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# Protein expression vector and secretion signal peptide optimization to drive the production, secretion, and functional expression of the bacteriocin enterocin A in lactic acid bacteria

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ABSTRACT

Replacement of the leader sequence (LS) of the bacteriocin enterocin A ($LS_{entA}$) by the signal peptides (SP) of the protein Usp45 ($SP_{usp45}$), and the bacteriocins enterocin P ($SP_{entP}$), and hiracin JM79 ($SP_{hirJM79}$) permits the production, secretion, and functional expression of EntA by different lactic acid bacteria (LAB). Chimeric genes encoding the $SP_{usp45}$, the $SP_{entP}$, and the $SP_{hirJM79}$ fused to mature EntA plus the EntA immunity genes (*entA* + *entiA*) were cloned into the expression vectors pNZ8048 and pMSP3545, under control of the inducible $P_{nisA}$ promoter, and in pMG36c, under control of the constitutive $P_{32}$ promoter. The amount, antimicrobial activity, and specific antimicrobial activity of the EntA produced by the recombinant *Lactococcus lactis*, *Enterococcus faecium*, *E. faecalis*, *Lactobacillus sakei* and *Pediococcus acidilactici* hosts varied depending on the signal peptide, the expression vector, and the host strain. However, the antimicrobial activity and the specific antimicrobial activity of the EntA produced by most of the LAB transformants was lower than expected from their production. The supernatants of the recombinant *L. lactis* NZ9000 (pNZUAI) and *L. lactis* NZ9000 (pNZHAI), overproducers of EntA, showed a 1.2- to 5.1-fold higher antimicrobial activity than that of the natural producer *E. faecium* T136 against different *Listeria* spp.

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## 1. Introduction

Ribosomally synthesized antimicrobial peptides are produced by many organisms, including mammals, birds, insects, plants, and microorganisms. In bacteria, such peptides are termed bacteriocins (Cotter et al., 2005), and those produced by lactic acid bacteria (LAB) attract considerable interest as natural and nontoxic food preservatives, for human and veterinary applications, and in the animal production field (Gálvez et al., 2008; Klostermann et al., 2008; Khan et al., 2010; Borrero et al., 2011a). The enterococci are among the dominant LAB in the intestinal flora of mammals and part of the food microbiota and produce a diverse and heterogeneous group of bacteriocins, called enterocins with different antimicrobial spectra, biochemical characteristics, and processing and secretion mechanisms (Franz et al., 2007; Nes et al., 2007). However, because enterocins may be produced by enterococcal species carrying antibiotic resistance genes and/or genes coding for potential virulence factors due to hygienic, safety, and biotechnological reasons, the production of enterocins in heterologous microbial hosts is being evaluated (Gutiérrez et al., 2006; Fernández et al., 2007; Sánchez et al., 2008; Borrero et al., 2011b).

Most bacteriocins produced by LAB are synthesized as biologically inactive precursors or prepeptides containing an N-terminal extension of the so-called double-glycine type (leader sequence) and are cleaved concomitantly with export across the cytoplasmic membrane by dedicated ATP-binding cassette transporters (ABC-transporters) and their accessory proteins (Håvarstein et al., 1995). However, many secreted prokaryotic proteins and a few bacteriocins, such as enterocin P (Cintas et al., 1997) and hiracin JM79 (Sánchez et al., 2007b), contain N-terminal extensions of the so-called Sec-type (signal peptide) which are proteolytically cleaved concomitantly with peptide externalization by the general secretory pathway (GSP) or Sec-dependent pathway. The Sec system is a universally conserved protein export system that translocates unfolded proteins across the cell membrane via a protein-conducting pore formed by the SecYEG complex and a molecular motor, the ATPase SecA. Secretory proteins are equipped with an N-terminal signal peptide (SP) that functions as a target and recognition signal for signal peptidases that remove the SP from the translocated protein, resulting in the extracellular release of the mature protein or peptide (Driessen and Nouwen, 2007; Natale

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et al., 2008). Accordingly, the N-terminal SP of secretory proteins could drive the access of the fused mature proteins or peptides to the GSP for their secretion by the heterologous producer cells.

Enterocin A (EntA) is a heat-stable, antilisterial bacteriocin with two disulfide bridges, and whose synthesis is directed by a gene cassette that encodes the *entAIFKRTD* operon. The genes *entA*, *entI*, and *entF* encode the enterocin A prepeptide synthesized as an 18-amino-acid leader sequence ($LS_{entA}$) and the 47-amino-acid mature bacteriocin, the immunity protein, and the induction factor prepeptide, respectively. The EntK and EntR proteins resemble the histidine kinase and response regulator proteins of the two-component signal transducing systems, and the EntT and EntD proteins are homologous to ABC-transporters and accessory factors involved in the processing and secretion of peptides across the bacterial membrane (Aymerich et al., 1996; Nilsen et al., 1998; O'Keefee et al., 1999). Furthermore, the potent antilisterial activity of EntA has driven interest for its biotechnological production by heterologous hosts (O'Keefee et al., 1999; Martínez et al., 2000; Klocke et al., 2005; Liu et al., 2008), and replacement of the leader sequence of EntA ($LS_{entA}$) by the signal peptide of enterocin P ($SP_{entP}$) permits the production and functional expression of EntA by *Lactococcus lactis* (Martín et al., 2007a).

In this work, of interest was to evaluate the replacement of the $LS_{entA}$ by different signal peptides and to determine their effect on the production, secretion, and functional expression of EntA using different protein expression vectors and LAB hosts. The *usp45* gene encodes the major extracellular protein Usp45 from *L. lactis* MG1363 and chimeras of the 27-amino-acid Usp45 signal peptide ($SP_{usp45}$), fused to mature proteins, have been used to drive their production by *L. lactis* (Mierau and Kleerebezem, 2005; Morello et al., 2008). The *entP* gene encodes the bacteriocin enterocin P (EntP), consisting of a 27-amino-acid SP ($SP_{entP}$) and the 44-amino-acid mature bacteriocin (Cintas et al., 1997), and chimeras of the $SP_{entP}$ fused to mature EntA or pediocin PA-1 (PedA-1), have driven their production by *L. lactis* (Martín et al., 2007a,b). The *hirJM79* gene encodes the bacteriocin hiracin JM79 (HirJM79), consisting of a 30-amino-acid SP ($SP_{hirJM79}$) and the 44-amino-acid mature bacteriocin (Sánchez et al., 2007b). We report in this work the use of chimeras of the $SP_{usp45}$, $SP_{entP}$, and $SP_{hirJM79}$ fused to the mature EntA structural plus immunity genes (*entA* + *entiA*), their cloning into the expression vectors pNZ8048, pMSP3545, and pMG36c and the use of derivatives of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *Enterococcus faecium*, *E. faecalis*, *Lactobacillus sakei* and *Pediococcus acidilactici* as the production hosts.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. faecium* T136 was used as the source of *entA* and *entiA*, whereas *L. lactis* MG1363, *E. faecium* P13, and *E. hirae* DCH5 were used as the source of the Usp45 signal peptide ($SP_{usp45}$), the EntP signal peptide ($SP_{entP}$), and the HirJM79 signal peptide ($SP_{hirJM79}$), respectively. The lactococcal strains were propagated at 32 °C in M17 broth (Oxoid Ltd., Basingstoke, UK) supplemented with 0.5% (w/v) glucose (GM17). All the enterococci, *Lb. sakei* Lb790, and *P. acidilactici* Ped$^-$ were grown in MRS broth (Oxoid) at 32 °C. *Escherichia coli* JM109 (Invitrogen S.A., Barcelona, Spain) was grown in LB broth (Sigma Chemical Co., St. Louis, MO, USA) at 37 °C with shaking. All *Listeria* strains, listed in Table 5, were cultured in BHI broth (Oxoid) at 32 °C. Agar plates were made by addition of 1.5% (w/v) agar (Oxoid) to the liquid media. When needed, chloramphenicol (Cm) (Sigma) was added to the cultures at 5 μg/ml, and erythromycin (Em) (Sigma) at 350 μg/ml and 5 μg/ml for *E. coli* JM109 and the LAB cultures, respectively. Cell dry weights of late exponential phase cultures expressed as cell dry mass were determined gravimetrically.

**Table 1**
Bacterial strains and plasmid used in this study.

| Strains or plasmids | Description[a] | Source and/or reference[b] |
|---|---|---|
| Strains | | |
| *E. faecium* | | |
| T136 | Enterocin A and B producer; control strain | DNBTA (Casaus et al., 1997) |
| P13 | Enterocin P producer; MPA and ADT indicator; EntA$^s$ | DNBTA (Cintas et al., 1997) |
| AR24 | Plasmid-free strain, non bacteriocin producer; EntA$^s$ | HRC |
| DCH37 | Plasmid free strain,, EntL50A, EntL50B, EntQ and EntP producer; EntA$^s$ | DNBTA (Sánchez et al., 2007) |
| *E. faecalis* JH2-2 | Plasmid-free strain, non bacteriocin producer; EntA$^s$ | Jacob and Hobbs (1974) |
| *E. hirae* DCH5 | Hiracin JM79 producer | DNBTA (Sánchez et al., 2007a) |
| *L. lactis* subsp. *cremoris* MG1363 | Plasmid-free strain, derivative of NCDO712; source of *usp45* | IFR (Gasson, 1983) |
| *L. lactis* subsp. *cremoris* NZ9000 | Plasmid-free strain, derivative of MG1363; pepN::*nisRK*, non bacteriocin producer; EntA$^r$ | NIZO (Kuipers et al., 1998) |
| *L. lactis* subsp. *lactis* IL1403 | Plasmid-free strain, derivative of IL594, non bacteriocin producer; EntA$^r$ | Chopin et al. (1984) |
| *Lb. sakei* Lb790 | Plasmid-free strain, non-bacteriocin producer; EntA$^s$ | Schillinger and Lucke (1989) |
| *P. acidilactici* Ped$^-$ | Plasmid-free strain, *P. acidilactici* 347 isogenic strain; non bacteriocin producer; EntA$^r$ | DNBTA (Martínez et al., 1998) |
| Plasmids | | |
| pNZ8048 | Cm$^r$; inducible expression vector carrying the *nisA* promoter | NIZO (Kuipers et al., 1998) |
| pMSP3545 | Em$^r$; inducible expression vector carrying the *nisA* promoter and the *nisR* and *nisK* genes | Bryan et al. (2000) |
| pMG36c | Cm$^r$, pMG36e derivative | RUG-MG (van de Guchte et al., 1989) |
| pNZUAI | Cm$^r$, pNZ8048 derivative carrying the PCR product NZUAI, containing the $SP_{usp45}$ | This work |
| pNZPAI | Cm$^r$, pNZ8048 derivative carrying the PCR product NZPAI, containing the $SP_{entP}$ | This work |
| pNZHAI | Cm$^r$, pNZ8048 derivative carrying the PCR product NZHAI, containing the $SP_{hirJM79}$ | This work |
| pMSUAI | Em$^r$, pMSP3545 derivative carrying the PCR product MSUAI, containing the $SP_{usp45}$ | This work |
| pMSPAI | Em$^r$, pMSP3545 derivative carrying the PCR product MSPAI, containing the $SP_{entP}$ | This work |
| pMSHAI | Em$^r$, pMSP3545 derivative carrying the PCR product MSHAI, containing the $SP_{hirJM79}$ | This work |
| pMGUAI | Cm$^r$, pMG36c derivative carrying the PCR product MGUAI, containing the $SP_{usp45}$ | This work |
| pMGPAI | Cm$^r$, pMG36c derivative carrying the PCR product MGPAI, containing the $SP_{entP}$ | This work |
| pMGHAI | Cm$^r$, pMG36c derivative carrying the PCR product MGHAI, containing the $SP_{hirJM79}$ | This work |

