite *E. faecium* (Efm) being less frequently found than *Enterococcus faecalis* (Efs) in clinical isolates, it is far more frequently resistant to vancomycin, one of the last-line intravenous antibiotic resources for therapy. However, although the rate of vancomycin-resistant *E. faecalis* (VREfs) has remained low, they are steadily increasing in both the US and in EU countries (http://www.cddep.org/ResistanceMap/bug-drug/EFe-VC) [3].

Vancomycin resistance among enterococci is mostly due to the spread of Tn*1546* (*vanA* genotype) and Tn*1549* (*vanB* genotype), which are generally identified on plasmids and chromosome, respectively [3]. The few studies in which plasmids carrying Tn*1546* from human or animal isolates were characterized

revealed they belong to plasmid families RepA_N (pheromone-responsive plasmids and derivatives of pRUM and pLG1), Inc18 and pHTβ [9–18] suggesting an apparent plasmid promiscuity of this transposon influencing its dissemination among enterococcal populations.

Recent analysis of enterococcal populations in the clinical setting depicts a rugged epidemiological profile, with successive waves of isolates causing infections, which belong to specific lineages of *E. faecium* (ST17, ST18 and ST78, previously considered within the same clonal complex (CC) 17), and *E. faecalis* (ST6, ST40) [19–21]. However, regional differences in the rates of VRE cannot be only explained by clonal replacement dynamics as suggested for other pathogens [22–24].

The aim of this study was to address the dynamics of vancomycin resistance among enterococci in Portugal, one of the developed countries with higher rates of both VREfm (21–23%) and VREfs (1.8–4.1%) (www.earss.rivm.nl; http://www.cddep.org/ResistanceMap/bug-drug/EFe-VC), and where VanA is prevalent over VanB [3,25–27], by analyzing the clonal and plasmid backgrounds influencing the spread and persistence of Tn*1546*. Our study suggests that clonalization, the local selection of distinct clonal variants giving rise to durable bacterial lineages, might result and be modified by the local spread and recombinatorial dynamics of mobile genetic elements, thus providing new clues about the local multi-hierarchical evolutionary biology of vancomycin resistance.

Results

Local dynamic landscape drives the spread and fixation of vancomycin resistance in Portuguese hospitals

We have determined that the enterococcal population from the Portuguese hospitals is formed by an ensemble of MLST/PFGE clones. Efm isolates fit in three out of six phylogenomic groups recently established by using Bayesian Analysis of Population Structure (BAPS), namely BAPS groups 2, 3 and 5 [19] (Figure 1). Most of the isolates cluster into the predominant BAPS group 3 [subgroup 3–3 comprising main human lineages ST18 (ST18 and ST132) and ST17 (ST16); and subgroup 3–1 comprising ST280], and the BAPS group 2 (including ST80 and ST656/ST78 lineage, ST5/CC5, ST190/CC9), which have been previously associated with isolates from humans and both animals and humans, respectively [10,19,25,28–30]. A number of clones cluster in the small Efm BAPS group 5 (ST366, ST367, ST369), which seems to comprise mosaic genomes [19]. Isolates of Efs belong to ST6/CC2, ST30, ST55, ST117, and ST159 lineages although, to the date of this publication, Efs population has not been clustered in different BAPS groups. Among all them, isolates within ST18 Efm and ST6 Efs lineages were predominant, in line with the intra- and interhospital spread of particular highly transmissible Efm and Efs clones recovered in Portuguese hospitals since the late 90s [22,25,31,32]. While ST6 Efs was widely disseminated in all hospitals analyzed in this country [26], specific Efm lineages were overrepresented in Coimbra (ST18) and Oporto (ST132, a single locus variant, SLV, of ST18). Strains belonging to ST18 (showing PFGE types H70 and H78), ST132 (PFGE type H88) and ST280 (with PFGE types 71 and H100) were spread in different hospitals (Figure 1 and Figure 2).

It is worthwhile to note the possible relatedness between isolates of different STs (Figure 1 and 2). They include some isolates linked to BAPS 3–3 subgroup as ST18, ST80, ST125, ST132, ST368, ST369, all SLVs of each other, with PFGE patterns differing in less than 8 bands difference. Similarly, strains identified as ST280 and ST391, both linked to BAPS group 3–1, showed related PFGE patterns despite being trilocus variants (≤ 8 bands difference).

vanA-Tn*1546* is located on highly transferable mosaic plasmids involving narrow host pRUM and pAD1 derivatives

The plasmid content of the isolates studied appears in Figure 2. Efm isolates carried a variable number of plasmids (n = 1–6) which contained specific sequences of different families including rolling-circle plasmids (RCR) related to pRI1 and small theta plasmids related to pCIZ2, RepA_N (pRUM-like, pLG1), pHTβ (present in all ST132 isolates), and Inc18 (pRE25 and pEF1-related). All Efs contained RCR plasmids and pheromone responsive-plasmids.

vanA-Tn*1546* was located on plasmids ranging from 30 to 150 kb, successfully transferred by conjugation in 95% (n = 71/75) of Efm and 97% (n = 28/29) of Efs, with a variable frequency (10^{-1}–10^{-8}). Transferable plasmids were identified as members of pRUM and Inc18 families or were mosaic plasmids of pRUM, Inc18 and pheromone plasmids (see sections below). Although some of these mosaic plasmids were detected in both Efm and Efs hosts, species-specific plasmid variants were predominant.

We have classified the enterococcal plasmids according to the content in rep/rel/TA systems, and RFLP profiles (Table 1, Figure 2). For the better interpretation of the results, we should keep in mind that members of the most common plasmid families classified in this and other studies as Inc18-like (pRE25, pIP501, pVEF1, pVEF2, pVEF3, pIP816, pEF1, pWZ909) or pRUM-like (pRUM, p5753cB, pS177) exhibit a high degree of *modular dissociability* or propensity for independent variation and shuffling, and may contain multiple replicons or be devoid of conjugation systems, thus making it very difficult to establish an accurate classification and to trace the origin of certain elements [9,33–40]. See Clewell *et al.* for a comprehensive updated revision of enterococcal plasmids [9]. In the following sections we will describe vancomycin resistant plasmids of Efm and Efs.

vanA plasmids of E. faecium

They were classified in two broad groups according to the plasmid replication modules and the background epidemiological context, i) pRUM-like variants ($Rep_{17.2/pRUM\text{-}like}$+ $Rel_{6/pEF1}$ ±$Rep_{1/pIP501}$± $Rep_{2/pRE25/pEF1}/TA_{Inc18}$), ii) mosaics of Inc18-pRUM-like ($Rep_{2/pRE25/pEF1}$ ± $Rep_{17.2/pRUM}/TA_{Axe\text{-}Txe}$). Highly transmissible pAD1-Inc18 mosaic plasmids from major Efs clones were also identified among Efm but they will be described in the next section.

i) *pRUM derivatives* ($rep_{17.2/pRUM\text{-}like}$+$rel_{6/pEF1}$) of variable size (30–120 kb) were detected since the mid 90 s from a diversity of clonal backgrounds. pRUM plasmids showing different *Cla*I-digested DNA RFLP patterns were identified carrying a whole copy of Tn*1546* (RFLP_1, RFLP_2, RFLP_20, 30–80 kb), IS*1216*::Tn*1546* (RFLP_8–12, 40–120 kb) or IS*Ef1*::Tn*1546* (RFLP_3–7, RFLP_13, 50–95 kb). Despite the heterogeneity of plasmid profiles, RFLP_3–6 or RFLP_8–10 shared a variable number of common bands that suggest a relationship among them (see Table 1 and Figure 3 for details about relationships among plasmids). pRUM-like plasmids exhibiting distinct RFLP profiles and carrying different transposon variants were isolated in early and recent isolates of different clonal backgrounds (Figure 2). They include ST190, carrying a 60 kb plasmid RFLP_1 type; ST670 carrying a 85 kb exhibiting a RFLP_4 plasmid type; ST656 carrying a 30 kb plasmid designated as RFLP_20, and ST18, ST132, ST280, carrying different

