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Use of the *usp45* lactococcal secretion signal sequence to drive the secretion and functional expression of enterococcal bacteriocins in *Lactococcus lactis*

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Abstract Replacement of the signal peptide (SP) of the bacteriocins enterocin P (EntP) and hiracin JM79 (HirJM79), produced by *Enterococcus faecium* P13 and *Enterococcus hirae* DCH5, respectively, by the signal peptide of Usp45 (SP_{usp45}), the major Sec-dependent protein secreted by *Lactococcus lactis*, permits the production, secretion, and functional expression of EntP and HirJM79 by *L. lactis*. Chimeric genes encoding the SP_{usp45} fused to either mature EntP (*entP*), with or without the immunity gene (*entiP*) or to mature HirJM79 (*hirJM79*), with or without the immunity gene (*hiriJM79*), were cloned into the expression vector pMG36c, carrying the P_{32} constitutive promoter, and into pNZ8048 under control of the inducible P*nisA* promoter. The production of EntP and HirJM79 by most of the *L. lactis* recombinant strains was 1.5- to 3.7-fold higher and up to 3.6-fold higher than by the *E. faecium* P13 and *E. hirae* DCH5 control strains, respectively. However, the specific antimicrobial activity of the recombinant EntP was 1.1- to 6.2-fold higher than that produced by *E. faecium* P13, while that of the HirJM79 was a 40% to an 89% of that produced by *E. hirae* DCH5. Chimeras of SP_{usp45} fused to mature EntP or HirJM79 drive the production and secretion of these bacteriocins in *L. lactis* in the absence of specific immunity and secretion proteins. The supernatants of the recombinant *L. lactis* NZ9000 strains, producers of EntP, showed a much higher antimicrobial activity against *Listeria* spp. than that of the recombinant *L. lactis* NZ9000 derivatives, producers of HirJM79.

Keywords Bacteriocins · Enterocin P · Hiracin JM79 · Heterologous production · Sec-dependent secretion · Lactic acid bacteria (BAL) · *Lactococcus lactis*

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Introduction

Many lactic acid bacteria (LAB) produce ribosomally synthesized antimicrobial peptides or proteins, more commonly referred to as bacteriocins (Nes et al. 1996; Eijsink et al. 2002). Furthermore, since bacteriocins produced by LAB display antimicrobial activity against a broad range of Gram-positive bacteria and, to a lesser extent, gram-negative bacteria, they are attracting interest for their potential use as natural and nontoxic food preservatives, for human and veterinary applications, and in the animal production field (Cintas et al. 2001; Deegan et al. 2006; Gálvez et al. 2007; Khan et al. 2010). However, the high cost of synthetic peptide bacteriocins and their low yields from many natural producers drive the exploration of alternative microbial systems for the heterologous production of bacteriocins. The ability of the enterococci, that are LAB members of the gut microbiota of humans and animals and part of the food microbiota, to produce antimicrobial peptides referred to as enterocins, is also widely recognized (Franz et al. 2007; Nes et al. 2007; Birri et al. 2010). However, since enterocins may be produced by enterococcal species carrying antibiotic resistance genes and/or genes coding for potential virulence factors, it would be desirable to produce and secrete their bacteriocins from alternative and safer microbial hosts (Gutiérrez et al. 2006; Liu et al. 2008; Sánchez et al. 2008).

In the native bacterial strains, most bacteriocins produced by LAB are synthesized as biologically inactive precursors or propeptides containing an N-terminal extension. The mature peptides are often cationic, amphiphilic, membrane-permeabilizing molecules that are classified in two major groups: the class I lantibiotics, containing post-translationally modified amino acids and the class II non-lantibiotics, containing non-modified amino acids (Cotter et al. 2005; Nes et al. 2007). The N-terminal extensions of most lantibiotics and non-lantibiotics are of the so-called double-glycine type (leader peptide) 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; Venema et al. 1995). However, some class II bacteriocins, such as enterocin P (Cintas et al. 1997; Herranz and Driessen 2005) 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 bacteriocin externalization by the general secretory pathway (GSP) or Sec-dependent pathway.