[a] EntA$^s$, enterocin A sensitive; EntA$^r$, enterocin A resistant; Cm$^r$, choramphenicol resistant; Em$^r$, erythromycin resistant.
[b] HRC, Departamento de Microbiología, Hospital Universitario Ramón y Cajal (Madrid, Spain); DNBTA, Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid (Madrid, Spain); IFR, Institute of Food Research (Norwich, United Kingdom); NIZO, Department of Biophysical Chemistry, NIZO Food Research (Ede, The Netherlands); RUG-MG, Department of Molecular Genetics, University of Groningen (Haren, The Netherlands).

### 2.2. Antimicrobial activity assays

The antimicrobial activity of individual colonies was examined by the stab-on-agar test (SOAT), as previously described (Cintas et al., 1997). Recombinant LAB cultures, transformed with either pNZ8048 or pMSP3545 derivatives, were induced for production of EntA at an optical density at 600 nm ($OD_{600}$) of 0.5, using nisin A (NisA) from the supernatant of *L. lactis* BB24 (NisA producer) at a final concentration of 10 ng/ml. The induced cultures were grown at 32 °C during 3 h. Cell-free culture supernatants were obtained by centrifugation of cultures at 12,000 × *g* at 4 °C for 10 min, adjusted to pH 6.2 with 1 M NaOH, filtered through 0.2 μm pore-size filters (Whatman Int. Ltd., Maidstone, UK), and stored at −20 °C until use. The antimicrobial activity of the supernatants was examined by an agar well diffusion test (ADT) and a microtiter plate assay (MPA), as previously described (Gutiérrez et al., 2006), using *E. faecium* P13 ($EntA^s$) as the indicator microorganism. With the MPA, growth inhibition of the sensitive culture was measured spectrophotometrically at 620 nm with a microtitre Labsystems iEMS plate reader (Labsystems, Helsinki, Finland). One bacteriocin unit (BU) was defined as the reciprocal of the highest dilution of the bacteriocin causing 50% growth inhibition (50% of the turbidity of the control culture without bacteriocin). The antimicrobial activity of some of the recombinant LAB was also tested against six *Listeria monocytogenes* and five *Listeria* spp., obtained from the CECT (Colección Española de Cultivos Tipo, Valencia, Spain), using the MPA.

### 2.3. Basic genetic techniques and enzymes

Total genomic DNA from *E. faecium* T136, *L. lactis* MG1363, *E. faecium* P13 and *E. hirae* DCH5 was isolated using the Wizard® DNA Purification Kit (Promega, Madison, WI, USA). Plasmid DNA isolation was carried out using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany), as suggested by the manufacturer, but with the cells suspended with lysozyme (40 mg/ml) and mutanolysin (500 U/ml) and incubated at 37 °C for 10 min before following the kit instructions. All DNA restriction enzymes were supplied by New England BioLabs (Beverly, MA, USA). Ligations were performed with the T4 DNA ligase (Roche Molecular Biochemicals, Mannheim, Germany). *E. coli* JM109 competent cells were transformed as described by the supplier. Electrocompetent LAB cells were transformed with a Gene Pulser™ and Pulse Controller apparatus (Bio-Rad Laboratories, Hercules, CA, USA) as described previously (Aukrust et al., 1995; Holo and Nes, 1989).

### 2.4. PCR amplification and nucleotide sequencing

Oligonucleotide primers were obtained from Sigma-Genosys Ltd., (Cambridge, UK). PCR-amplifications were performed in 50 μl reaction mixtures containing 1 μl of purified DNA, 70 pmol of each primer, and 1 U of Platinum® *Pfx* DNA Polymerase (Invitrogen S.A., Barcelona, Spain). Samples were subjected to an initial cycle of denaturation (97 °C for 2 min), followed by 35 cycles of denaturation (94 °C for 45 s), annealing (50–63 °C for 30 s), and elongation (68 °C for 40 s), ending with a final extension step at 68 °C for 7 min in a DNA thermal cycler Techgene (Techne, Cambridge, UK). The PCR-generated fragments were purified by a NucleoSpin® Extract II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) before cloning into the vector, and for nucleotide sequencing. Nucleotide sequencing of purified PCR products was done using the ABI PRISM® BigDye™ Terminator cycle sequence reaction kit and the automatic DNA sequencer ABI PRISM, model 377 (Applied Biosystems, Foster City, CA, USA) at the DNA Sequencing Service (Sistemas Genómicos, Valencia, Spain).

### 2.5. Recombinant plasmids derived from pNZ8048

The primers and inserts used for construction of the recombinant plasmids are listed in Table 2. Plasmid derivatives were constructed as follows: primers USPNC-F/USPEA-R were used for PCR-amplification from total genomic DNA of *L. lactis* MG1363 of a 127-bp *Nco*I fragment (NZU) containing the signal peptide of the Usp45 protein ($SP_{Usp45}$) and the N-terminal nucleotide sequence of the mature EntA structural gene (*entA*). Primers EPNC-F/PPIA-R were used for PCR-amplification from total genomic DNA of *E. faecium* P13 of a 127-bp *Nco*I fragment (NZP) containing the signal peptide of the EntP protein ($SP_{EntP}$) and the N-terminal nucleotide sequence of the mature *entA* structural gene. Primers HNZNC-F/EAPSH-R were used for PCR-amplification from total genomic DNA of *E. hirae* DCH5 of a 135-bp *Nco*I fragment (NZH) containing the signal peptide of the HirJM79 protein ($SP_{HirJM79}$) and the N-terminal nucleotide sequence of the mature *entA* structural gene. Primers ENTAM-F and LAMI-R were used for PCR-amplification from total genomic DNA of *E. faecium* T136 of a 467-bp *Hind*III fragment (AI) containing the mature *entA* structural gene with the immunity gene (*entiA*). Mixtures of fragments NZU and AI, of fragments NZP and AI, and that of fragments NZH and AI were used as templates to amplify by PCR two 567-bp *Nco*I–*Hind*III fragments (NZUAI and NZPAI), and a 575-bp *Nco*I–*Hind*III fragment (NZHAI) using the primer pairs USPNC-F/LAMI-R, EPNC-F/LAMI-R and HNZNC-F/LAMI-R, respectively. Fragments NZUAI, NZPAI and NZHAI were digested with the indicated restriction enzymes and inserted into pNZ8048, digested with the same enzymes. The ligation mixtures were used to transform *L. lactis* NZ9000 competent cells. The plasmid derivatives pNZUAI, pNZPAI and pNZHAI, respectively, were checked by bacteriogenicity tests, PCR and sequencing of the inserts.

### 2.6. Recombinant plasmids derived from pMSP3545

To construct the recombinant plasmids derived from pMSP3545, the purified pNZUAI, pNZPAI and pNZHAI vectors were used as the PCR targets with the primer pairs USPNC-F/EAXBAI-R, EPNC-F/EAXBAI-R and HNZNC-F/EAXBAI-R, respectively, for generation of two 567-bp fragments (MSUAI and MSPAI), and a 575-bp fragment (MSHAI). The purified inserts were digested with the *Nco*I–*Xba*I restriction enzymes and inserted into vector pMSP3545, previously digested with the same enzymes, and the ligation mixtures were used to transform *L. lactis* NZ9000, *L. lactis* IL1403, *E. faecium* AR24, *E. faecalis* JH2-2, *Lb. sakei* Lb790 and *P. acidilactici* $Ped^-$ competent cells. The plasmid derivatives pMSUAI, pMSPAI and pMSHAI, were checked by bacteriogenicity tests, PCR and sequencing of the inserts.

### 2.7. Recombinant plasmids derived from pMG36c

To construct the recombinant plasmids derived from pMG36c, the purified pNZUAI, pNZPAI and pNZHAI plasmids were used, respectively, as the PCR targets with the primer pairs USP-F/LAMI-R, LJ1-F/LAMI-R and HPJ-F/LAMI-R, for generation of two 585-bp fragment (MGUAI and MGPAI), and a 593-bp fragment (MGHAI), all containing the $P_{32}$ ribosome binding site (RBS). The purified inserts were digested with the *Sac*I–*Hind*III restriction enzymes and inserted into plasmid pMG36c, previously digested with the same enzymes, and the ligation mixtures were used to transform *L. lactis* NZ9000, *L. lactis* IL1403, *E. faecium* AR24, *E. faecalis* JH2-2, *L. sakei* Lb790, *P. acidilactici* $Ped^-$, *E. faecium* DCH37, and *E. faecium* T136 competent cells. The plasmid derivatives pMGUAI, pMGPAI and pMGHAI, were checked by bacteriogenicity tests, PCR and sequencing of the inserts.