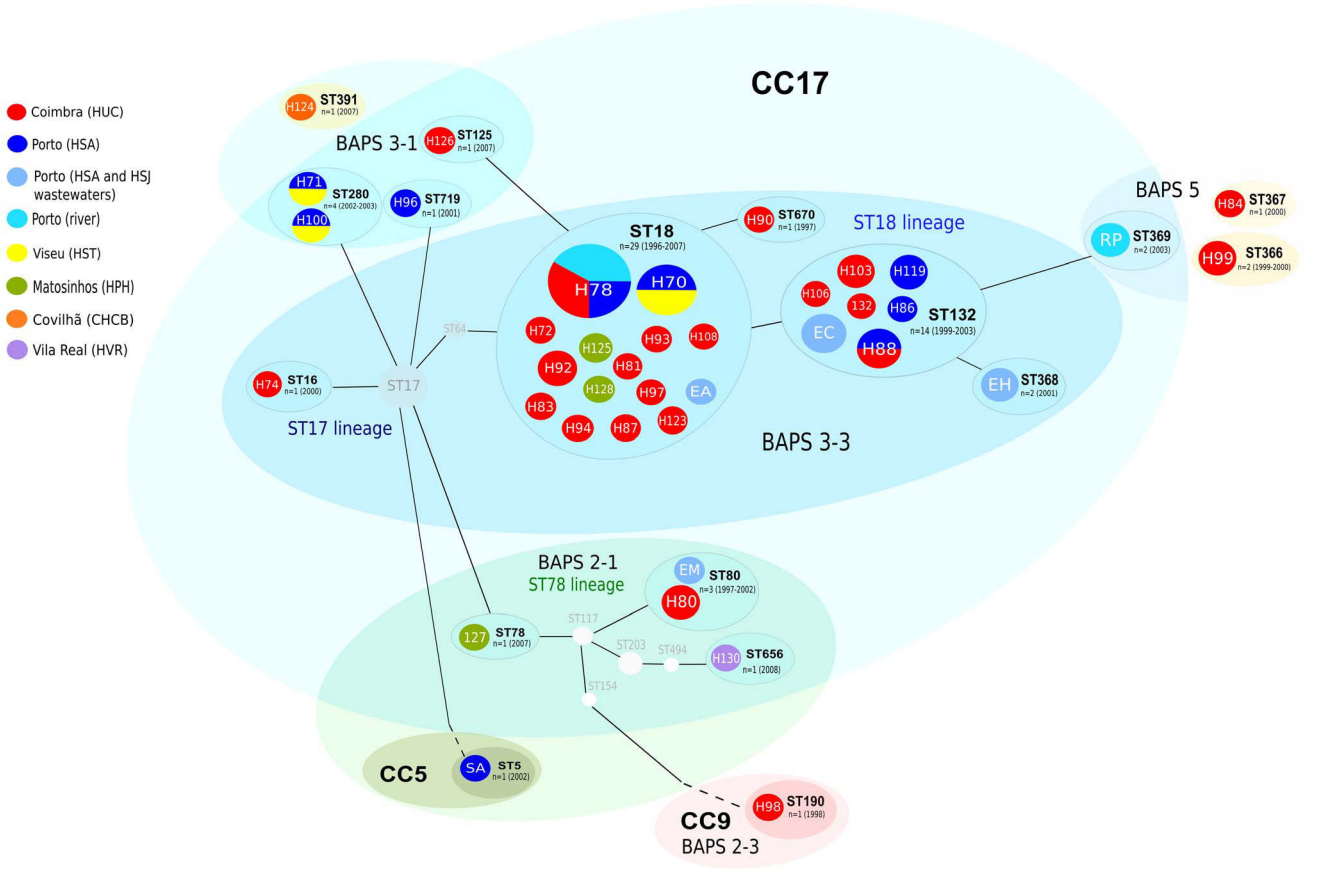

Figure 1. Population of vancomycin resistant *Enterococcus faecium*. Abbreviations: ST, sequence types; CC, clonal complex; BAPS, Bayesian Analysis of Population Structure; HUC, Hospital Universitário de Coimbra; HSA, Hospital Santo António; HSJ, Hospital São João; HST, Hospital São Teotónio; HPH, Hospital Pedro Hispano; CHCB, Centro Hospitalar da Cova da Beira; HVR, Hospital S. Pedro. A colored circle represents each PFGE type (white numbers/letters; H for hospital, SW for sewage, R for river and S for swine clones) and each PFGE type is associated with the corresponding sequence type (STs are represented in black letter and in colored elipses grouping different PFGE types) and BAPS group (in colored elipses grouping different STs). The size of the colored circles corresponds to the number of isolates. CC17 (in light blue), CC5 (in light green), CC9 (in light red) and the singletons ST366, ST367 and ST391 (light yellow) are represented according to the eBURST algorithm (download on 26th January 2012) with black lines joining single locus variants (SLV). STs that were not identified in this study are represented as light grey nodes to link the sequence types identified in this study accordingly to eBURST. ST18 strains (H70, H78, H87, H93, H108, H125) and most ST132 strains (H86, H88, H106, SWC) were clonally related by PFGE ($\leq$ 7 bands difference). Remarkable relationships among PFGE banding patterns of strains belonging to different STs were observed (H125/ST18 and H126/ST125; H124/ST391 and H71/ST280, SWM/ST80 and H86/H88/H106/H119/SWC/ST132, and isolates SWA/ST18 and SWC/ST132 ($\leq$ 8 bands difference). This figure drawn up was performed in the "Open Source vector graphics editor Inkscape" (version Inkscape-0.48.2–1).
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transposon variants. These results suggest multiple independent acquisitions of pRUM-like plasmids and further rearrangements with other elements, some plasmid variants being efficiently transferred among a diversity of different clones. It is of interest to highlight that epidemic ST18 PFGE types H83 (1996) and H92 (2000) harboured two pRUM-like plasmids. One was the $rep_{17.2/pRUM\text{-}like}$::$rel_{6/pEF1}$ vancomycin resistant plasmid showing RFLP_2 and RFLP_12 and the other was a 25 kb carrying a $rep_{17.1/pRUM}$ gene and a copy of the Axe-Txe toxin-antitoxin system ($rep_{17.1/pRUM}$+$TA_{Axe\text{-}Txe}$) identical to the pRUM derivatives described to date (pRUM, p5753cB and pS177) (GenBank accession number GQ900487; Figure 2) [12,38,39] and other vancomycin resistant plasmids circulating at international level (Freitas *et al.*, unpublished data). Diversification in the Rep sequences of these pRUM-like plasmids (homology of 96% at nucleotide level and 95% at protein level) might have resulted in the compatibility with similar (but not identical) plasmids in the same clonal background along extended periods of time.

ii) *Inc18 plasmids and mosaic Inc18-pRUM plasmids.* Clonally related ST132 and ST18 Efm isolates from Oporto contained Inc18 plasmids ($Rep_{1/pIP501}$ ± $Rep_{2/pRE25/pEF1}$, RFLP_14–15) or mosaic plasmids of Inc18 and pRUM ($Rep_{2/pRE25/pEF1}$+$Rep_{1/pIP501}$+TA_{Inc18}+$Rep_{17.2/pRUM\text{-}like}$+$Rel_{pEF1}$, RFLP_16–19), all carrying IS*1216*-Tn*1546* variants. Plasmids showing RFLP types 16–19 were highly similar (5 bands/12 bands in common), RFLP_19 being persistently recovered from clonally related ST132, ST368 and ST369 isolates, collected from hospitalized patients of HSA near by sewage plant and the river Douro from 2001 to 2003. This RFLP_19 has been also identified in a VREfm isolate recovered from swine in 2007 (Tn*1546* type "PP-31", RFLP_19.1), highlighting the remarkable stability of particular VanA Inc18 plasmids in ensembles of related clones able to spread in different hosts [10]. A diversity of Tn*1546*::IS*1216*