The Sec-dependent pathway is a universally conserved 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 et al. 2008). Accordingly, the N-terminal SP of secretory proteins may drive the access of the fused mature proteins or peptides to the GSP for their secretion by the heterologous producer cells. The bacteriocin enterocin P (EntP), produced by *Enterococcus faecium* P13, is synthesized as a 71-amino-acid peptide consisting of a 27-amino-acid SP (SP_{EntP}) and the 44-amino-acid mature bacteriocin (Cintas et al. 1997), whereas hiracin JM79 (HirJM79), synthesized by *Enterococcus hirae* DCH5, consists of a 74-amino-acid peptide containing a 30-amino-acid SP ($SP_{HirJM79}$) and the 44-amino-acid mature bacteriocin (Sánchez et al. 2007b). The *ups45* gene encodes the major extracellular protein Usp45 from *Lactococcus lactis* MG1363, and chimeras of the 27-amino-acid Usp45 signal peptide (SP_{usp45}), fused to a number of mature proteins and peptides have been used to drive their secretion by *L. lactis* (van Asseldonk et al. 1990; Mierau and Kleerebezem 2005; Kuipers et al. 2006; Morello et al. 2008). The potent antimicrobial activity of the bacteriocins EntP and HirJM79 against pathogenic bacteria such as *Listeria monocytogenes* has driven interest for their biotechnological production by heterologous hosts (Gutiérrez et al. 2006; Sánchez et al. 2008). We report in this work the production, secretion, and functional expression of the bacteriocins EntP and HirJM79 by constructing chimeras of the SP_{usp45} fused to mature *entP* and *hirJM79* with or without their corresponding immunity genes, their cloning into different lactococcal expression vectors, and the use of derivatives of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* as the production hosts.

Material and methods

Bacterial strains, plasmids, growth conditions, and antimicrobial activity assays

LAB strains and plasmids used in this work are listed in Table 1. *E. faecium* P13 and *E. hirae* DCH5 were used as the source of EntP (Cintas et al. 1997) and HirJM79 (Sánchez et al. 2007a), respectively. *E. faecium* T136 ($EntP^S$, $HirJM79^S$) (Casaus et al. 1997) and *L. lactis* MG1363 ($EntP^R$, $HirJM79^R$, $NisA^S$) were used as indicator strains for determination of antimicrobial activities. The primary amino acid and nucleotide sequence of the structural *entP* and *hirJM79* genes and their bacteriocin immunity genes in *E. faecium* P13 and *E. hirae* DCH5, are given by the GenBank accession numbers AF005276 and DQ664500, respectively. The lactococcal strains were grown in M17 medium (Oxoid Ltd., Basingstoke, UK) supplemented with 0.5% (*w*/*v*) glucose (GM17) at 32°C. Enterococcal strains were grown in MRS broth (Oxoid) at 32°C. Cell dry weights of late exponential phase cultures were determined gravimetrically. All *Listeria* strains were grown in BHI broth (Oxoid) at 32°C. Agar plates were made by addition of 1.5% (*w*/*v*) agar (Oxoid) to the liquid media. Chloramphenicol (Sigma Chemical Co., St. Louis, MO, USA) was added to the cultures of recombinant *L. lactis* at 5 $\mu g ml^{-1}$. The antimicrobial activity of individual colonies was examined by the stab-on-agar test (SOAT), as previously described (Cintas et al. 1997). Recombinant cultures of *L. lactis* NZ9000 were induced for production of EntP and HirJM79 when they reached an optical density at 600 nm (OD_{600}) of 0.5 using as inducer nisin A (NisA) from the supernatant of *L. lactis* BB24 (NisA producer) at a final concentration of 10 $ng ml^{-1}$. 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 supernatants was examined by an agar well diffusion test (ADT) and a microtiter plate assay (MPA), as previously described (Gutiérrez et al. 2006). With the MPA, growth inhibition of sensitive culture was measured spectrophotometrically at 620 nm with a microtiter Labsystems iEMS plate reader (Labsystems,