**Table 2**
Primers and PCR products used in this study.

| Primers or PCR products | Nucleotide sequence (5′–3′) or description[a] | Amplification fragments |
|---|---|---|
| Primers | | |
| ENTAM-F | ACCACTCATAGTGGAAAATATTATGGAAATGG | AI |
| LAMI-R | ATAAGTTAAGCTTGTATTAAAATTGAGATTTATCTCCATAATCTGCTCG | AI, NZUAI, NZPAI, NZHAI, MGUAI, MGPAI, MGHAI |
| EAXBAI-R | ATAAGTTTCTAGATATTTAAAATTGAGATTTATCTCCATAATCTGC | MSUAI, MSPAI, MSHAI |
| USPNC-F | GAATTCTCACCATGGGAAAAAAAAAGATTATCTCAGCTATTTTAATGTCTAC | NZU, NZUAI, MSUAI |
| USPEA-R | CCATTTCCATAATATTTTCCAGTATGAGTGGTAGCGTAAACACCTGACAACGGG | NZU |
| EPNC-F | GAATTCTCACCATGGGAAGAAAAAAATTATTTAGTTTAGCTCTTATTGG | NZP, NZPAI, MSPAI |
| PPIA-R | TCCATAATATTTTCCAGTATGAGTGGTTGCATCAACTTTTGTACCAAAATTTGTC | NZP |
| HNZNC-F | GAATTCTCACCATGGGAAAAAAGAAAGTATTAAAACATTGTGTTATTCTAGG | NZH, NZHAI, MSHAI |
| EAPSH-R | TCCATAATATTTTCCACTATGAGTGGTTGCATCAACTTTTATTCCTGTACCG | NZH |
| USP-F | CATAGAGCTCTCTAA**GGAGG**ATTTTTGAAATGAAAAAAAAGATTATCTCAGCTAT | MGUAI |
| LJ1-F | AATTATAGAGCTCTC**GGAGG**AATTTTGAAATGAGAAAAAAATTATTTAGTTTAGC | MGPAI |
| HPJ-F | CATAGAGCTCTGTAA**GGAGG**ATTTTTGAAATGAAAAAGAAAGTATTAAAACATTG | MGHAI |
| PCR products | | |
| AI | 467-bp *Hind*III fragment containing the mature enterocin A (*ent*A) and immunity (*enti*A) genes | |
| NZU | 127-pb *Nco*I fragment containing the *usp45* signal peptide (SP$_{usp45}$) and the beginning of *entA* | |
| NZP | 127-pb *Nco*I fragment containing the *entP* signal peptide (SP$_{entP}$) and the beginning of *entA* | |
| NZH | 135-pb *Nco*I fragment containing the *hirJM79* signal peptide (SP$_{hirJM79}$) and the beginning of *entA* | |
| NZUAI | 567-bp *Nco*I/*Hind*III fragment containing the SP$_{usp45}$ fused to *ent*A and *entiA* | |
| NZPAI | 567-bp *Nco*I/*Hind*III fragment containing the SP$_{entP}$ fused to *ent*A and *entiA* | |
| NZHAI | 575-bp *Nco*I/*Hind*III fragment containing the SP$_{hirJM79}$ fused to *ent*A and *entiA* | |
| MSUAI | 567-bp *Nco*I/*Xba*I fragment containing the SP$_{usp45}$ fused to *ent*A and *entiA* | |
| MSPAI | 567-bp *Nco*I/*Xba*I fragment containing the SP$_{entP}$ fused to *ent*A and *entiA* | |
| MSHAI | 575-bp *Nco*I/*Xba*I fragment containing the SP$_{hirJM79}$ fused to *ent*A and *entiA* | |
| MGUAI | 585-bp *Sac*I/*Hind*III fragment containing the $P_{32}$ ribosome binding site and the SP$_{usp45}$ fused to *ent*A and *entiA* | |
| MGPAI | 585-bp *Sac*I/*Hind*III fragment containing the $P_{32}$ ribosome binding site and the SP$_{entP}$ fused to *ent*A and *entiA* | |
| MGHAI | 593-bp *Sac*I/*Hind*III fragment containing the $P_{32}$ ribosome binding site and the SP$_{hirJM79}$ fused to *ent*A and *entiA* | |

[a] Cleavage site for restriction enzymes is underlined in the primers; $P_{32}$ ribosome binding site is shown in bold.

### *2.8. Production of specific anti-EntA polyclonal antibodies and ELISA*

The peptide fragment EAJB ($NH_2$-GGFLGGAIPGKC-COOH), deduced from the C-terminal amino acid sequence of EntA, was selected as the antigen for the generation of antibodies of predetermined specificity against EntA. The synthetic peptide EAJB was synthesized by Invitrogen Ltd. (Paisley, Scotland, UK), with a peptide purity of >95%. The peptide EAJB was conjugated to the keyhole limpet hemocyanin (KLH) carrier protein as an EAJB-KLH conjugate, 1:2 (w/w), using the components of an inject maleimide-activated mariculture KLH kit (Perbio Science, Rockford, IL) for use as the immunogen. Rabbits (New Zealand White Females) were immunized with EAJB-KLH, as described previously (Gutiérrez et al., 2004). Serum was obtained from blood samples incubated overnight at 4 °C, centrifuged at 1000 × *g* at room temperature for 15 min, and stored at −20 °C until use. The enzyme-linked immunosorbent assay (ELISA) procedures for determination of antiserum specificity and sensitivity were performed as described previously (Martínez et al., 1998; Gutiérrez et al., 2004). A noncompetitive indirect ELISA (NCI-ELISA) was developed to detect and quantify EntA in the supernatant of the producer cells. Briefly, wells of flat-bottom polystyrene microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight (4 °C) with supernatants from *E. faecium* T136 or the recombinant LAB hosts. After addition of the anti-EntAJB-KLH serum and the goat anti-rabbit immunoglobulin G peroxidase conjugate (Cappel Laboratories, West Chester, PA, USA), bound peroxidase was determined with ABTS (2,2′-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]) (Sigma) as the substrate by measuring the absorbance of the wells at 405 nm with a Labsystems iEMS reader (Labsystems) with a built-in software package for data analysis.

### *2.9. Purification of EntA and mass spectrometry analysis*

EntA was purified from *E. faecium* T136 and *L. lactis* NZ9000 transformed with pNZUAI, pNZPAI and pNZHAI as previously described (Gutiérrez et al., 2006; Sánchez et al., 2008). Briefly, supernatants from early stationary phase 1-l cultures were subjected to precipitation with ammonium sulfate, desalted by gel filtration, and further subjected to cation-exchange and hydrophobic-interaction chromatography, followed by reverse-phase chromatography in a fast-protein liquid chromatography system (RP-FPLC) (Amersham Biosciences Europe, Madrid, Spain). Final concentrations of purified EntA were estimated using the extinction coefficient of the bacteriocin (an $A_{280}$ of 2.1 corresponds to 1 mg/ml of EntA) and the NCI-ELISA. Purified fractions from the last reverse-phase chromatography step were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, as described (Gutiérrez et al., 2006). Briefly, 1 μl sample was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 ml of a 3 mg/ml α-cyano-4-hydroxy-transcinnamic acid matrix (Sigma) in 50% acetonitrile was added to the dried sample, which was allowed again to air-dry at room temperature. MALDI-TOF analyses were performed in a Voyager-DE STR Instrument (PerSeptive Biosystems, Foster City, CA, USA) fitted with a nitrogen laser and operated in reflector mode, with an accelerating voltage of 25,000 V.

## 3. Results

### *3.1. Construction of the recombinant plasmids for production of EntA*

Cloning of chimeras containing the SP$_{usp45}$, the SP$_{entP}$, and the SP$_{hirJM79}$ fused to mature *entA* + *entiA* into the expression vectors pNZ8048, pMS3545, and pMG36c resulted in the recombinant plasmids described in Tables 3 and 4.

### *3.2. Heterologous production and functional expression of EntA by different LAB strains*

When derivatives of plasmid pNZ8048 were used to transform *L. lactis* subsp. *cremoris* NZ9000, and derivatives of pMSP3545

**Table 3**
Bacteriocin production and antimicrobial activity of supernatants from recombinant LAB strains transformed with derivatives of pNZ8048 and pMSP3545.

| Strain | Bacteriocin production (μg EntA/mg cell dry weight)[a] | Antimicrobial activity (BU/mg cell dry weight)[b] | Specific antimicrobial activity (BU/μg EntA)[c] |
|---|---|---|---|
| *L. lactis* subsp. *cremoris* | | | |
| NZ9000 (pNZ8048) | NP | NA | NE |
| NZ9000 (pNZUAI) | 35.2 | 3,527 | 100 |
| NZ9000 (pNZPAI) | 16.7 | 701 | 42 |
| NZ9000 (pNZHAI) | 32.4 | 4,775 | 147 |
| *L. lactis* subsp. *cremoris* | | | |
| NZ9000 (pMSP3545) | NP | 21 | NE |
| NZ9000 (pMSUAI) | 6.4 | 726 | 220 |
| NZ9000 (pMSPAI) | 6.1 | 829 | 136 |
| NZ9000 (pMSHAI) | 6.5 | 875 | 134 |
| *L. lactis subsp. lactis* | | | |
| IL1403 (pMSP3545) | NP | 22 | NE |
| IL1403 (pMSUAI) | 6.5 | 822 | 126 |
| IL1403 (pMSPAI) | 6.3 | 793 | 126 |
| IL1403 (pMSHAI) | 10.1 | 1,297 | 129 |
| *E. faecium* | | | |
| AR24 (pMSP3545) | NP | 35 | NE |
| AR24 (pMSUAI) | 1.7 | 117 | 69 |
| AR24 (pMSPAI) | 1.6 | 160 | 100 |
| AR24 (pMSHAI) | 1.8 | 124 | 69 |
| *E. faecalis* | | | |
| JH2-2 (pMSP3545) | NP | 33 | NE |
| JH2-2 (pMSUAI) | 1.5 | 60 | 40 |
| JH2-2 (pMSPAI) | 1.6 | 602 | 376 |
| JH2-2 (pMSHAI) | 1.5 | 243 | 162 |
| *Lb. sakei* | | | |
| Lb790 (pMSP3545) | NP | 33 | NE |
| Lb790 (pMSUAI) | 6.8 | 202 | 29 |
| Lb790 (pMSPAI) | 4.8 | 89 | 19 |
| Lb790 (pMSHAI) | 4.9 | 113 | 23 |
| *P. acidilactici* | | | |
| Ped⁻ (pMSP3545) | NP | 36 | NE |
| Ped⁻ (pMSUAI) | 1.8 | 77 | 43 |
| Ped⁻ (pMSPAI) | 1.9 | 241 | 127 |
| Ped⁻ (pMSHAI) | 1.9 | 304 | 160 |
| *E. faecium*[d] | | | |
| T136 | 1.9 | 721 | 379 |