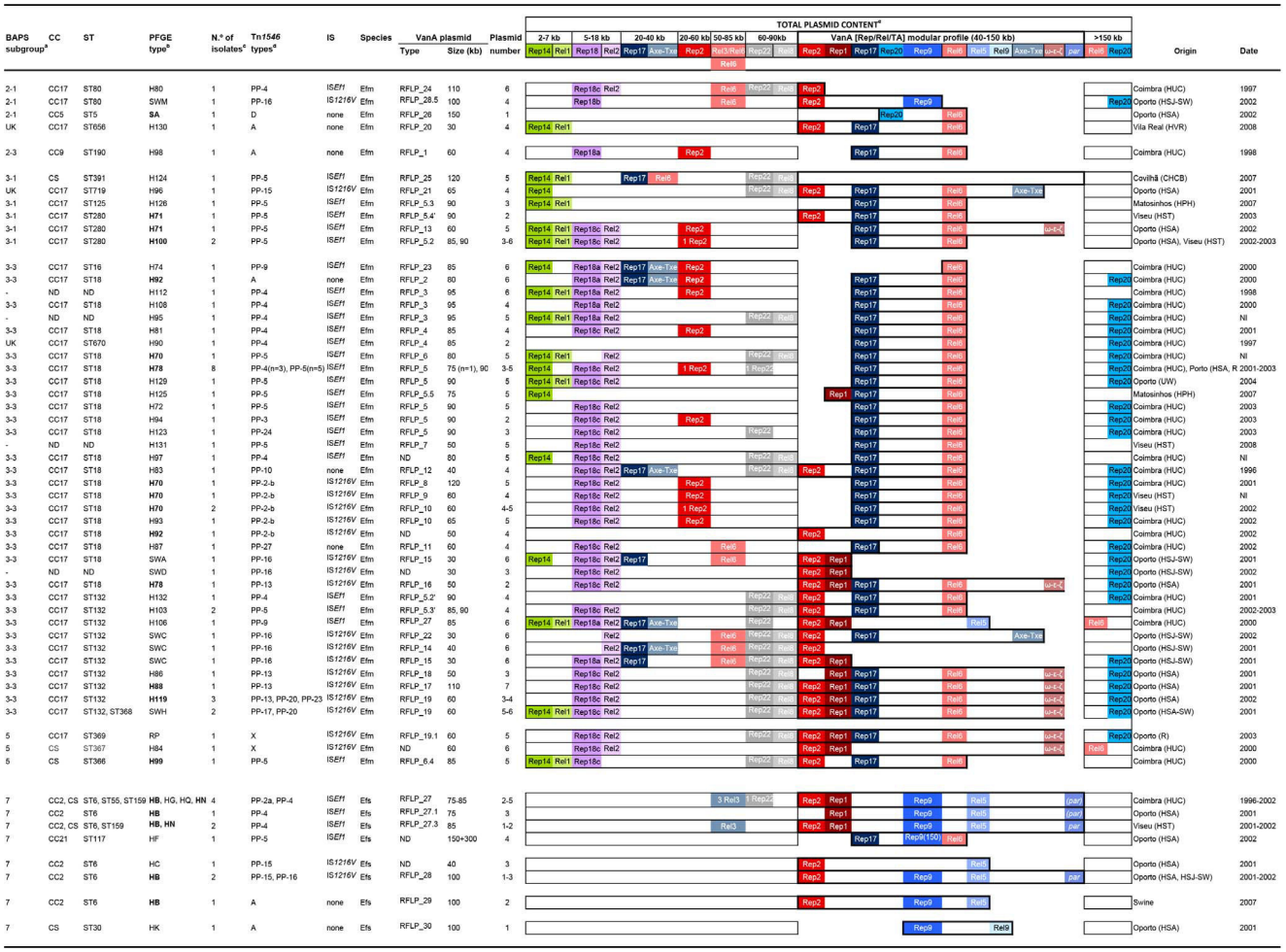

Figure 2. Clonal and plasmid diversity among VREfm and VREFs from Portugal. Abbreviations: IS, insertion sequence; Efm, *Enterococcus faecium*; Efs, *Enterococcus faecalis*; kb, kilobases; BAPS, Bayesian Analysis of Population Structure; ST, sequence type; CC, clonal complex; rep (replicases); rel (relaxases); TA (toxin-antitoxin system); HUC, Hospital Universitário de Coimbra; HSA, Hospital Santo António; HSJ, Hospital São João; HST, Hospital São Teotónio; HPH, Hospital Pedro Hispano; CHCB, Centro Hospitalar da Cova da Beira; HVR, Hospital S. Pedro; SW, sewage wastewaters; UW, urban wastewaters; R, river; ND, not determined; NI, not identified; UK, unknown. [a]The distribution of the different isolates is shown by BAPS subgroups as described [19]. [b]PFGE types shown in bold represented widespread clones in Portuguese hospitals and/or aquatic surroundings over years. [c]Most Efm isolates expressed resistance to vancomycin, teicoplanin, erythromycin, ampicillin, ciprofloxacin (92–100%) and to a lesser extent to high levels of kanamycin (65%), gentamicin (41%), streptomycin and tetracycline (28% each). While *acm* was identified in different CC17 and non-CC17 lineages (76%), *esp* was detected in CC17 isolates (35%, ST132 and its SLVs ST368, ST369) and *hyl* was sporadically found (9%, ST18, ST125, ST132, SLVs of each other, and ST280 isolates) [25]. Efs isolates (mostly ST6) showed resistance to vancomycin, teicoplanin, erythromycin, ciprofloxacin, high levels of gentamicin and kanamycin (82–100%), tetracycline and chloramphenicol (65% each) and high levels of streptomycin (46%), and mostly contained *gelE* and *agg* (>90%), *cyl* (82%) and *esp* (46%) [26]. [d]Tn*1546* designation is based on the results obtained by a PCR assay described by Woodford *et al.* consisting on the amplification of overlapped fragments covering the whole Tn*1546* [68]. Fragments of unexpected length were further analysed by sequencing (this study) [27]. [e]The total rep/rel/TA content of isolates is represented according to its location on plasmids of different size ranges. Rep (normal cells), rel (cells with dots) and TA (cells with diagonal stripes) genes belonging to the same plasmid are represented with the same color and that belonging to the same plasmid family with the same range of colors. The content of VanA plasmids including rep, rel, and TA genes is indicated according to the plasmid type in which they were identified, as well as by the numeric nomenclature used by Jensen *et al.* [72] for replicases (rep_1, rep_2, rep_9, rep_{14}, rep_{17}, rep_{18a}), given new and consistent designations to replicases non described in reference 72 (rep_{18b}, rep_{18c}, rep_{20}, rep_{22}). Relaxases were designated per numerical order as designed by M. V. Francia (unpublished data). Rolling-Circle plasmids are represented in green ($rep_{14/pRI1\text{-}like}$, $rel_{1/pRI1}$), small-theta replicating plasmids in violet ($rep_{18a/pEF418}$, $rep_{18b/pB82}$, $rep_{18c/pCIZ2}$, $rel_{2/pCIZ2}$), Inc18-like plasmids in different red tones ($rep_{1/pIP501}$, $rep_{2/pRE25/pEF1}$, $rel_{6/pEF1}$, $TA_{Inc18\text{-}\omega\text{-}\varepsilon\text{-}\zeta}$), RepA_N plasmids in different blue tones, pRUM in dark blue ($rep_{17/pRUM}$, $rel_{3/pRUM}$, $TA_{pRUM\text{-}Axe\text{-}Txe}$), pLG1 in turquoise ($rep_{20/pLG1}$), pheromone-responsive plasmids in light blue ($rep_{9/pAD1}$, $rel_{5/pAD1}$, $rel_{9/pCF10}$, par_{pAD1}), and pHTβ/pMG1 plasmids in grey ($rep_{22/pHT\beta}$, $rel_{8//pHT\beta}$). Rep families are named $Rep_{\tilde{n}}$ where "ñ indicates the number assigned to different rep-families according to Jensen *et al.* [72]. The name of the most representative plasmid of the family is also represented for a better follow-up of the results (e.g. $rep_{17/pRUM}$, rep17 from pRUM and related plasmids p5753cB and pS177; $rep_{1/pIP501}$ rep1 linked to Inc18 plasmids as pIP501, pIP816 and pRE25; $rep_{9/pAD1}$, rep9 linked to pCF10, pAD1, pTEF1, pTEF2, pBEE99, pMG2200; $rep_{14/pRI1\text{-}like}$, rep14 associated with RCR plasmids pEFNP1, pJS42 and/or pRI1; $rep_{18a/pEF418}$, rep18 from pEF418; and $rep_{22/pHT\beta}$, rep of both pHTβ and pMG1 plasmids). We further specified the name of different plasmids associated with a given group if necessary. For example, it results helpful for Inc18 family given the number of plasmids containing the same rep gene. These plasmids are increasingly identified among isolates of different origins (e.g. $rep_{2/pRE25/pEF1}$ for designing rep2, as rep and rel modules of pEF1, a plasmid originally identified in olives [35], seems to be widely present in all Efm clinical isolates). Sequencing identified the different variants within these families (see text). Rep_{18b}, rep_{18c} and rep_{20} were not included in Jensen's scheme [72] and the numbers were assigned in this paper following that numeration ($rep_{18b/pB82}$, rep from pB82; $rep_{18c/pCIZ2}$, rep from pCIZ2; $rep_{20/pLG1}$, rep from pLG1). Rel genes were arbitrarily

designated with numbers corresponding to different plasmid types [9] (Francia *et al*, unpublished data): Rel_1, pJS42, pRI1; Rel_2, rel from p200B, pCIZ2 and/or pB82 plasmids; Rel_3, pRUM; Rel_5, rel from pAD1, pTEF1, pAM373 and the pathogenicity island of V583; Rel_6, pEF1; Rel_8, pHTβ and pMG1; Rel_9, pCF10. Toxin-antitoxin systems included Axe-Txe from pRUM, ω-ε-ζ from Inc18 plasmids and *par* from pAD1. Genes hybridizing in the same band as *vanA* plasmids appear in bold rectangles.
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variants (PP-13, PP-17, PP-20, PP-23, PP-31 and X) which differed in the number of IS*1216* copies, the presence of insertions identified as short regions of Inc18-like plasmids or duplicated Tn*1546* sequence fragments in different orientations, were identified among related plasmids showing the RFLP_19 pattern (Table 1, Figure 4). These results illustrate the possibility of efficient intraclonal and intraplasmid diversification of Tn*1546*::IS*1216* variants. Acquisition of a *vanA*-Inc18 ($rep_{2/pRE25/pEF1}$ + $rep_{1/pIP501}$+ ω-ε-ζ) plasmid carrying a Tn*1546*::IS*1216* "variant", predominant among poultry from Europe [41] by Portuguese strains containing VanA-pRUM ($rep_{17.2/pRUM\text{-}like}$+$rel_{6/pEF1}$) plasmids cannot be excluded. Recombination between pRUM::Tn*1546* and Inc18::Tn*1546* would explain duplicated Tn*1546* regions.

iii) *Megaplasmids*. Tn*1546* type "D" was located on a megaplasmid carrying $rep_{20/pLG1}$ and $rel_{6/pEF1}$ from isolates of a CC5 Efm clone spreading among swine and humans of different continents. This transposon has been previously associated with isolates from swine which frequently exhibit the G8234T mutation. The variable size (150–190 kb) of *vanA* megaplasmids linked to CC5 lineage has been previously reported [10].

E. faecalis vanA plasmids

The *vanA* Efs plasmids were Inc18-pheromone-responsive mosaics, further classified in four main types on the basis of their RFLP patterns (RFLP_27–30), rep-rel/TA content, and replicase sequences. These plasmids have been documented in different Portuguese hospitals since the mid 90 s [26].

Plasmids showing highly related patterns designated as RFLP_27 (carrying IS*Ef1*-Tn*1546)* or RFLP_28 (carrying IS*1216*-Tn*1546*) were recovered from both Efs (ST6, ST55, ST159) and Efm (ST80, ST132). However, despite the similarity of their RFLP patterns, they differed in the rep/rel/TA content and transposon variant content (Table 1). Conversely, the finding of an ST117 Efs isolate from Oporto with two different *vanA* plasmids of 150 kb and 300 kb indicates acquisition and further recombination of widespread pRUM-*vanA* plasmids from Efm with narrow host pheromone responsive plasmids of Efs.

The observed differences in transposon variants and plasmid modules reflect frequent rearrangements during transfer of plasmids between Efs and/or Efm clonal backgrounds and also highlight the connectivity of these enterococcal populations resulting in the acquisition and generation of plasmids with enhanced host range.

Fixation of *vanA*-Tn*1546* variants is associated with plasmid connectivity

Tn*1546* backbones were classified in three main groups corresponding to Tn*1546* with no insertion sequences ("type A" and "type D") and variants containing IS*Ef1* (5 types) or IS*1216* (11 types) at different locations of the Tn*1546* backbone (Figure 4). Variants with a single copy of IS*Ef1* within the *vanX-vanY* region at nt 9044 were located on early (1996–1997) Efm plasmids identified as Inc18 and pRUM lacking Axe-Txe, and also on early (1996) Efs Inc18-pAD1 mosaics. Some of them were isolated from strains for more than one decade, which can be explained by their successful long-term recovered clonal and plasmid backgrounds.