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source and/or reference[b]
Strains		
E. faecium		
T136	Enterocin A and B producer; MPA and ADT indicator	DNBTA, Casaus et al. (1997)
P13	Enterocin P producer; control strain	DNBTA, Cintas et al. (1997)
DCH5	Hiracin JM79 producer; control strain	DNBTA, Sánchez et al. (2007b)
L. lactis subsp. *lactis*		
IL1403	Plasmid-free derivative of IL594	Chopin et al. (1984)
DPC5598	Plasmid-free derivative of DPC4268	DPC, Trotter et al. (2002)
BB24	Nisin A producer	DNBTA, Cintas et al. (1998)
L. lactis subsp. *cremoris*		
NZ9000	MG1363 pepN::*nisRK*	NIZO, Kuipers et al. (1998)
MG1363	Plasmid-free derivative of NCDO712; source of *usp45*; ADT indicator	IFR, Gasson (1983)
Plasmids		
pMG36c	Cm^r, pMG36e derivative	RUG-MG, Van de Guchte et al. (1989)
pNZ8048	Cm^r; inducible expression vector carrying the *nisA* promoter	NIZO, Kuipers et al. (1998)
pMUPE	Cm^r, pMG36c derivative carrying the PCR product D (P_{32} ribosome binding site and the SP_{usp45} fused to mature *ent*P)	This work
pMUPEI	Cm^r, pMG36c derivative carrying the PCR product E (P_{32} ribosome binding site and the SP_{usp45} fused to mature *ent*P and *enti*P)	This work
pNUPE	Cm^r, pNZ8048 derivative carrying the PCR product F (SP_{usp45} fused to mature *ent*P gene)	This work
pNUPEI	Cm^r, pNZ8048 derivative carrying the PCR product G (SP_{usp45} fused to mature *ent*P+*enti*P)	This work
pMUHE	Cm^r, pMG36c derivative carrying the PCR product K (P_{32} ribosome binding site and the SP_{usp45} fused to mature *hirJM79*)	This work
pMUHEI	Cm^r, pMG36c derivative carrying the PCR product L (P_{32} ribosome binding site and the SP_{usp45} fused to mature *hirJM79*+*hiriJM79*)	This work
pNUHE	Cm^r, pNZ8048 derivative carrying the PCR product M (SP_{usp45} fused to mature *hirJM79*)	This work
pNUHEI	Cm^r, pNZ8048 derivative carrying the PCR product N (SP_{usp45} fused to mature *hirJM79*+*hiriJM79*)	This work

[a] *ADT* agar well diffusion test, *MPA* microtiter plate asay, *Cm^r^* chloramphenicol resistance, [b] *DNBTA* Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid (Madrid, Spain), *DPC* Teagasc Dairy Products Research Centre, Moorepark, Fermoy, Co. (Cork, Ireland), *NIZO* Department of Biophysical Chemistry, NIZO Food Research (Ede, The Netherlands), *IFR* Institute of Food Research (Norwich, United Kingdom), *RUG-MG* Department of Molecular Genetics, University of Groningen (Haren, The Netherlands)

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 recombinant *L. lactis* hosts was also tested against six *L. monocytogenes* and five *Listeria* spp., obtained from the Colección Española de Cultivos Tipo, Valencia, Spain (CECT).

Basic genetic techniques and enzymes

Total genomic DNA from *E. faecium* P13, *E. hirae* DCH5, and *L. lactis* MG1363 was isolated using the Wizard® DNA Purification Kit (Promega, Madison, WI, USA). Plasmid DNA isolation from *L. lactis* was carried out using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany), but with the *L. lactis* cells previously suspended

with lysozyme (40 mg ml^{-1}) and mutanolysin (500 U ml^{-1}) and incubated at 37°C for 10 min before following the kit instructions. All DNA restriction enzymes were purchased from New England BioLabs (Beverly, MA, USA). Ligations were performed with the T4 DNA ligase (Roche). Electrocompetent *L. lactis* cells were transformed according to the method of Holo and Nes (1989), with a Gene Pulser™ and Pulse Controller apparatus (Bio-Rad Laboratories, Hercules, CA, USA).

PCR amplification and nucleotide sequencing

Oligonucleotide primers were obtained from Sigma-Genosys Ltd. (Cambridge, UK). PCR amplifications of inserts 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) with proofreading 3′ to 5′ exonuclease activity. 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°C to 60°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 the NucleoSpin® Extract II Kit (Macherey-Nagel GmbH, Düren, Germany) for cloning and 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).

Recombinant plasmids derived from pMG36c

The primers and inserts used for construction of the recombinant plasmids are listed in Table 2. Derivatives of plasmid pMG36c were constructed as follows: primers USP-F and USPEP-R were used for PCR amplification from total genomic DNA of *L. lactis* MG1363 of a 110-bp *Sac*I fragment (A) containing the P_{32} ribosome binding site (RBS), the signal peptide of the Usp45 protein (SP_{usp45}), and the N-terminal nucleotide sequence of the mature EntP structural gene (*entP*). Primers EP-F and LJ2-R were used for PCR amplification from total genomic DNA of *E. faecium* P13 of a 147-bp *Hind*III fragment (B) containing the mature *entP* structural gene. Primers EP-F and LJ3A-R were used for PCR amplification from the same DNA target of a 407-bp fragment (C) containing the mature *entP* structural gene with the immunity gene (*entiP*). Mixtures of fragments A and B and that of fragments A and C were used as templates to amplify by PCR a 230-bp *Sac*I–*Hind*III fragment (D) and a 490-bp *Sac*I–*Hind*III fragment (E), using the primer pairs USP-F-F/LJ2-R and USP-F/LJ3A-R, respectively. Similarly, primers USP-F and USPHA-R were used for PCR amplification of the *L. lactis* MG1363 DNA, obtaining a fragment similar to SUP (H) except for the substitution of the N-terminal nucleotide sequence of *entP* by that of the mature HirJM79 structural gene (*hirJM79*). Primers HA-F and HPJE-R were used for PCR amplification from total genomic DNA of *E. hirae* DCH5 of a 151-bp *Hind*III fragment (I) containing the *hirJM79* structural gene. Primers HA-F and HPJEI-R were used for PCR amplification from the same DNA target of a 464-bp fragment (J) containing the *hirJM79* structural gene with the immunity gene (*hiriJM79*). Mixtures of fragments H and I and that of fragments H and J were used as templates to amplify by PCR a 232-bp *Sac*I–*Hind*III fragment (K) and a 545-bp *Sac*I–*Hind*III fragment (L) using, respectively, the primer pairs USP-F-F/HPJE-R and USP-F/HPJEI-R. Fragments D, E, K, and L were digested with the indicated restriction enzymes and inserted into pMG36c, digested with the same enzymes. The ligation mixtures were used to transform *L. lactis* IL1403, *L. lactis* NZ9000, and *L. lactis* DPC5598 competent cells. The plasmid derivatives pMUPE, pMUPEI, pMUHE, and pMUHEI were checked by bacteriogenicity tests, PCR, and sequencing of the inserts.