Most of the data are mean from two independent determinations in triplicate.
NP, no production, NA, no activity, NE, not evaluable.
[a] Production of EntA was calculated by using a NCI-ELISA with polyclonal antibodies specific for EntA.
[b] Antimicrobial activity was calculated against *E. faecium* P13 (EntA$^s$).
[c] Specific antimicrobial activity refers to the antagonistic activity against *E. faecium* P13 divided by the EntA produced.
[d] Culture of *E. faecium* T136 used as control for the production and antimicrobial activity of EntA.

and pMG36c were used to transform the *L. lactis* subsp. *cremoris* NZ9000, *L. lactis* subsp. *lactis* IL1403, *E. faecium* AR24, *E. faecalis* JH2-2, *Lb. sakei* Lb790 and *P. acidilactici* Ped⁻ hosts, the supernatants of all the recombinant strains showed antagonistic activity against the indicator strain *E. faecium* P13 (Fig. 1). The largest halos of inhibition were observed with *L. lactis* NZ9000 and *L. lactis* IL1403, transformed with derivatives of pNZ8048 and pMSP3545 whose promoters are inducible by NisA (Fig. 1A). Smaller but variable halos of inhibition were observed by supernatants of *E. faecium* AR24, *E. faecalis* JH2-2, *Lb. sakei* Lb790, and *P. acidilactici* Ped⁻ hosts, transformed with derivatives of pMSP3545 (Fig. 1A). Halos of inhibition of variable size were also observed by the recombinant LAB transformed with derivatives of pMG36c with chimeras of the different signal secretion sequences fused to *entA* + *entiA* under control of a constitutive promoter (Fig. 1B).

The production and functional expression of the EntA produced by the recombinant LAB hosts was further quantified using specific anti-EntA antibodies in an NCI-ELISA, and by a microtitre plate assay (MPA). The results of Table 3 show that the heterologous extracellular production of EntA by *L. lactis* NZ9000, transformed with the pNZ8048 derivatives, was 8.7- to 18.5-fold higher than that of *E. faecium* T136. Furthermore, *L. lactis* NZ9000 (pNZUAI) and *L. lactis* NZ9000 (pNZHAI) showed a higher production of EntA than *L. lactis* NZ9000 (pNZPAI). The production of EntA by *L. lactis* NZ9000, *L. lactis* IL1403, and *Lb. sakei* Lb790, transformed with the pMSP3545 derivatives, was 2.5- to 5.3-fold higher than by *E. faecium* T136, whereas the production of EntA by the *E. faecium* AR24, *E. faecalis* JH2-2, and *P. acidilactici* Ped⁻ hosts, transformed with the former vectors, was similar (0.8- to 1.0-fold) to that of *E. faecium* T136. Finally, the production of EntA by the recombinant *L. lactis* NZ9000, *L. lactis* IL1403, and *Lb. sakei* Lb790 strains, transformed with the pMG36c derivatives, was 1.5- to 5.7-fold higher than the production of EntA by *E. faecium* T136. For the above cited *L. lactis* NZ9000 transformants the production of EntA was higher by those transformed with pMGUAI and pMGHAI than with pMGPAI. For the rest of the LAB hosts the production of EntA was 0.4- to 1.2-fold higher than by *E. faecium* T136 (Table 4).

When supernatants of the recombinant LAB strains, transformed with derivatives of pNZ8048 and pMSP3545 (Table 3), were evaluated for their antimicrobial activity against *E. faecium* P13, the antagonistic activity of *L. lactis* NZ9000 (pNZUAI) and *L. lactis* NZ9000 (pNZHAI) were 4.9- and 6.6-fold higher, respectively, than that of *E. faecium* T136. Similarly, the antagonistic activity of *L. lactis* NZ9000 and *L. lactis* IL1403, transformed with derivatives of pMSP3545, was up to 1.8-fold higher than that of *E. faecium* T136 (Table 3). The antimicrobial activity of supernatants from the rest

**Table 4**
Bacteriocin production and antimicrobial activity of supernatants from recombinant LAB strains transformed with derivatives of pMG36c.

| Strain | Bacteriocin production (μg EntA/mg cell dry weight)[a] | Antimicrobial activity (BU/mg cell dry weight)[b] | Specific antimicrobial activity (BU/μg EntA)[c] |
|---|---|---|---|
| *L. lactis* subsp. *cremoris* | | | |
| NZ9000 (pMG36c) | NP | NA | NE |
| NZ9000 (pMGUAI) | 5.7 | 142 | 25 |
| NZ9000 (pMGPAI) | 2.8 | 99 | 35 |
| NZ9000 (pMGHAI) | 6.0 | 331 | 55 |
| *L. lactis* subsp. *lactis* | | | |
| IL1403 (pMG36c) | NP | NA | NE |
| IL1403 (pMGUAI) | 7.2 | 455 | 63 |
| IL1403 (pMGPAI) | 2.6 | 131 | 50 |
| IL1403 (pMGHAI) | 8.0 | 577 | 75 |
| *E. faecium* | | | |
| AR24 (pMG36c) | NP | NA | NE |
| AR24 (pMGUAI) | 1.3 | 142 | 109 |
| AR24 (pMGPAI) | 1.3 | 424 | 316 |
| AR24 (pMGHAI) | 1.3 | 276 | 212 |
| *E. faecalis* | | | |
| JH2-2 (pMG36c) | NP | 38 | NE |
| JH2-2 (pMGUAI) | 1.8 | 124 | 68 |
| JH2-2 (pMGPAI) | 1.9 | 1,379 | 726 |
| JH2-2 (pMGHAI) | 1.2 | 83 | 69 |
| *Lb. sakei* | | | |
| Lb790 (pMG36c) | NP | NA | NE |
| Lb790 (pMGUAI) | 10.9 | 151 | 14 |
| Lb790 (pMGPAI) | 7.6 | 220 | 29 |
| Lb790 (pMGHAI) | 10.8 | 172 | 16 |
| *P. acidilactici* | | | |
| Ped⁻ (pMG36c) | NP | 22 | NE |
| Ped⁻ (pMGUAI) | 2.4 | 53 | 22 |
| Ped⁻ (pMGPAI) | 2.0 | 151 | 76 |
| Ped⁻ (pMGHAI) | 2.1 | 125 | 60 |
| *E. faecium*[c] | | | |
| T136 (pMG36c) | 1.3 | 734 | 407 |
| T136 (pMGPAI) | 2.4 | 946 | NE |
| DCH37 (pMG36c) | NP | 4,243 | NE |
| DCH37 (pMGPAI) | 0.8 | 5,301 | NE |
| *E. faecium*[d] | | | |
| T136 | 1.9 | 721 | 379 |

Most of the data are mean from two independent determinations in triplicate.
NP, no production, NA, no activity, NE, not evaluable.
[a] Production of EntA was calculated by using a NCI-ELISA with polyclonal antibodies specific for EntA.
[b] Antimicrobial activity was calculated against *E. faecium* P13 (EntA$^s$).
[c] Specific antimicrobial activity refers to the antagonistic activity against *E. faecium* P13 divided by the EntA produced.
[d] Culture of *E. faecium* T136 used as control for the production and antimicrobial activity of EntA.

of the LAB hosts, transformed with derivatives of pMSP3545, was always lower (8–83%) than that of *E. faecium* T136. The antimicrobial activity of supernatants of the recombinant LAB strains, transformed with derivatives of pMG36c (Table 4), was always lower (8–80%) than that of *E. faecium* T136, except for the supernatants of *E. faecalis* JH2-2 (pMGPAI), *E. faecium* DCH37 (pMGPAI), and *E. faecium* T136 (pMGPAI) whose antimicrobial activity was 1.9-, 1.2-, and 1.3-fold higher, respectively, than that of *E. faecium* T136.

However, in spite of the high extracellular production of EntA by *L. lactis* NZ9000, transformed with derivatives of pNZ8048, a lower specific antimicrobial activity was observed (11–38%) as compared to that of *E. faecium* T136 (Table 3). A lower specific antimicrobial activity (25–99%) was also observed for the LAB strains, transformed with derivatives of pMSP3545 and pMG36c, except for *E. faecalis* JH2-2 (pMGPAI), that showed a 1.9-fold higher specific antimicrobial activity than that of *E. faecium* T136 (Tables 3 and 4).

### 3.3. Antimicrobial activity of supernatants from recombinant L. lactis against different Listeria spp.

Supernatants of the recombinant LAB hosts transformed with derivatives of pNZ8048, pMSP3545 and pMG36c, displaying a high EntA production or a high antimicrobial activity, were evaluated for their antagonistic activity against different *L. monocytogenes*, *L. ivanovii*, *L. grayii*, *L. welshimeri*, *L. seeligeri*, and *L. innocua* strains (Table 5). The supernatants of *L. lactis* NZ9000 (pNZUAI) and *L. lactis* NZ9000 (pNZHAI), overproducers of EntA, showed a 1.2- to 5.1-fold higher antimicrobial activity than that of *E. faecium* T136 against all *Listeria* spp. evaluated, whereas that of *L. lactis* NZ9000 (pNZPAI) showed a 16–46% antagonistic activity against the same *Listeria* spp., as compared to that of *E. faecium* T136. The supernatants of *L. lactis* NZ9000 (pMSHAI) and *L. lactis* IL1403 (pMSHAI) showed a lower or a slightly higher antilisterial activity than that of *E. faecium* T136. The supernatant of *E. faecalis* JH2-2 (pMSPAI) showed a 26–67% antilisterial activity, and that of *E. faecalis* JH2-2 (pMGPAI) showed a 0.7- up to a 1.4-fold higher antilisterial activity than the control enterococcal strain. The supernatants of *E. faecium* DCH37 (pMGPAI) showed a 0.7- up to a 1.4-fold higher antilisterial activity, whereas the antimicrobial activity of *E. faecium* T136 (pMGPAI) was improved when compared with that of the control strain *E. faecium* T136 for most of the *Listeria* spp. strains tested.