Variants containing IS*1216* were mostly located on Inc18 plasmids or on mosaic plasmids Inc18-pRUM or Inc18-pAD1. Most variants contained the IS*1216* at 8839nt of the transposon (PP13, PP17, PP20, PP23, PP30) similarly to other Tn*1546* variants previously described in Europe [42]. Some of them also harboured different insertions corresponding to unknown sequences (X, PP23) or RCR plasmid sequences (PP10) [43] suggesting frequent recombination between acquired genes/plasmids and housekeeping Efm and Efs plasmids (Figure 4). Tn*1546* type D was specifically linked to megaplasmids from CC5 Efm from swine of different continents (Figure 2, Figure 4).

The presence of early plasmids carrying Tn*1546* belonging to different families suggests independent acquisitions of the transposon by pRUM and Inc18 plasmids, which would have been acquired by diverse Efm and Efs populations. Local fixation would be influenced by connectivity of plasmid and population backgrounds enabling further evolvability of transposon variants.

Discussion

This paper shows the local dynamics of Tn*1546*-vanA among *Enterococci* is shaped by horizontal genetic transfer of pRUM and Inc18 plasmids and by recombination-driven evolution of them within and between Efs and Efm clones. The clonal diversity reported in this study has also been observed in areas where the spread of VRE has been documented [44]. Recent retrospective analysis of enterococcal populations suggests that the temporal evolution of the population biology of *Enterococci* is driven by a succession of epidemic waves of enterococcal human specific lineages, Efm ST78 and Efs ST6 emerging in the last decade at global scale similarly to that reported for other pathogens [19,23,24]. In Portugal, the population structure of VRE analysed in this study comprises isolates of main human Efm lineages, ST18 (ST18, ST132) being much more abundant than ST17 (represented by a single isolate of early ST16 lineage) [31], or ST78 (represented by sporadic ST80 and ST656, the first one linked to early VRE outbreaks) [25,29]. It is worthwhile highlighting the recent detection of isolates of another Efm lineage in hospitals of the Oporto area (http://www.mlst.net) as ST117 Efm (ST78 lineage), which would reflect the increasing trend of isolates belonging to the ST78 lineage at international level. However, regional differences in the rates of VRE cannot be fully explained by clonal replacement dynamics since similar enterococcal clones appear widely distributed in areas with high and low rates of VRE (Tedim AP *et al.*, unpublished data). Instead, local conditions, including type and density of hosts, antibiotic usage, and transmission facilities, may influence regional differences in the proportions of VRE, as suggested by mathematical modelling studies on local trends of antibiotic resistance [45,46]. Clones can locally evolve by variation, drift and short-distance migration, leading to changes in colonization ability, pathogenicity or even host range, the fittest clonal variants being able to facilitate the spread of antibiotic resistance [23,47–50]. The observed clonal heterogeneity of the predominant ST18 lineage which comprises particular ST18 and ST132 strains widespread in different cities, highlights the role of certain efficiently transmissible clones in the

Table 1. Plasmids identified in this study.

RFLP type	VanA modular profile	Size	No. isolates	Tn*1546*	PFGE type	City	Year
RFLP_1	$Rep_{17.2}$::Rel_6	60	1	A	ST190_H98	Coimbra	1998
RFLP_2	$Rep_{17.2}$::Rel_6	80	1	A	ST18_H92	Coimbra	2000
RFLP_8 [c]	$Rep_{17.2}$::Rel_6	120	1	PP2b	ST18_H70	Coimbra	2001
RFLP_9 [c]	$Rep_{17.2}$::Rel_6	60	1	PP2b	ST18_H70	Viseu	NI
RFLP_10 [c]	$Rep_{17.2}$::Rel_6	60	3	PP2b	ST18_H70, H93	Coimbra, Viseu	2002
RFLP_11	$Rep_{17.2}$::Rel_6	60	1	PP27	ST18_H87	Coimbra	2002
RFLP_3 [b]	$Rep_{17.2}$::Rel_6	95	3	PP4	ST18_H108	Coimbra	1998–2000-NI
RFLP_4 [b]	$Rep_{17.2}$::Rel_6	85	2	PP4	ST670_H90; ST18_H81	Coimbra	1997–2001
RFLP_7	$Rep_{17.2}$::Rel_6	50	1	PP5	NI	Viseu	2008
RFLP_6 [b]	$Rep_{17.2}$::Rel_6	80	1	PP5	ST18_H70	Coimbra	NI
RFLP_5 [b]	$Rep_{17.2}$::Rel_6	90	12	PP3, PP4, PP5, PP24	ST18_H78, H72, H94, H123, H129	Coimbra, Porto, Matosinhos	2001–2007
RFLP_5.2[b]	$Rep_{17.2}$::Rel_6	90	2	PP5	ST280_ H100	Porto, Viseu	2002–2003
RFLP_5.3[b]	$Rep_{17.2}$::Rel_6	90	1	PP5	ST125_H126	Matosinhos	2007
RFLP_5.3′[b]	$Rep_{17.2}$::Rep_2:: Rel_6	85	2	PP5	ST132_H103	Coimbra	2002–2003
RFLP_5.2′[b]	$Rep_{17.2}$::Rep_2:: Rel_6	90	1	PP4	ST132_H132	Coimbra	2001
RFLP_5.4′[b]	$Rep_{17.2}$::Rep_2:: Rel_6	90	1	PP5	ST280_ H71	Viseu	2003
RFLP_5.5[b]	$Rep_{17.2}$::Rep_2:: Rel_6	75	1	PP5	ST18_H125	Matosinhos	2007
RFLP_6.4	$Rep_{17.2}$::Rep_2:: Rel_6	85	1	PP5	ST366_H99	Coimbra	2000
RFLP_20	$Rep_{17.2}$::Rep_2:: Rel_6	30	1	A	ST656_H130	Vila Real	2008
RFLP_12 [a]	$Rep_{17.2}$::Rep_2:: Rel_6	40	1	PP10	ST18_H83	Coimbra	1996
RFLP_13 [b]	$Rep_{17.2}$:: Rel_6::TA_{Inc18}[a]	60	1	PP5	ST280_ H71	Oporto	2002
RFLP_18	$Rep_{17.2}$:: Rep_1:: Rel_6:: TA_{Inc18}	50	1	PP13	ST132_H86	Oporto	2001
RFLP_16	$Rep_{17.2}$:: Rep_1:: Rep_2:: Rel_6::TA_{Inc18}	50	1	PP13	ST18_H78	Oporto	2001
RFLP_17	$Rep_{17.2}$:: Rep_1:: Rep_2:: Rel_6::TA_{Inc18}	110	1	PP13	ST132_H88	Oporto	2001
RFLP_19	$Rep_{17.2}$:: Rep_1:: Rep_2:: Rel_6::TA_{Inc18}	60	3	PP13, PP20, PP23	ST132_H119	Oporto	2002
RFLP_19	$Rep_{17.2}$:: Rep_1:: Rep_2:: Rel_6::TA_{Inc18}	60	2	PP17, PP20	ST368_SWH	Oporto	2001
RFLP_19.1	$Rep_{17.2}$:: Rep_1:: Rep_2:: Rel_6::TA_{Inc18}	60	1	X	ST369_RP	Oporto	2003
RFLP_21	$Rep_{17.2}$:: Rep_2:: Rel_6:: TA_{pRUM}	65	1	PP15	ST719_H96	Oporto	2001
RFLP_22	$Rep_{17.2}$:: Rep_2:: Rel_6:: TA_{pRUM}	30	1	PP16	ST132_SWC	Oporto	2002
RFPL_27[d]	Rep_9: Rep_2: Rep_1:: Rel_5::TA_{pAD1}	75–85	4	PP2a, PP4	ST6_HB, ST55_HG, ST159_HN	Coimbra	1996–2002
RFPL_27.3[d]	Rep_9: Rep_2: Rep_1:: Rel_5::TA_{pAD1}	85	2	PP4	ST6_HB, ST159_HN	Viseu	2001–2002
RFPL_27.1[d]	Rep_9: Rep_1:: Rel_5:: TA_{pAD1}	75	1	PP4	ST6_HB	Oporto	2001
RFPL_27[d]	Rep_9: Rep_2: Rep_1:: Rel_5	85	1	PP9	ST132_H106	Coimbra	2000
RFPL_28.5	Rep_9: Rep_2	100	1	PP16	ST80_SWM	Oporto	2002
RFPL_28	Rep_9: Rep_2: Rel_5:: TA_{pAD1}	100	2	PP15, PP16	ST6_HB	Oporto	2001–2002
RFLP_29	Rep_9: Rep_2: Rel_5	100	1	A	ST6_HB	Swine	2007
RFLP_30	Rep_9: Rel_9	100	1	A	ST30_HK	Oporto	2001
RFLP_24	Rep_2	110	1	PP4	ST80_H80	Coimbra	1997
RFLP_14	Rep_2	40	1	PP16	ST132_SWC	Oporto	2002
RFLP_15	Rep_1:: Rep_2	30	2	PP16	ST18_SWA, ST132_SWC	Oporto	2001
RFLP_23	Rel_6	85	1	PP9	ST16_H74	Coimbra	2000
RFLP_26	Rep_{20}:: Rel_6	150	1	D	ST5_SA	Oporto	2002
RFLP_25	–	120	1	PP5	ST391_H124	Covilhã	2007

Abbreviations: RFLP, restriction fragment length polymorphism; ST, sequence type; NI, not identified.

[a]Plasmid type RFLP_12 ($Rep_{17.2/pRUM\text{-}like}$ + $Rep_{2/pRE25/pEF1}$ + $Rel_{6/pEF1}$) contains a partial sequence of the replication gene of the RCR plasmid pEFNP1 (GenBank accession number AB038522), suggesting the integration of this RCR plasmid on the mobile element carrying Tn*1546* involving truncation of the $rep_{14/pRI1/pEFNP1}$.

[b]Plasmid types RFLP_3, _4, _5, _6 and _13 ($Rep_{17.2/pRUM\text{-}like}$ + $Rel_{6/pEF1}$ and eventually containing $Rep_{1/pIP501}$, $Rep_{2/pRE25/pEF1}$ or TAI_{nc18}) shared common bands and were identified in the same or different clonal backgrounds in different cities for extended periods of time.