### 3.4. Purification of EntA and mass spectrometry analysis

MALDI-TOF-MS analysis of the EntA purified (data not shown) from the supernatants of *E. faecium* T136 and *L. lactis* NZ9000 transformed with derivatives of pNZ8048 showed a major peak of about

(A) Inducible promoter (B) Constitutive promoter

Signal Peptide (SP)

Control, $SP_{usp45}$, $SP_{entP}$, $SP_{hirJM79}$ (A); Control, $SP_{usp45}$, $SP_{entP}$, $SP_{hirJM79}$ (B)

(A): *L. lactis* NZ9000 (pNZ8048); *L. lactis* NZ9000 (pMSP3545); *L. lactis* IL1403 (pMSP3545); *E. faecium* AR24 (pMSP3545); *E. faecalis* JH2-2 (pMSP3545); *Lb. sakei* Lb790 (pMSP3545); *P. acidilactici* Ped⁻ (pMSP3545); *E. faecium* T136

(B): *L. lactis* NZ9000 (pMG36c); *L. lactis* IL1403 (pMG36c); *E. faecium* AR24 (pMG36c); *E. faecalis* JH2-2 (pMG36c); *Lb. sakei* Lb790 (pMG36c); *P. acidilactici* Ped⁻ (pMG36c); *E. faecium* T136 (pMG36c); *E. faecium* DCH37 (pMG36c)

**Fig. 1.** Antimicrobial activity of supernatants from the recombinant LAB strains. (A) Strains transformed with derivatives of pNZ8048 and pMSP3545 with the chimeric genes under control of an inducible promoter: (1) *L. lactis* NZ9000 (pNZ8048), (2) *L. lactis* NZ9000 (pNZUAI), (3) *L. lactis* NZ9000 (pNZPAI), (4) *L. lactis* NZ9000 (pNZHAI), (5) *L. lactis* NZ9000 (pMSP3545), (6) *L. lactis* NZ9000 (pMSUAI), (7) *L. lactis* NZ9000 (pMSPAI), (8) *L. lactis* NZ9000 (pMSHAI), (9) *L. lactis* IL1403 (pMSP3545), (10) *L. lactis* IL1403 (pMSUAI), (11) *L. lactis* IL1403 (pMSPAI), (12) *L. lactis* IL1403 (pMSHAI), (13) *E. faecium* AR24 (pMSP3545), (14) *E. faecium* AR24 (pMSUAI), (15) *E. faecium* AR24 (pMSPAI), (16) *E. faecium* AR24 (pMSHAI), (17) *E. faecalis* JH2-2 (pMSP3545), (18) *E. faecalis* JH2-2 (pMSUAI), (19) *E. faecalis* JH2-2 (pMSPAI), (20) *E. faecalis* JH2-2 (pMSHAI), (21) *Lb. sakei* Lb790 (pMSP3545), (22) *Lb. sakei* Lb790 (pMSUAI), (23) *Lb. sakei* Lb790 (pMSPAI), (24) *Lb. sakei* Lb790 (pMSHAI), (25) *P. acidilactici* Ped⁻ (pMSP3545), (26) *P. acidilactici* Ped⁻ (pMSUAI), (27) *P. acidilactici* Ped⁻ (pMSPAI), (28) *P. acidilactici* Ped⁻ (pMSHAI), and (29) *E. faecium* T136. (B) Strains transformed with derivatives of pMG36c with the chimeric genes under control of a constitutive promoter: (1) *L. lactis* NZ9000 (pMG36c), (2) *L. lactis* NZ9000 (pMGUAI), (3) *L. lactis* NZ9000 (pMGPAI), (4) *L. lactis* NZ9000 (pMGHAI), (5) *L. lactis* IL1403 (pMG36c), (6) *L. lactis* IL1403 (pMGUAI), (7) *L. lactis* IL1403 (pMGPAI), (8) *L. lactis* IL1403 (pMGHAI), (9) *E. faecium* AR24 (pMG36c), (10) *E. faecium* AR24 (pMGUAI), (11) *E. faecium* AR24 (pMGPAI), (12) *E. faecium* AR24 (pMGHAI), (13) *E. faecalis* JH2-2 (pMG36c), (14) *E. faecalis* JH2-2 (pMGUAI), (15) *E. faecalis* JH2-2 (pMGPAI), (16) *E. faecalis* JH2-2 (pMGHAI), (17) *Lb. sakei* Lb790 (pMG36c), (18) *Lb. sakei* Lb790 (pMGUAI), (19) *Lb. sakei* Lb790 (pMGPAI), (20) *Lb. sakei* Lb790 (pMGHAI), (21) *P. acidilactici* Ped⁻ (pMG36c), (22) *P. acidilactici* Ped⁻ (pMGUAI), (23) *P. acidilactici* Ped⁻ (pMGPAI), (24) *P. acidilactici* Ped⁻ (pMGHAI), (25) *E. faecium* T136 (pMG36c), (26) *E. faecium* T136 (pMGPAI), (27) *E. faecium* DCH37 (pMG36c), and (28) *E. faecium* DCH37 (pMGPAI). The antimicrobial activity was determined by an agar well diffusion test with *E. faecium* P13 as the indicator microorganism.

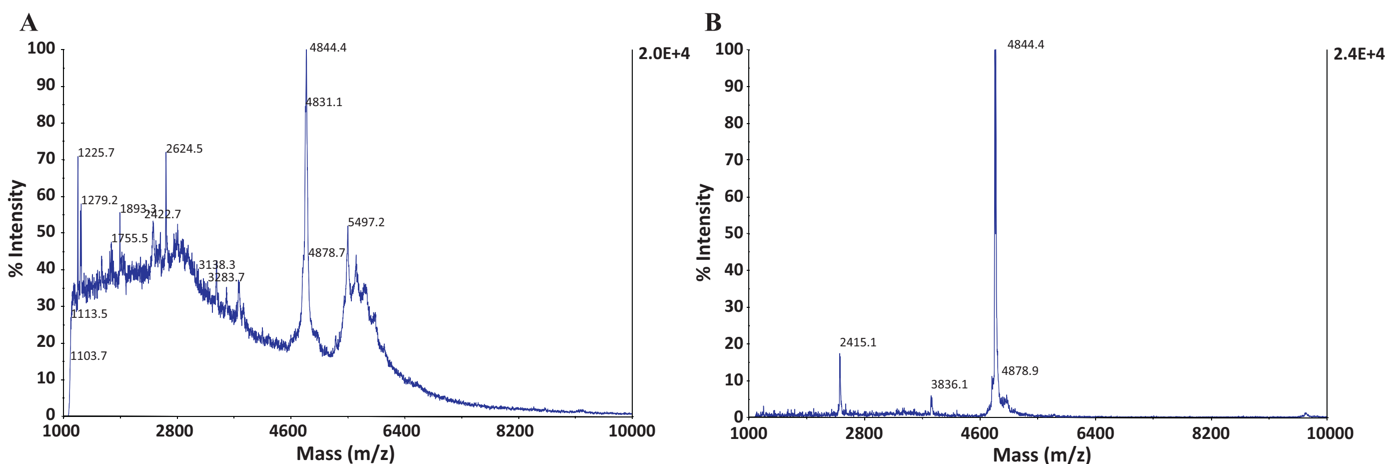


**Fig. 2.** Mass spectrometry analysis of the purified enterocin A from (A) *E. faecium* T136, and (B) *L. lactis* NZ9000 (pNZHAI).

4.844 Da, as shown for the EntA purified from *E. faecium* T136 (Fig. 2A) and *L. lactis* (pNZHAI) (Fig. 2B).

## 4. Discussion

Many bacteriocins show a broad spectrum of antimicrobial activity and are more active than conventional antibiotics against pathogenic and drug-resistant Gram-positive bacteria yet display no toxicity towards eukaryotic cells (Sit and Vederas, 2008). Furthermore, with the emergence of bacterial antibiotic resistance, bacteriocins produced by LAB may find their use as natural antimicrobial peptides in food, medical, veterinary, and animal production applications. However, the high cost of synthetic bacteriocin synthesis, their low yields, and the production of potential virulence factors from many natural producers drive the exploration of recombinant microbial systems for the biotechnological production of bacteriocins. The production of bacteriocins in heterologous LAB hosts may be essentially based on the expression of native biosynthetic genes, by exchange or replacement of leader peptides and/or dedicated processing and secretion systems (ABC transporters) or by fusion of mature bacteriocins to signal peptides that act as secretion signals (Gutiérrez et al., 2006; Martín et al., 2007a,b; Borrero et al., 2011b).

Previous studies have shown the heterologous production of EntA by cloning and expression of the *entAITD* cassette under control of a constitute promoter in *L. lactis* (O'Keefee et al., 1999). A very low-level of co-production of EntA and PedA-1 was quantified in *L. lactis* IL1403 (pMJ04) with the co-linearly arranged *entA-pedA* structural genes under control of a constitutive promoter (Martínez et al., 2000). The heterologous expression of EntA fused to a cellulose-binding domain in *E. coli* resulted in a fused protein with antilisterial activity (Klocke et al., 2005). When plasmid pEnt02, containing the *entAITD* genes, was introduced into a starter *L. lactis* strain a decrease in the *L. monocytogenes* counts was achieved during manufacture of Cottage cheese (Liu et al., 2008). However, the secretion of Sec-dependent enterocins from structural genes expressed in recombinant *L. lactis* hosts (Herranz and Driessen, 2005; Gutiérrez et al., 2006; Sánchez et al., 2008), and the use of chimeras of heterologous signal peptides fused to mature bacteriocins to drive their production and secretion in *L. lactis* in the absence of specific immunity and secretion proteins (Martín et al., 2007a,b; Borrero et al., 2011b), prompted us to evaluate the construction of chimeras containing heterologous signal peptides fused to mature EntA and its immunity protein and their cloning into different expression vectors to drive the production and functional expression of EntA by different LAB hosts.