[c]Plasmids types RFLP 8, _9 and _10 also shared a variable number of common bands.

[d]Plasmids showing patterns related to RFLP_27 (75–85 kb; $rep_{9/pAD1}$ + $rel_{5/pAD1}$ + $rep_{1/pIP501}$ + par_{pAD1} and/or $rep_{2/pRE25/pEF1}$) initially recovered from the widespread ST6-CC2 Efs clone in Coimbra in 1996 and other Efs (ST55 and ST159) and Efm clones contained similar IS*Ef1*-Tn*1546* variants (PP-2a, PP-4, PP-9). Other highly related mosaic Inc18-pAD1-related plasmids carrying IS*1216*-Tn*1546* were recovered from ST6 VREfs and ST80 VREfm isolates (type″II_{Efs}″, $rep_{9/pAD1}$ +$rel_{5/pAD1}$ + par_{pAD1} + $rep_{2/pRE25/pEF1}$ *versus* type″II_{Efm}″, $rep_{9/pAD1}$ + $rep_{2/pRE25/pEF1}$).

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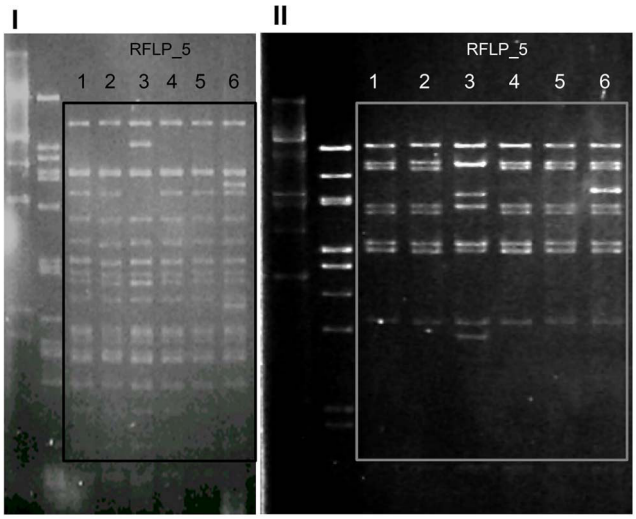

Figure 3. Restriction fragment length polymorphism patterns of plasmids showing RFLP_5 profiles after digestion with *Cla*I (I) and *Eco*RI (II) restriction enzymes (New England Biolabs Inc, UK). Lane 1, RFLP_5 (PFGE H78, ST18 Efm,); lane 2, RFLP_5 (PFGE H72, ST18 Efm); lane 3, RFLP 5.2 (PFGE H100, ST280 Efm), lane 4, RFLP 5 (PFGE H78, ST18 Efm); lane 5, RFLP_5 (PFGE H78, ST18 Efm); lane 6, RFLP_5.2′ (PFGE H132, ST132 Efm).
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dissemination of antibiotic resistance. Succesful clones can eventually be able to disseminate at international level as strains of ST6 Efs or ST280 Efm within main Efm human lineages driving or contributing the spread of different traits as Tn*1546* or Tn*1549* [51]. One remarkable fact is the similarity among PFGE patterns of isolates with different STs. Given the high content of plasmids and transposons of the isolates studied, and the frequent rearrangements identified among Efm and/or Efs isolates [21], chromosomal transfer can not be discarded. Recent phylogenomic analysis based on the degree of admixture among a diversity of isolates studied suggests that recombination is restricted to isolates within specific BAPS groups [19]. Most plasmids coding for vancomycin resistance are found in similar clonal backgrounds. This observation suggests that recombination does occur within isolates of similar BAPS groups as recently described [19]. However, the observed mosaicism and enhanced host range of particular plasmid variants indicates the existence of an unexpectedly high degree of connectivity between phylogenetically distant enterococcal populations and/or in bacterial genetic exchange communities integrating enterococci.

Broad host and narrow host plasmids carrying vancomycin resistance would have a high "*betweenness centrality*", which is a pivotal index in network theory useful for measuring the load placed on the given node in the network as well as the node's importance to the network than just connectivity [52]. A recent *in silico* network analysis of all plasmid sequences available at the GenBank databases confirms very high" *betweenness*" values for some Inc18 plasmids as pVEF3 (an Inc18 derivative highly spread among Efm from animals in Europe) [13,37], and also for a pheromone-responsive plasmid pTEF1 (a plasmid recovered from ST6_Efs strain V583, highly related to the ST6 described in this work) [53] (unpublished data). Other plasmids with a high degree of *modular dissociability*, would be pRUM-like elements, which may enhance their complexity resulting in new configurations with enhanced *betweenness*. It is tempting to suggest that plasmid variability has contributed to intra-clonal diversification both in Efm and Efs, giving rise to a local wealth of clonal variants able to fully explore the local adaptive landscape. In fact, this and other studies demonstrate that selected variants of Inc18, pAD1, and pRUM plasmids can determine differences in the dynamics of VRE in different areas, further influencing the plasmid host range and the selection of specific clones within human adapted lineages. Examples of widespread plasmid variants of Inc18 or pRUM plasmids coding for vancomycin resistance have been reported recently. They included Inc18 widespread among Efm poultry isolates from Europe [13] or among Efs clinical isolates from the USA, the last one being able to transfer Tn*1546* to *S. aureus* [15]; and mosaics of pRUM variants containing Axe-Txe and Inc18 from humans in different continents (Freitas AR *et al.* unpublished data). The identification of chimeric pRUM-Inc18 plasmids containing rep/rel/TA of Inc18 sequences and Tn*1546* variants widely observed in poultry, hospitals and hospital sewage in the Oporto area reflects genetic exchanges between enterococci from different origins and highlights the need to enforce barriers to avoid the spread of multidrug resistance human pathogens to the environment and viceversa.

In this scenario, the genetic context of Tn*1546* seems to greatly influence the evolvability of the transposon and explains the high diversity of variants found in this and other studies [1,27,42,54]. The frequent presence of insertions in the backbone of Tn*1546* and the abundance of IS*1216* and IS*Ef1* in enterococcal genomes [9,55] makes homoplasic evolution of Tn*1546* in different backgrounds possible. However, other IS (IS*1251*, IS*1542*, IS*1476*, IS*19* and IS*1485*) linked to different plasmid and clonal backgrounds [9,40] have been identified at different sites of Tn*1546*, thus suggesting that chance and selection are responsible to differences in variants collected in different areas. The widespread of Inc18 plasmids with a common origin in Europe [13,56] indicates local fixation of Tn*1546* influenced by a founder effect and further connectivity of plasmid and population backgrounds enabling further evolvability of transposon variants as reported in this study.

Our results suggest that VRE spread is facilitated by selected clones of different lineages through strong interactive processes of clonalization and plasmid diversification that might occur at local scales. Despite the maintenance of significant gene flow, a sympatric, or more probably, parapatric bacterial clonalization process (when diverging populations share a common or neighbouring environment), might contribute to the formation of temporary genetic mosaics and the preservation of ecologically important genomic traits [57]. Such micro-evolutionary process will result in an array of clonal complexes forming a population structure able to exploit the local spatio-temporal patch heterogeneities [58]. Note that exploitation of connected microenvironments should accelerate evolution of antibiotic resistance [59]. The expected result of such a successful population structure is the local persistence of antibiotic resistant clones, and eventually the local fixation [60] of vancomycin-resistance [46].

In summary, this study highlights the relevance of studying the local microecology of genes, elements, lineages and populations to decipher the robustness of the trans-hierarchical networks connecting these evolutionary elements in order to describe and predict the local evolvability of vancomycin-resistance [61]. Traditional surveillance studies are *one-off cross* sectional surveys focused on single traits as epidemic strains, genes or mobile genetic elements over limited periods of time which only gives *one shot* view that precludes addressing the long-term dynamics of antibiotic resistance. The more comprehensive approach described in this study is needed for understanding in depth the evolution of complexity in multihierarchical systems as those involved in the