The results obtained in this work suggest that the production, secretion, and antimicrobial activity of the EntA produced by the recombinant LAB strains depend on the signal peptide, the expression vector, and the host strain (Fig. 1). Although *entA* is the minimum requirement for synthesis and secretion of EntA by recombinant *L. lactis* immune to its antimicrobial activity (Martín et al., 2007a), *entiA* is required for secretion of EntA by recombinant enterococci and lactobacilli sensitive to its antagonistic effect ($EntA^s$). The production of EntA by the $EntA^s$-LAB hosts is explained assuming that they express the EntiA protein. It is known that bacteriocin producers are protected from their own bacteriocins by the concomitant expression of a cognate immunity protein, and that bacteriocins of the class IIa, such as the EntA, use components of the mannose phosphotransferase system (Man-PTS) of the susceptible cells as the target/receptor. The immunity proteins form a strong complex with the receptor proteins, thereby preventing producer cells from being killed (Diep et al., 2007; Kjos et al., 2010).

In this work, the generation of antibodies of predetermined specificity for EntA and an NCI-ELISA have permitted the detection and quantification of this bacteriocin in the supernatants of the recombinant LAB strains, and to determine its specific antimicrobial activity. Some other characteristics of the expression vectors used to drive the recombinant production of the cloned EntA are the following. Plasmids pNZ8048 and pMSP3545 are shuttle vectors that replicate in *E. coli* and LAB. Plasmid pNZ8048 contains the high-copy number heterogramic replicon of the lactococcal plasmid pSH71 with a unique *Nco*I cleavage site, downstream of the *nisA* ribosomal binding sequence (RBS), used for translational fusions inducible by nisin A (NisA) (De Ruyter et al., 1996; Kuipers et al., 1998). Plasmid pMSP3545 contains the ColE1 origin of replication from pSP73, the low-copy number pAMβ1 replication origin of *E. faecalis*, the $P_{nisA}$ with a unique *Nco*I cleavage site and the polylinker fragment from pNZ8048, and the genes encoding NisR and NisK the two-component signaling mechanism for activating transcription from $P_{nisA}$ in the presence of NisA (Bryan et al., 2000). The expression vector pMG36c is a shuttle vector, based on the low copy replication origin of pWV01, able to replicate in *E. coli*, *B. subtilis* and LAB, whereas the strong $P_{32}$ promoter drives the constitutive transcription of inserted genes into the multicloning site (MCS) of pUC18 (van de Guchte et al., 1989).

The enhanced production of EntA by the recombinant LAB would depend, among other factors, on the expression vector size, plasmid stability, and copy number differences between pNZ8048, pMSP3545, and pMG36c (Bryan et al., 2000; Kim and Mills, 2007) but, more likely, might be caused by the promoters used to drive gene expression. Of interest is the 7.8- to 18.5-fold higher production of EntA by *L. lactis* NZ9000 transformed with derivatives of pNZ8048 containing nisin-inducible gene expression (NICE) constructs, as compared to other recombinant LAB hosts and *E. faecium* T136 (Table 3). In this respect, for optimization of protein production, inducible systems are often considered superior to constitutive expression systems since the former enable achievement of sufficient biomass prior to initiation of target protein expression (Kim and Mills, 2007). The short induction time for bacteriocin production from nisin-inducible systems most probably prevents EntA from attaching to cell walls, forming aggregates, and/or undergoing protease degradation (Gutiérrez et al., 2006). The lower production of EntA by the pMSP3545 derivatives as compared to the pNZ8048 derivatives, despite the use of the NICE system during protein expression, may in part be due to the low copy number of the pAMβ1 origin of replication of pMSP3545 (Kim and Mills, 2007). Nevertheless, the production of EntA by *L. lactis* IL1403 and *Lb. sakei* Lb790, transformed with derivatives of pMSP3545, was 2.5- to 5.3-fold higher than by *E. faecium* T136, while the production of EntA by the *E. faecium* AR24, *E. faecalis* JH2-2, and *P. acidilactici* $Ped^-$ hosts was similar to that of *E. faecium* T136 (Table 3). The construction of derivatives of pMG36c, with the constitutive $P_{32}$ promoter, also permitted production of EntA by the recombinant LAB strains, in some cases, at levels above those obtained with the nisin-inducible constructs (Table 4). It may occur that the *nisA* promoter is not recognized by the transcription initiation machinery of the heterologous LAB hosts as it is in *L. lactis*. Alternatively, it could be that the *nisA* promoter-derived transcripts are recognized with reduced efficiency by the translation machinery of the heterologous LAB hosts compared to that of *L. lactis* (Kleeberezem et al., 1997). However, besides promoter choice, other factors such as mRNA stability and secondary structure may steer protein production from the recombinant LAB hosts (Diep et al., 2009).

The secretion of recombinant proteins in the growth medium reduces the efforts and costs of protein purification. Therefore, protein secretion is a preferred means of protein expression in the development of LAB as cell factories for production of biologically active compounds (Mathiesen et al., 2008). The results of this work indicate that when the $SP_{usp45}$ and $SP_{hirJM79}$ drive the secretion of EntA in *L. lactis* NZ9000, *L. lactis* IL1403, and *Lb.*

**Table 5**
Antimicrobial activity of supernatants from recombinant LAB strains against *Listeria* spp.[a]

| Strain | *L. ivanovii* | *L. grayii* | *L. welshimeri* | *L. seeligeri* | *L. innocua* | *L. monocytogenes* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 913 | 931 | 919 | 917 | 910 | 911 | 935 | 936 | 939 | 4031 | 4032 |
| *L. lactis* subsp. cremoris | | | | | | | | | | | |
| NZ9000 (pNZUAI) | 13,714 | 12,377 | 15,277 | 35,231 | 5,701 | 7,531 | 5,746 | 19,398 | 17,982 | 11,851 | 22,731 |
| NZ9000 (pNZPAI) | 3,487 | 749 | 1,050 | 2,238 | 1,008 | 730 | 777 | 1,585 | 1,628 | 960 | 1,444 |
| NZ9000 (pNZHAI) | 13,431 | 12,416 | 12,030 | 31,630 | 8,524 | 7,375 | 6,122 | 19,402 | 14,389 | 11,816 | 19,192 |
| NZ9000 (pMSHAI) | 10,314 | 2,638 | 6,740 | 12,398 | 3,334 | 5,597 | 2,421 | 4,033 | 2,135 | 1,700 | 3,831 |
| *L. lactis* subsp. lactis | | | | | | | | | | | |
| IL1403 (pMSHAI) | 10,580 | 4,094 | 9,395 | 12,068 | 4,181 | 6,059 | 2,700 | 6,747 | 3,462 | 2,240 | 6,901 |
| *E. faecalis* | | | | | | | | | | | |
| JH2-2 (pMSPAI) | 5,025 | 1,261 | 3,441 | 3,569 | 2,070 | 1,154 | 1,599 | 2,850 | 2,364 | 1,360 | 3,117 |
| JH2-2 (pMGPAI) | 9,555 | 4,900 | 6,697 | 16,833 | 7,544 | 4,612 | 5,197 | 7,515 | 4,930 | 3,037 | 7,204 |
| *E. faecium* | | | | | | | | | | | |
| DCH37 (pMGPAI) | 9,713 | 4,344 | 4,002 | 12,450 | 5,660 | 3,707 | 4,941 | 7,450 | 4,556 | 3,301 | 6,992 |
| T136 (pMGPAI) | 9,822 | 5,988 | 5,612 | 22,217 | 6,796 | 5,090 | 6,093 | 9,513 | 5,960 | 5,012 | 7,941 |
| *E. faecium*[b] | | | | | | | | | | | |
| T136 | 9,668 | 4,599 | 5,555 | 13,419 | 4,582 | 4,165 | 3,876 | 5,663 | 3,495 | 4,996 | 5,096 |

Most of the data are mean from two independent determinations in triplicate.
[a] Antimicrobial activity expressed in BU per milligrams cell dry weight.
[b] Culture of *E .faecium* T136 used as control for the production and antimicrobial activity of EntA.

*sakei* Lb790, the production of EntA is usually higher than when its secretion is driven by the $SP_{entP}$ (Tables 3 and 4). Targeting of a Sec-dependent protein to the membrane, the translocation process itself, and its subsequent processing by a signal peptidase represent the major bottlenecks for efficient translocation, and thus production of heterologous proteins (Degering et al., 2010). In our work, MALDI-TOF MS analysis revealed that EntA, purified from *L. lactis* NZ9000 transformed with the pNZ8048 derivatives, had a molecular mass identical to that of the EntA produced by *E. faecium* T136 (Fig. 2), suggesting that the EntA produced by the recombinant *L. lactis* NZ9000 hosts was adequately processed and secreted. The successful production and secretion of non-Sec-dependent bacteriocins by heterologous LAB hosts still raises the question of why most LAB bacteriocins have a dedicated and processing system when they can access the GSP if provided with an adequate SP. It is possible that differences in the amino acid sequence of the homologous and heterologous SPs evaluated in this work may provide a better recognition by the lactococcal and lactobacillal secretion machinery, including signal peptidases and chaperones, as compared to that of the enterococci (Mathiesen et al., 2008). However, since no prediction method for the right combination of SP and target protein is currently applicable with success (Ravn et al., 2003; Brockmeier et al., 2006), screening for the correct SP-protein combination for overproduction and secretion of the protein or peptide of interest is still necessary (Degering et al., 2010; Stammen et al., 2010).

The antimicrobial activity and the specific antimicrobial activity of most of the LAB transformants was lower than expected from their production of EntA, including those of the *L. lactis* NZ9000 hosts overproducers of EntA (Table 3). Is important to consider that not always a higher bacteriocin production reports a higher antimicrobial activity and that chimeras of signal secretion peptides (SP) fused to mature EntA drive the production and secretion of these bacteriocins in different LAB in the absence of specific secretion proteins. However, a higher antimicrobial activity was shown by *E. faecalis* JH2-2 (pMGPAI), which also displayed the highest specific antimicrobial activity, by *E. faecium* DCH37 (pMGPAI), and by *E. faecium* T136 (pMGPAI), as compared to that of *E. faecium* T136 (Table 4). The lack of production by *E. faecalis* JH2-2 (pMGPAI) of the proteinases gelatinase E (GelE) and serine proteinase E (SprE) (Mohamed and Murray, 2006) could be responsible of the higher antimicrobial activity of the secreted EntA. Similarly, the higher antagonistic activity of the EntA secreted by *E. faecium* DCH37 (pMGPAI) could be ascribed to a synergistic action with the bacteriocins enterocin L50 (EntL50A and EntL50B), enterocin P, and enterocin Q produced by this isolate (Sánchez et al., 2007a). The higher production of EntA by *E. faecium* T136 (pMGPAI) could be responsible for its slightly higher antimicrobial activity against *E. faecium* P13.

The lower specific antimicrobial activity of the EntA produced by most of the recombinant LAB hosts may depend on many factors, which are difficult to determine. It is possible that the synergistic effect of the EntA and enterocin B, produced by *E. faecium* T136 (Casaus et al., 1997), increase the specific antimicrobial activity of the produced EntA or that regulatory responses to a secretion stress, due to production of EntA by the recombinant LAB producers, activate quality control networks involving folding factors and housekeeping proteases (Darmon et al., 2002). Differences in components of the Sec-dependent translocation and Sec machinery in the different LAB strains or differences in protein folding and turnover or conformational modifications of the bacteriocin to a less extracellular active form, may also account for differences in the antagonistic activity of the secreted EntA (Sarvas et al., 2004). The EntA contains four cysteine residues that are involved in the formation of two disulfide bonds (DSB) which are known to improve its antimicrobial activity (Eijsink et al., 1998). DSB formation is a universally conserved mechanism for stabilizing extracytoplasmic proteins, carried out by thiol-disulfide-oxidoreductases (TDORs), and plays an important role in the folding, structural integrity, and activity of numerous proteins (Kouwen et al., 2007). However, DSB formation in heterologous hosts may be a handicap for production of biologically active peptides and proteins by bacterial cell factories (Freitas et al., 2005). Proteins present in *E. faecium* T136, but not in the recombinant LAB hosts, may be necessary to generate correct DSB, as suggested during production of PedA-1 in natural and heterologous LAB hosts (Fimland et al., 2000). Besides, EntA contains a methionine residue that may change to an apparently less active form due to its oxidation to methionine sulfoxide (Johnsen et al., 2000). In proteins, mainly methionine residues are oxidized, but oxidation of cysteines is becoming of interest. The lower specific antimicrobial activity of the extracellular EntA may be also ascribed to differences in protein folding efficiency and turnover, and to bacteriocin self-aggregation (Diep et al., 2009; Borrero et al., 2011b).

Concerning the role of the host cells in the production and functional expression of EntA, the *L. lactis* NZ9000 derivatives showed

the highest bacteriocin production and antimicrobial activity, followed by the *L. lactis* IL1403 hosts. The *Lb. sakei* Lb790 hosts are high producers of EntA with a low antimicrobial activity while the *E. faecalis* JH2-2 derivatives, in spite of its low EntA production, display the highest specific antimicrobial activity. The production of EntA in the supernatants of *L. lactis*, a microorganism Generally Recognized As Safe (GRAS) and with a Qualified Presumption of Safety (QPS) (Casalta and Montel, 2008), may facilitate the biotechnological use of this bacteriocin, partially purified or purified to homogeneity, as a natural antimicrobial peptide in food, pharmaceutical, veterinary, and medical applications. The generation of recombinant *L. lactis*, *Lb. sakei*, and *P. acidilactici* hosts producing biologically active EntA confirms the feasibility of developing safer bacteriocin-producing LAB strains potentially useful as probiotics with antilisterial or other antiinfective effects in humans and animals (Corr et al., 2007; Gillor et al., 2008). In this context, the optimization of gene expression and protein secretion in lactobacilli and pediococci is another aspect in further development of LAB as cell factories for delivery and production of many other proteins and peptides of biotechnological interest. The supernatants of the recombinant *E. faecium* DCH37 (pMGPAI) and *E. faecium* T136 (pMGPAI) strains showed also a higher antimicrobial activity than that of their respective control strains, suggesting that efforts towards production of bacteriocins in heterologous hosts would also lead to increased bacteriocin production and construction of multibacteriocinogenic strains with higher antimicrobial activity.

## 5. Conclusions

If the use of bacteriocins as natural antimicrobial agents in food, veterinary, and medical applications is ever to meet the high expectations of the research community, then high level production of active bacteriocin is essential. In this study, the production of EntA by *L. lactis* NZ9000, transformed with derivatives of pNZ8048, was 8.7- to 18.5-fold higher, respectively, than by *E. faecium* T136. The production of EntA by *L. lactis* NZ9000, *L. lactis* IL1403 and *Lb. sakei* Lb790, transformed with derivatives of pMSP3545, was 2.5- to 5.3-fold higher than by *E. faecium* T136 while the production of EntA by the *E. faecium* AR24, *E. faecalis* JH2-2, and *P. acidilactici* Ped$^-$ hosts, was similar to that of *E. faecium* T136. The production of EntA by the recombinant LAB strains, transformed with derivatives of pMG36c, was a 0.4- to 5.7-fold higher than by *E. faecium* T136. However, the production of EntA and its biological activity, either against *E. faecium* P13 or *Listeria* spp., varied depending on the signal peptide, the expression vector, and the host strain in the sense that the choice of each component must confer the successful production and functional expression of EntA. Since apparently, the effectiveness of the expression systems is difficult to predict, further research efforts are required for a more efficient genetically engineered EntA production.

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## References

Aukrust, T.W., Brurberg, M.B., Nes, I.F., 1995. Transformation of *Lactobacillus* by electroporation. Methods Mol. Biol. 47, 201–208.

Aymerich, T., Holo, H., Håvarstein, L.S., Hugas, M., Garriga, M., Nes, I.F., 1996. Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin-family of bacteriocins. Appl. Environ. Microbiol. 62, 1676–1682.

Borrero, J., Brede, D.A., Skaugen, M., Diep, D.B., Herranz, C., Nes, I.F., Cintas, L.M., Hernández, P.E., 2011a. Characterization of garvicin ML, a novel circular bacteriocin produced by *Lactococcus garvieae* DCC43, isolated from Mallard ducks (*Anas platyrhynchos*). Appl. Environ. Microbiol. 77, 369–373.

Borrero, J., Jiménez, J.J., Gútiez, L., Herranz, C., Cintas, L.M., Hernández, P.E., 2011b. Use of the usp45 lactococcal secretion signal sequence to drive the secretion and functional expression of enterococcal bacteriocins in *Lactococcus lactis*. Appl. Microbiol. Biotechnol. 89, 131–143.

Brockmeier, U., Caspers, M., Freudl, R., Jockwer, A., Noll, T., Eggert, T., 2006. Systematic screening of all signal peptides from *Bacillus subtilis*: a powerful strategy in optimizing heterologous protein secretion in Gram-positive bacteria. J. Mol. Biol. 362, 393–402.

Bryan, E.M., Bae, T., Kleerebezem, M., Dunny, G.M., 2000. Improved vectors for nisin-controlled expression in gram-positive bacteria. Plasmid 44, 183–190.

Casalta, E., Montel, M.C., 2008. Safety assessment of dairy microorganisms: the *Lactococcus* genus. Int. J. Food Microbiol. 126, 271–273.

Corr, S.C., Li, Y., Riedel, C.U., O'Toole, P.W., Hill, C., Gahan, C.G.M., 2007. Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. Proc. Natl. Acad. Sci. U.S.A. 104, 7617–7621.

Casaus, P., Nilsen, T., Cintas, L.M., Nes, I.F., Hernández, P.E., Holo, H., 1997. Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. Microbiology 143, 2287–2294.

Chopin, M.C., Chopin, A., Moillo-Bott, A., Langella, P., 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. Plasmid 11, 260–263.

Cintas, L.M., Casaus, P., Håvarstein, L.S., Hernández, P.E., Nes, I.F., 1997. Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. 63, 4321–4330.

Cotter, P.D., Hill, C., Ross, R.P., 2005. Bacteriocins: developing innate immunity for food. Nat. Rev. Microbiol. 3, 777–788.

Darmon, E., Noone, D., Masson, A., Bron, S., Kuipers, O.P., Devine, K.M., van Dijl, J.M., 2002. A novel class of heat and secretion stress-responsive genes is controlled by the autoregulated CssRS two-component system of *Bacillus subtilis*. J. Bacteriol. 184, 5661–5671.

De Ruyter, P.G.G.A., Kuipers, O.P., de Vos, W.M., 1996. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol. 62, 3662–3667.

Degering, C., Eggert, T., Puls, M., Bongaerts, J., Evers, S., Maurer, K.H., Jaeger, K.E., 2010. Optimization of protease secretion in *Bacillus subtillis* and *Bacillus licheniformis* by screening of homologous and heterologous signal peptides. Appl. Environ. Microbiol. 76, 6370–6376.

Diep, D.B., Skaugen, M., Salehian, Z., Holo, H., Nes, I.F., 2007. Common mechanisms of target cell recognition and immunity for class II bacteriocins. Proc. Natl. Acad. Sci. U.S.A. 104, 2384–2389.

Diep, D.B., Mathiesen, G., Eijsink, V.G.H., Nes, I.F., 2009. Use of lactobacilli and their pheromone based regulatory mechanism in gene expression and drug delivery. Curr. Pharm. Biotechnol. 10, 62–73.

Driessen, A.J.M., Nouwen, N., 2007. Protein translocation across the bacterial cytoplasmic membrane. Annu. Rev. Biochem. 77, 643–667.

Eijsink, V.G.H., Skeie, M., Middelhoven, P.H., Brurberg, M.B., Nes, I.F., 1998. Comparative studies of class II bacteriocins of lactic acid bacteria. Appl. Environ. Microbiol. 64, 3275–3281.

Fernández, M., Martínez-Bueno, M., Martín, M.C., Valdivia, E., Maqueda, M., 2007. Heterologous expression of enterocin AS-48 in several strains of lactic acid bacteria. J. Appl. Microbiol. 102, 1350–1361.

Fimland, G., Johnsen, L., Axelsson, L., Brurberg, M.B., Nes, I.F., Eijsink, V.G.H., Nissen-Meyer, J., 2000. A C-terminal disulfide bridge in pediocin-like bacteriocins renders bacteriocin activity less temperature dependent and is a major determinant of the antimicrobial spectrum. J. Bacteriol. 182, 2643–2648.