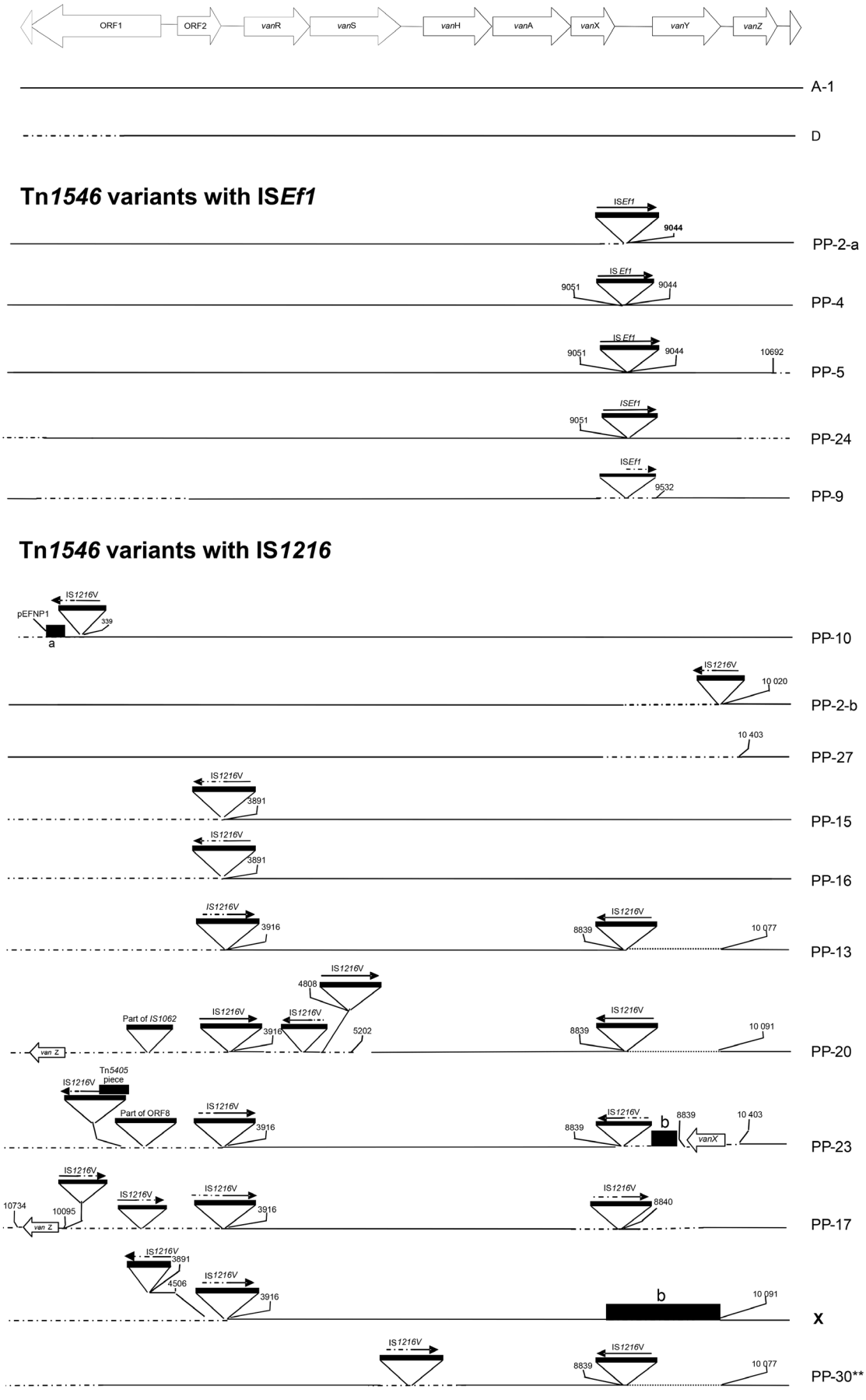
ORF1
ORF2
vanR
vanS
vanH
vanA
vanX
vanY
vanZ
A-1
D
Tn1546 variants with ISEf1
ISEf1
9044
PP-2-a
9051
PP-4
10692
PP-5
PP-24
9532
PP-9
Tn1546 variants with IS1216
IS1216V
pEFNP1
339
a
PP-10
10 020
PP-2-b
10 403
PP-27
3891
PP-15
PP-16
3916
8839
10 077
PP-13
4808
Part of IS1062
5202
van Z
10 091
PP-20
Tn5405 piece
Part of ORF8
b
vanX
PP-23
10734
10095
8840
PP-17
4506
x
PP-30**

Figure 4. Genetic maps of Tn*1546* variants. Tn*1546* variants are represented as previously described by Novais et *al.* [27] although grouped differently and specific types have been further explored (PP10, PP30): Tn*1546* prototype A corresponds to the original sequence described by Arthur *et al.* [77] and D corresponds to Tn*1546* variants from animals. Tn*1546* variants with IS*Ef1* within *vanX-vanY* intergenic region (PP2a, PP4, PP5, PP9, PP24) and Tn*1546* variants with IS*1216* insertions at different positions (PP10, PP2b, PP13, PP15, PP16, PP17, PP20, PP23, PP27, PP30, X) are represented. The positions of genes and open reading frames and the direction of transcription are depicted with open arrows. IS elements are represented by triangles; other sequences are designated by rectangles. DNA insertions are represented highlighting the first nucleotide upstream and downstream from the insertion sites whenever known. Deletions are indicated by dots and discontinuous lines indicate sequences that were not characterized. ([a]) DNA sequence with homology to ORF3 (unknown protein product) and ORF1 (replication protein) of pEFNP1 plasmid (GenBank accession number AB038522). ([b]) DNA sequence with no match to any sequence available in GenBank. (*) PP23 was identified in an isolate susceptible to teicoplanin; this variant contained an insertion in the *vanY* gene that would affect the transcription of *vanZ* and it might explain the susceptibility to this glycopeptide as previously reported [27]. (**) PP30 was identified in an ST78 isolate susceptible to both glycopeptides (MIC against vancomycin and teicoplanin of 4 mg/L) carrying *vanA*-Tn*1546*. This variant contained alterations within the *vanS-vanH* intergenic region (an IS*1216* insertion), which is involved in the expression and regulation of the resistance to vancomycin, and it constitutes the first description of a *vanA* isolate phenotypically susceptible to vancomycin in Portugal.
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spread of antibiotic resistance among the populations of bacterial human pathogens.

Materials and Methods

Bacterial strains and epidemiological background

One hundred four VRE clinical isolates carrying Tn*1546* from different regions of Portugal, 75 VREfm and 29 VREfs, were analyzed in this study. They included: i) clinical isolates from hospitals of Coimbra (Hospital Universitário de Coimbra, HUC), Oporto (Hospital Santo António, HSA), Viseu (Hospital de São Teotónio, HST); Matosinhos (Hospital Pedro Hispano, HPH), Vila Real (Hospital S. Pedro, HVR) and Covilhã (Centro Hospitalar da Cova da Beira, CHCB) located in Northern and Central Portugal (62 Efm and 26 Efs; 1996–2008); ii) isolates from waste waters of hospitals (HSA and Hospital de São João, HSJ) (10 Efm and 3 Efs), and iii) isolates from the estuary of the River Douro (3 Efm) recovered in the Oporto area during 2001–2003. Part of the isolates analyzed in this work corresponds to strains from previous surveillance studies [25–27,62]; this paper constitutes the first description of isolates obtained during 2007 and 2008. Contemporary Portuguese VRE isolates of animal origin were used for comparative analysis of lateral transfer events [10].

Susceptibility against 15 antibiotics was determined by the agar dilution method following CLSI standard guidelines. Clonal relatedness was established by pulsed-field gel electrophoresis (PFGE), banding patterns were interpreted according to criteria previously suggested for long-term studies, and multilocus sequence typing (MLST) as described elsewhere (http://efaecium.mlst.net) [25,63–65].

The presence of putative virulence traits [collagen-binding adhesin (*acm*), enterococcal surface protein (*esp*), hyaluronidase ($hyl_{E.\ faecium}$), cytolysin/hemolysin (*cyl*), gelatinase (*gelE*) and aggregation substance (*agg*)] was searched by using PCR as described [66,67].

Genetic context of Tn*1546*

Characterization of Tn*1546* backbone was determined by amplification of overlapping transposon fragments and further sequencing of PCR products [27,68]. We have accomplished the analysis for the isolates not studied in previous surveys and have interpreted the resulting transposon diversity (this study) [27], under the light of the plasmid and clonal backgrounds identified in this geographical area.

Plasmid analysis

Isolates (n = 62 Efm and n = 13 Efs) representing the clonal diversity observed in both species were selected for plasmid characterization (Table 1, Figure 2). The content and size of plasmids from transconjugants obtained by filter mating were determined by using either the technique described by Barton *et al.* (plasmids >10 kb) or the alkaline lysis extraction method of Kado & Liu (plasmids <10 kb) [51,69,70]. Classification of *E. faecium* plasmids was based on the presence of specific modules for replication (rep-initiator proteins), mobilization (relaxases) and stability (toxin-antitoxin systems). *Relaxases* (rel) were sought by a multiplex-PCR-based relaxase typing method which differentiates relaxases of the MOBQ, MOBP, MOBC and MOBV families related to 27 known plasmids [9,71] (Francia MV, unpublished data). *Replication initiator proteins* (rep) were investigated by amplification of 24 replicons, which allows discriminating among DNA sequences from more than 100 published Gram-positive plasmids [9,72]. Designation of rep sequences pointed out the plasmid type in which they were initially identified, as well as the numeric nomenclature originally used by Jensen *et al.* (Figure 2s footnote) [72]. *Toxin-antitoxin systems* (TA) previously identified among streptococci and enterococci (Axe-Txe, ω-ε-ζ *par*, *maz*EF) or Gram-negative bacteria (*rel*BE) were detected by PCR [73]. PCR products were sequenced in order to confirm the specificity of the method and to analyze similarities with other well-characterized plasmids. Genomic location of the Tn*1546* and the rel/rep/TA sequences was determined by hybridization of *vanA* and *rel/rep/TA* specific probes obtained by PCR from DNA from reference plasmids with S1 or I-*Ceu*I digested genomic DNA from representative strains [51,69]. Structural relationship between plasmids of similar size was established by comparison of their RFLP patterns obtained after digestion with different restriction enzymes (*Eco*RI, *Hind*III and *Cla*I; see Figure 3). Plasmid DNA was obtained by using a modified protocol based on the alkaline lysis method described by Handwerger *et al.* [74] consisting of increasing two-fold the volume of lysozyme, SDS/NaOH and acetate potassium solutions, extending the incubation period in potassium acetate solution for at least three hours, precipitating the supernatant obtained after extraction with phenol-chloroform using ethanol-acetate potassium solution (2:0.1 vol/vol) at 25°C for at least 2 hours, and resuspending final DNA pellets in 30 µl of water for further enzyme digestion analysis.

Molecular techniques

Southern blot DNA transfer and hybridization were performed by standard procedures [75]. The *vanA* and rep/rel/TA/bac probes used in the hybridization assays were generated by PCR using well known positive controls as template DNA. Labelling and detection were carried out using Gene Images Alkphos Direct Labelling system kit, following the manufacturer's instructions (Amersham GB/GE Healthcare Life Sciences UK Limited). PFGE was performed as described previously [76] using the

following conditions: switch time of 5 s to 25 s for 6 h, followed by 30 s to 45 s for 18 h (S1 nuclease); 5 s to 30 s for 22 h, 14°C, and 6 V/cm^2 (I-*Ceu*I) and 1 s to 20 s for 26 h, 14°C, and 6 V/cm^2 (*Sma*I).