Franz, C.M., van Belkum, M.J., Holzapfel, W.H., Abriouel, H., Gálvez, A., 2007. Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. FEMS Microbiol. Rev. 31, 293–310.

Freitas, D.A., Leclerc, S., Miyoshi, A., Oliveira, S.C., Sommer, P.S.M., Rodrigues, L., Correa, A., Gautier, M., Langella, P., Azevedo, V.A., Le Loir, Y., 2005. Secretion of *Streptomyces tendae* antifungal protein 1 by *Lactococcus lactis*. Braz. J. Med. Biol. Res. 38, 1585–1592.

Gálvez, A., López, R.L., Abriouel, H., Valdivia, E., Omar, N.B., 2008. Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. Crit. Rev. Biotechnol. 28, 125–152.

Gasson, M.J., 1983. Plasmid complements of *Streptococcus lactis* NCDO712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. 154, 1–9.

Gillor, O., Etzion, A., Riley, M.A., 2008. The dual role of bacteriocins as anti- and probiotics. Appl. Microbiol. Biotechnol. 81, 591–606.

Gutiérrez, J., Criado, R., Citti, R., Martín, M., Herranz, C., Fernández, M.F., Cintas, L.M., Hernández, P.E., 2004. Performance and applications of polyclonal antipeptide antibodies specific for the enterococcal bacteriocin enterocin P. J. Agric. Food Chem. 52, 2247–2255.

Gutiérrez, J., Larsen, R., Cintas, L.M., Kok, J., Hernández, P.E., 2006. High-level heterologous production and functional expression of the Sec-dependent enterocin P from *Enterococcus faecium* P13, in *Lactococcus lactis*. Appl. Microbiol. Biotechnol. 72, 41–51.

Håvarstein, L.S., Diep, D.B., Nes, I.F., 1995. A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitantly with export. Mol. Microbiol. 16, 229–240.

Herranz, C., Driessen, A.J.M., 2005. Sec-mediated secretion of enterocin P by *Lactococcus lactis*. Appl. Environ. Microbiol. 71, 1959–1963.

Holo, H., Nes, I.F., 1989. High-frequency transformation by electroporation of *Lactococcus lactis* subsp. cremoris grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. 55, 3119–3123.

Jacob, A., Hobbs, S.J., 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. J. Bacteriol. 117, 360–372.

Johnsen, L., Fimland, G., Eijsink, V., Nissen-Meyer, J., 2000. Engineering increased stability in the antimicrobial peptide pediocin PA-1. Appl. Environ. Microbiol. 66, 4798–4802.

Khan, H., Flint, S., Yu, P.L., 2010. Enterocins in food preservation. Int. J. Food Microbiol. 141, 1–10.

Kim, J.H., Mills, D.A., 2007. Improvement of a nisin-inducible expression vector for use in lactic acid bacteria. Plasmid 58, 275–283.

Kjos, M., Salehian, Z., Nes, I.F., Diep, D.B., 2010. An extracellular loop of the mannose phosphotransferase system component IIC is responsible for specific targeting by class IIa bacteriocins. J. Bacteriol. 192, 5906–5913.

Kleeberezem, M., Beerthuyzen, M., Vaughan, E.E., de Vos, W.M., Kuipers, O.P., 1997. Controlled gene expression systems for lactic acid bacteria: transferable nisin-inducible expression cassettes for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp. Appl. Environ. Microbiol. 63, 4581–4584.

Klocke, M., Mundt, K., Idler, F., Jung, S., Bachausen, J.E., 2005. Heterologous expression of enterocin A, a bacteriocin from *Enterococcus faecium*, fused to a cellulose-binding domain in *Escherichia coli* results in a functional protein with inhibitory activity against *Listeria*. Appl. Microbiol. Biotechnol. 67, 532–538.

Klostermann, K., Crispie, F., Flynn, J., Ross, R.P., Hill, C., Meaney, W., 2008. Intramammary infusion of a live culture of *Lactococcus lactis* for treatment of bovine mastitis: comparison with antibiotic treatment in field trials. J. Dairy Res. 75, 365–373.

Kouwen, T.R.H.M., van der Goot, A., Dorenbos, R., Winter, T., Antelmann, H., Plaisier, M.C., Quax, W.J., van Dijl, J.M., Dubois, J.Y.F., 2007. Thiol-disulphide oxidoreductase molecules in the low-GC Gram-positive bacteria. Mol. Microbiol. 64, 984–999.

Kuipers, O.P., de Ruyter, P.G., Kleerevezem, M., de Vos, W.M., 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. J. Biotechnol. 64, 15–21.

Liu, L., O'Conner, P., Cotter, P.D., Hill, C., Ross, R.P., 2008. Controlling *Listeria monocytogenes* in Cottage cheese through heterologous production of enterocin A by *Lactococcus lactis*. J. Appl. Microbiol. 104, 1059–1066.

Martín, M., Gutiérrez, J., Criado, R., Herranz, C., Cintas, L.M., Hernández, P.E., 2007a. Cloning, production and expression of the bacteriocin enterocin A produced by *Enterococcus faecium* PLBC21 in *Lactococcus lactis*. Appl. Microbiol. Biotechnol. 76, 667–675.

Martín, M., Gutiérrez, J., Criado, R., Herranz, C., Cintas, L.M., Hernández, P.E., 2007b. Chimeras of mature pediocin PA-1 fused to the signal peptide of enterocin P permits the cloning, production, and expression of pediocin PA-1 in *Lactococcus lactis*. J. Food Prot. 70, 2792–2798.

Martínez, J.M., Martínez, M.I., Suárez, A.M., Herranz, C., Casaus, P., Cintas, L.M., Rodríguez, J.M., Hernández, P.E., 1998. Generation of polyclonal antibodies of predetermined specificity against pediocin PA-1. Appl. Environ. Microbiol. 64, 4536–4545.

Martínez, J.M., Kok, J., Sanders, J.W., Hernández, P.E., 2000. Heterologous coproduction of enterocin A and pediocin PA-1 by *Lactococcus lactis*: detection by specific peptide-directed antibodies. Appl. Environ. Microbiol. 66, 3543–3549.

Mathiesen, G., Sveen, A., Piard, J.C., Axelsson, L., Eijsink, V.G.H., 2008. Heterologous protein secretion by *Lactobacillus plantarum* using homologous signal peptides. J. Appl. Microbiol. 105, 215–226.

Mierau, I., Kleerebezem, M., 2005. 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. Appl. Microbiol. Biotechnol. 68, 705–717.

Mohamed, J.A., Murray, B.E., 2006. Influence of the fsr locus on biofilm formation by *Enterococcus faecalis* lacking *gelE*. J. Med. Microbiol. 55, 1747–1750.

Morello, E., Bermúdez-Humarán, L.G., Llull, D., Solé, V., Miraglio, N., Langella, P., Poquet, I., 2008. *Lactococcus lactis*, an efficient cell factory for recombinant protein production and secretion. J. Mol. Microbiol. Biotechnol. 14, 48–58.

Natale, P., Brüsser, T., Driessen, A.J., 2008. Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane: distinct translocases and mechanisms. Biochim. Biophys. Acta 1778, 1735–1756.

Nes, I.F., Diep, D.B., Holo, H., 2007. Bacteriocin diversity in *Streptococcus* and *Enterococcus*. J. Bacteriol. 189, 1189–1198.

Nilsen, T., Nes, I.F., Holo, H., 1998. An exported inducer regulates bacteriocin production in *Enterococcus faecium* CTC492. J. Bacteriol. 180, 1848–1854.

O'Keefee, T., Hill, C., Ross, R.P., 1999. Characterization and heterologous expression of the genes encoding enterocin A production, immunity and regulation in *Enterococcus faecium* DPC1146. Appl. Environ. Microbiol. 65, 1506–1515.

Ravn, P., Arnau, J., Madsen, S.M., Vrang, A., Israelsen, H., 2003. Optimization of signal peptide SP310 for heterologous protein production in *Lactococcus lactis*. Microbiology 149, 2193–2201.

Sánchez, J., Basanta, A., Gómez-Sala, B., Herranz, C., Cintas, L.M., Hernández, P.E., 2007a. Antimicrobial and safety aspects, and biotechnological potential of bacteriocinogenic enterococci isolated from Mallard ducks (*Anas platyrhynchos*). Int. J. Food Microbiol. 117, 295–305.

Sánchez, J., Diep, D.B., Herranz, C., Nes, I.F., Cintas, L.M., Hernández, P.E., 2007b. Amino acid and nucleotide sequence, adjacent genes, and heterologous expression of hiracin JM79, a Sec-dependent bacteriocin produced by *Enterococcus hirae* DCH5, isolated from Mallard ducks (*Anas platyrhynchos*). FEMS Microbiol. Lett. 270, 227–236.

Sánchez, J., Borrero, J., Gómez-Sala, B., Basanta, A., Herranz, C., Cintas, L.M., Hernández, P.E., 2008. Cloning and heterologous production of hiracin JM79, a Sec-dependent bacteriocin produced by *Enterococcus hirae* DCH5, in lactic acid bacteria and *Pichia pastoris*. Appl. Environ. Microbiol. 8, 2471–2479.

Sarvas, M., Harwood, C.R., Bron, S., van Dijl, J.M., 2004. Post-translocational folding of secretory proteins in Gram-positive bacteria. Biochim. Biophys. Acta 1694, 311–327.

Schillinger, U., Lucke, F.K., 1989. Antibacterial activity of *Lactobacillus sake* isolated from milk. Appl. Environ. Microbiol. 55, 1901–1906.

Sit, C.S., Vederas, J.C., 2008. Approaches to the discovery of new antibacterial agents based on bacteriocins. Biochem. Cell Biol. 86, 116–123.

Stammen, S., Müller, B.K., Korneli, C., Biedendieck, R., Gamer, M., Franco-Lara, E., Jahn, D., 2010. High-yield intra- and extracellular protein production using *Bacillus megaterium*. Appl. Environ. Microbiol. 76, 4037–4066.

van de Guchte, M., van der Vossen, J.M., Kok, J., Venema, G., 1989. Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. lactis. Appl. Environ. Microbiol. 55, 224–228.