Plasmid sequences

Analysis of nucleotide and aminoacid sequences revealed two types of sequences amplified with primers used for identification of $rep_{17/pRUM}$. They were 100% (designated as $Rep_{17.1/pRUM}$) or 97% (96% identity at amino acid level; designated as $Rep_{17.2/pRUM\text{-}like}$) homologous to that of RepA_pRUM (GenBank accession number AF507977). Most $Rep_{1/pIP501}$ aminoacid sequences were 98%–100% identical to RepE_pIP816, a member of the Inc18 family (GenBank accession number AM932524), and to a lesser degree to pRE25, pTEF1 or pSM19035; and $Rep_{2/pRE25/pEF1}$ showed 96%–100% amino acid identity to that of pEF1 (GenBank acc. no. DQ198088). Sequences identified as $Rel_{6/pEF1}$ showed 98%–100% homology to orf34_pEF1. Relaxases of the *E. faecalis* pheromone-responsive plasmids identified in this study displayed a high homology with those of known enterococcal pheromone plasmids pAD1, pAM373 and pTEF1 (orf57_pAD1, GenBank acc. no. AAL59457; EFA0025_pTEF1, GenBank AE016833; and EP0019_pAM373, GenBank acc. no. NC_002630). That of plasmid showing RFLP_27 showed a 67–84% homology with the above mentioned pheromone enterococcal plasmids but 94% identity with a MobC relaxase (annotated as a hypothetical protein) from a vancomycin-resistant *S. aureus* strain (GenBank acc. no. EIK35827).

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Author Contributions

Performed most experimental work: ARF. Partially contributed to clonal and transposon characterization: CN APT. Conceived and designed the experiments: ARF LP TMC. Analyzed the data: ARF CN APT MVF FB LP TMC. Contributed reagents/materials/analysis tools: ARF MVF LP TMC. Wrote the paper: ARF FB TMC.

References

1. Bonten MJM, Willems RJ, Weinstein RA (2001) Vancomycin resistant enterococci: why are they here, and where do they come from? Lancet Infect Dis 1: 314-325.
2. Murray BE (2000) Vancomycin-resistant enterococcal infections. N Engl J Med 342: 710-721.
3. Werner G, Coque TM, Hammerum AM, Hope R, Hryniewicz W, et al. (2008) Emergence and spread of vancomycin resistance among enterococci in Europe. Euro Surveill 13:pii = 19046.
4. De Lencastre H, Brown AE, Chung M, Armstrong D, Tomasz A (1999) Role of the transposon Tn*5482* in the epidemiology of vancomycin-resistant *Enterococcus faecium* in the pediatric oncology unit of a New York City hospital. Microb. Drug Resist 5: 113-129.
5. Martone WJ (1998) Spread of vancomycin resistant enterococci: why did it happen in the United States? Infect Control Hosp Epidemiol 19: 539-545.
6. Thal L, Donabedian S, Robinson-Dunn B, Chow JW, Dembry L, et al. (1998) Molecular analysis of glycopeptide-resistant *Enterococcus faecium* isolates collected from Michigan hospitals over a 6-year period. J Clin Microbiol 36: 3303-3308.
7. Kawalec M, Gniadkowski M, Hryniewicz W (2000) Outbreak of vancomycin-resistant enterococci in a hospital in Gdansk, Poland, due to horizontal transfer of different Tn*1546*-like transposons variants and clonal spread of several strains. J Clin Microbiol 38: 3317-3322.
8. Stampone L, Del Grosso M, Boccia D, Pantosti A (2005) Clonal spread of a vancomycin-resistant *Enterococcus faecium* strain among bloodstream-infecting isolates in Italy. J Clin Microbiol 43: 1575-1580.
9. Clewell DB, Weaver KE, Dunny GM, Coque TM, Francia MV, et al. (2013) Extrachromosomal and Mobile Elements in Enterococci. Transmission, Maintenance, and Epidemiology. In: Clewell DB, Shankar N, Ike Y, and Gilmore M, editors. The Enterococci: Pathogenesis, Molecular Miology and Antibiotic Resistance. *In revision*.
10. Freitas AR, Coque TM, Novais C, Hammerum AM, Lester CH, et al. (2011) Human and swine hosts share vancomycin-resistant *Enterococcus faecium* CC17, CC5, and *Enterococcus faecalis* CC2 clonal clusters harboring Tn*1546* on indistinguishable plasmids. J Clin Microbiol 49: 925-931.
11. Laverde Gomez JA, Van Schaik W, Freitas AR, Coque TM, Weaver KE, et al. (2010) A multiresistance megaplasmid pLG1 bearing a $hyl_{E.\ faecium}$ genomic island in hospital *Enterococcus faecium* isolates. Int J Med Microbiol 301: 165-175.
12. Rosvoll TC, Pedersen T, Sletvold H, Johnsen PJ, Sollid JE, et al. (2010) PCR-based plasmid typing in *Enterococcus faecium* strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHTbeta-related replicons associated with glycopeptide resistance and stabilizing toxin-antitoxin systems. FEMS Immunol Med Microbiol 58: 254-268.
13. Sletvold H, Johnsen PJ, Wikmark OG, Simonsen GS, Sundsfjord A, et al. (2010) Tn*1546* is part of a larger plasmid-encoded genetic unit horizontally disseminated among clonal *Enterococcus faecium* lineages. J Antimicrob Chemother 65: 1894-1906.
14. Tomita H, Pierson C, Lim SK, Clewell DB, Ike Y (2002) Possible connection between a widely disseminated conjugative gentamicin resistance (pMG1-like) plasmid and the emergence of vancomycin-resistance in *Enterococcus faecium*. J Clin Microbiol 40: 3326-3333.
15. Zhu W, Murray PR, Huskins WC, Jernigan JA, McDonald LC, et al. (2010) Dissemination of an *Enterococcus* Inc18-Like *vanA* plasmid associated with vancomycin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 54: 4314-4320.
16. Valdezate S, Miranda C, Navarro A, Freitas AR, Cabrera JJ, et al. (2012) Tn*1546* plasmid in a haemo-oncology ward of a Spanish hospital. J Antimicrob Chemother 67: 832-836.
17. Garcia-Migura L, Sanchez-Valenzuela AJ, Jensen LB (2011) Presence of glycopeptides-encoding plasmids in enterococcal isolates from food and humans in Denmark. Foodborne Pathog Dis 8: 1191-1197.
18. Qu TT, Yang Q, Shen P, Wei ZQ, Yu YS (2012) Novel vancomycin-resistance transposon, plasmid replicon types, and virulence factors of vancomycin-resistant Enterococci in Zhejiang, China. Microb Drug Resist 18: 183-188.
19. Willems R, Top J, van Schaik W, Leavis H, Bonten M, et al. (2012) Restricted gene flow among hospital subpopulations of *Enterococcus faecium*. MBio 3(4):e00151-12.
20. McBride SM, Fischetti VA, Leblanc DJ, Moellering RC Jr, Gilmore MS (2007) Genetic diversity among *Enterococcus faecalis*. PLoS One 2(7):e582.
21. Manson JM, Hancock LE, Gilmore MS (2010) Mechanism of chromosomal transfer of *Enterococcus faecalis* pathogenicity island, capsule, antimicrobial resistance, and other traits. Proc Natl Acad Sci U S A 107(27):12269-74.
22. Leavis HL, Bonten MJ, Willems RJ (2006) Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. Curr Opin Microbiol 9: 454-460. Review.
23. Wyllie D, Paul J, Crook D (2011) Waves of trouble: MRSA strain dynamics and assessment of the impact of infection control. J Antimicrob Chemother 66: 2685-2688.
24. Chambers HF, Deleo FR (2009) Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol 7(9):629-41. Review.
25. Freitas AR, Novais C, Ruiz-Garbajosa P, Coque TM, Peixe L (2009) Dispersion of multidrug-resistant *Enterococcus faecium* isolates belonging to major clonal complexes in different Portuguese settings. Appl Environ Microbiol 75: 4904-4908.
26. Freitas AR, Novais C, Ruiz-Garbajosa P, Coque TM, Peixe L (2009) Clonal expansion within clonal complex 2 and spread of vancomycin-resistant plasmids among different genetic lineages of *Enterococcus faecalis* from Portugal. J Antimicrob Chemother 63: 1104-1111.
27. Novais C, Freitas AR, Sousa JC, Baquero F, Coque TM, et al. (2008) Diversity of Tn*1546* and its role in the dissemination of vancomycin-resistant enterococci in Portugal. Antimicrob Agents Chemother 52: 1001-1008.
28. Willems RJ, Hanage WP, Bessen DE, Feil EJ (2011) Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance. FEMS Microbiol Rev 35: 872-900.
29. Mato R, Almeida F, Pires R, Rodrigues P, Ferreira T, et al. (2009) Assessment of high-level gentamicin and glycopeptide-resistant *Enterococcus faecalis* and *E. faecium* clonal structure in a Portuguese hospital over a 3-year period. Eur J Clin Microbiol Infect Dis 28: 855-859.
30. Araújo C, Torres C, Gonçalves A, Carneiro C, López M, et al. (2011) Genetic detection and multilocus sequence typing of *vanA*-containing *Enterococcus* strains from mullets fish (*Liza ramada*). Microb Drug Resist 17: 357-361.
31. Galloway-Peña JR, Nallapareddy SR, Arias CA, Eliopoulos GM, Murray BE (2009) Analysis of clonality and antibiotic resistance among early clinical isolates of *Enterococcus faecium* in the United States. J Infect Dis 200: 1566-1573.

32. Willems RJ, van Schaik W (2009) Transition of *Enterococcus faecium* from commensal organism to nosocomial pathogen. Future Microbiol 4:1125-1135.
33. Weaver KE, Kwong SM, Firth N, Francia MV (2009) The RepA_N replicons of Gram-positive bacteria: a family of broadly distributed but narrow host range plasmids. Plasmid 61: 94-109.
34. Schwarz FV, Perreten V, Teuber M (2001) Sequence of the 50-kb conjugative multiresistance plasmid pRE25 from *Enterococcus faecalis*RE25. Plasmid 46: 170-187.
35. Ruiz-Barba JL, Floriano B, Maldonado-Barragán A, Jiménez-Díaz R (2007) Molecular analysis of the 21-kb bacteriocin-encoding plasmid pEF1 from *Enterococcus faecium* 6T1a. Plasmid. 57: 175-181.
36. Sletvold H, Johnsen PJ, Simonsen GS, Aasnaes B, Sundsfjord A, et al. (2007) Comparative DNA analysis of two *vanA* plasmids from *Enterococcus faecium* strains isolated from poultry and a poultry farmer in Norway. Antimicrob Agents Chemother 51: 736-739.
37. Sletvold H, Johnsen PJ, Hamre I, Simonsen GS, Sundsfjord A, et al. (2008) Complete sequence of *Enterococcus faecium* pVEF3 and the detection of an omega-epsilon-zeta toxin-antitoxin module and an ABC transporter. Plasmid 60: 75-85.
38. Grady R, Hayes F (2003) Axe-Txe, a broad-spectrum proteic toxin- antitoxin system specified by a multidrug-resistant, clinical isolate of *Enterococcus faecium*. Mol Microbiol 47: 1419-1432.
39. Halvorsen EM, Williams JJ, Bhimani AJ, Billings EA, Hergenrother PJ (2011) Txe, an endoribonuclease of the enterococcal Axe-Txe toxin-antitoxin system, cleaves mRNA and inhibits protein synthesis. Microbiology 157: 387-397.
40. Coque TM, Freitas AR, Novais C, Peixe L, Baquero F (2012) Mobile Genetic Elements and Lateral Genetic Transfer in Enterococci. *In*: *Enterococcus and Safety*. Series: Advances in Food Safety and Food Microbiology. Semedo-Lemsaddek T, Barreto-Crespo MT, Tenreiro R (*eds*). Nova Publishers. ISBN: 978-1-61470-569-7.
41. Sørum M, Johnsen PJ, Aasnes B, Rosvoll T, Kruse H, et al. (2006) Prevalence, persistence, and molecular characterization of glycopeptide-resistant enterococci in Norwegian poultry and poultry farmers 3 to 8 years after the ban on avoparcin. Appl Environ Microbiol 72: 516-521.
42. Schouten MA, Willems RJ, Kraak WA, Top J, Hoogkamp-Korstanje JA, et al. (2001) Molecular analysis of Tn*1546*-like elements in vancomycin-resistant enterococci isolated from patients in Europe shows geographic transposon type clustering. Antimicrob Agents Chemother. 45: 986-989.
43. Garcia-Migura L, Hasman H, Jensen LB (2009) Presence of pRI1: a small cryptic mobilizable plasmid isolated from *Enterococcus faecium* of human and animal origin. Curr Microbiol 58: 95-100.
44. Zheng B, Tomita H, Xiao YH, Wang S, Li Y, et al. (2007) Molecular characterization of vancomycin-resistant *Enterococcus faecium* isolates from mainland China. J Clin Microbiol 45: 2813-2818.
45. McCormick AW, Whitney CG, Farley MM, Lynfield R, Harrison LH, et al. (2003) Geographic diversity and temporal trends of antimicrobial resistance in *Streptococcus pneumoniae* in the United States. Nat Med 9: 424-430.
46. Johnsen PJ, Townsend JP, Bøhn T, Simonsen GS, Sundsfjord A, et al. (2009) Factors affecting the reversal of antimicrobial-drug resistance. Lancet Infect Dis. 9: 357-364.
47. Price LB, Stegger M, Hasman H, Aziz M, Larsen J, et al. (2012) *Staphylococcus aureus* CC398: Host Adaptation and Emergence of Methicillin Resistance in Livestock. MBio 3: pii = e00305-11.
48. Robinson DA, Kearns AM, Holmes A, Morrison D, Grundmann H, et al. (2005) Re-emergence of early pandemic *Staphylococcus aureus* as a community-acquired meticillin-resistant clone. Lancet 365: 1256-1258.
49. de Regt MJ, van Schaik W, van Luit-Asbroek M, Dekker HA, van Duijkeren E, et al. (2012) Hospital and community ampicillin-resistant *Enterococcus faecium* are evolutionarily closely linked but have diversified through niche adaptation. PLoS One 7: e30319.
50. Li M, Du X, Villaruz AE, Diep BA, Wang D, et al. (2012) MRSA epidemic linked to a quickly spreading colonization and virulence determinant. Nat Med 18: 816-819.
51. Freitas AR, Tedim AP, Novais C, Ruiz-Garbajosa P, Werner G, et al. (2010) Global spread of the $hyl_{(Efm)}$ colonization-virulence gene in megaplasmids of the *Enterococcus faecium* polyclonal subcluster. Antimicrob Agents Chemother 54: 2660-2665.
52. Freeman LC (1977) A set of measures of centrality based on betweenness. Sociometry 40: 35-41.
53. Tamminen M, Virta M, Fani R, Fondi M (2012) Large-scale analysis of plasmid relationships through gene-sharing networks. Mol Biol Evol 29: 1225-1240.
54. Willems RJ, Top T, van den Braak N, Van Belkum A, Mevius DJ, et al. (1999) Molecular diversity and evolutionary relationships of Tn*1546*-like elements in enterococci from humans and animals. Antimicrob Agents Chemother 43: 483-491.
55. Paulsen IT, Banerjei L, Myers GS, Nelson KE, Seshadri R, et al. (2003) Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science. 299: 2071-2074.
56. Garcia-Migura L, Hasman H, Svendsen C, Jensen LB (2008) Relevance of hot spots in the evolution and transmission of Tn*1546* in glycopeptide-resistant *Enterococcus faecium* (GREF) from broiler origin. J Antimicrob Chemother 62: 681-687.
57. Via S (2009) Natural selection in action during speciation. Proc Natl Acad Sci U S A 106: 9939-9946.
58. Levin SA, Paine RT (1974) Disturbance, patch formation, and community structure. Proc Natl Acad Sci U S A 71: 2744-2747.
59. Zhang Q, Lambert G, Liao D, Kim H, Robin K, et al. (2011) Acceleration of emergence of bacterial antibiotic resistance in connected environments. Science 333: 1764-1767.
60. Aguilée R, Claessen D, Lambert A (2009) Allele fixation in a dynamic metapopulation: founder effects vs refuge effects. Theor Pop Biol 76: 105-117.
61. Baquero F, Tedim AP, Coque TM (2013) Antibiotic resistance shaping multilevel population biology of bacteria. Frontiers in Antimicrobials, Resistance and Chemotherapy. *In press*. ISSN:664-302.
62. Novais C, Coque TM, Ferreira H, Sousa JC, Peixe L (2005) Environmental contamination with vancomycin-resistant enterococci from hospitalsewage in Portugal. Appl Environ Microbiol 71: 3364-3368.
63. Novais C, Sousa JC, Coque TM, Peixe LV (2005) Molecular characterization of glycopeptide-resistant *Enterococcus faecium* isolates from Portuguese hospitals. Antimicrob Agents Chemother 49: 3073-3079.
64. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, et al. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 33: 2233-2239.
65. Morrison D, Woodford N, Barrett SP, Sisson P, Cookson BD (1999) DNA banding pattern polymorphism in vancomycin-resistant *Enterococcus faecium* and criteria for defining strains. J Clin Microbiol 37: 1084-1091.
66. Nallapareddy SR, Weinstock GM, Murray BE (2003) Clinical isolates of *Enterococcus faecium* exhibit strain-specific collagen binding mediated by Acm, a new member of the MSCRAMM family. Mol Microbiol 47: 1733-1747.
67. Vankerckhoven V, van Autgaerden T, Vael C, Lammens C, Chapelle S, et al. (2004) Development of a multiplex PCR for the detection of *asa1*, *gelE*, *cylA*, *esp* and *hyl* genes in enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. J Clin Microbiol 42: 4473-4479.
68. Woodford N, Adebiyi AMA, Palepou MFI, Cookson BD (1998) Diversity of VanA glycopeptide resistance elements in enterococci from humans and nonhuman sources. Antimicrob Agents Chemother 42: 502-508.
69. Barton BM, Harding GP, Zuccarelli AJ (1995) A general method for detecting and sizing large plasmids. Anal Biochem 226: 235-240.
70. Kado CI, Liu ST (1981) Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 145: 1365-1373.
71. Garcillan-Barcia MP, Francia MV, de la Cruz F (2009) The diversity of conjugative relaxases and its application in plasmid classification. FEMS Microbiol Rev 33: 657-687.
72. Jensen LB, Garcia-Migura L, Valenzuela AJ, Løhr M, Hasman H, et al. (2010) A classification system for plasmids from enterococci and other Gram-positive bacteria. J Microbiol Methods 80: 25-43.
73. Moritz EM, Hergenrother PJ (2007) Toxin-antitoxin systems are ubiquitous and plasmid-encoded in vancomycin-resistant enterococci. Proc Natl Acad Sci U S A 104: 311-316.
74. Handwerger S, Skoble J (1995) Identification of chromosomal mobile element conferring high-level vancomycin resistance in *Enterococcus faecium*. Antimicrob Agents Chemother 39: 2446-2453.
75. Sambrook J, Frisch E, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
76. Kaufmann ME (1998) Pulsed-Field Gel Electrophoresis. In: Woodford N and Johnson AP, editors. Molecular Bacteriology: protocols and clinical applications. Humana Press: Inc Totowa NJ. pp. 17-31.
77. Arthur M, Molinas C, Depardieu F, Courvalin P (1993) Characterization of Tn*1546*, a Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J Bacteriol 175: 117-127.