Recombinant plasmids derived from pNZ8048

To construct the recombinant plasmids derived from pNZ8048, the purified pMUPE, pMUPEI, pMUHE, and pMUHEI plasmids were used, respectively, as the PCR templates with the primer pairs USPBS-F/LJ2-R, USPBS-F/LJ3A-R, USPBS-F/HPJE-R, and USPBS-F/HPJEI-R, for generation of a 224-bp fragment (F), a 484-bp fragment (G), a 226-bp fragment (M), and a 539-bp fragment (N). The purified inserts were ligated as *Bsph*I–*Hind*III fragments into plasmid pNZ8048, previously digested with the enzymes *Nco*I and *Hind*III, and the ligation mixtures were used to transform the *L. lactis* NZ9000 competent cells. The plasmid derivatives pNUPE, pNUPEI, pNUHE, and pNUHEI were checked by bacteriogenicity tests, PCR, and sequencing of the inserts.

ELISA for detection and quantification of EntP and HirJM79

Polyclonal antibodies with predetermined specificity for EntP (Gutiérrez et al. 2004) and HirJM79 (Sánchez et al. 2008) and a non-competitive indirect enzyme-linked immunosorbent assay (NCI-ELISA) were used to detect and quantify the EntA and HirJM79 of the supernatants of the producer cells, as previously described (Gutiérrez et al. 2004). Briefly, wells of flat-bottom polystyrene microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated

Table 2 Primers and PCR products used in this study

Primer or PCR product	Nucleotide sequence (5′–3′) or description[a]	PCR product amplified
Primers		
USP-F	CATA*GAGCTC*TGTAA**GGAGG**ATTTTTGAAATGAAAAAAAAGATTATCTCAGCTAT	A, D, E, H, K and L
USPEP-R	AACACCATTACCATATGAACGCGTAGCAGCGTAAACACCTGACAACGGG	A
EP-F	GCTACGCGTTCATATGGTAATGGTGTTTATTGT	B and C
LJ2-R	ATAAGTT*AAGCTT*GTATTAATG TCCCATACCTGCCAAACCAG	B, D and F
LJ3A-R	ATAAGTT*AAGCTT*GTATCAAAGTCCCGACCATGCTTTGG	C, E and G
USPBS-F	ATAAACTCA*TCATGA*AAAAAAAGATTATCTCAGCTATTTTAATGTCTAC	F, G, M and N
USPHA-R	CAATAAAGACCATTTCCATAGTAAGTAGCAGCGTAAACACCTGACAACGGG	H
HA-F	GCTACTTACTATGGAAATGGTCTTTATTGTAACAAAG	I and J
HPJE-R	ATAAGTT*AAGCTT*GTACTACCTTCTAGGTGCCCATGGACC	I, K and M
HPJEI-R	ATAAGTT*AAGCTT*GTATTAACTCCAAATACCAATAGAAGCCCATC	J, L and N
PCR product		
A	110-bp *Sac*I fragment containing the P_{32} ribosome binding site and the SP_{usp45}	
B	147-bp *Hind*III fragment containing mature *entP*	
C	407-bp *Hind*III fragment containing mature *entP* and *entiP*	
D	230-bp *SacI*/*Hind*III fragment containing the P_{32} ribosome binding site and the SP_{usp45} fused to mature *entP*	
E	490-bp *SacI*/*Hind*III fragment containing the P_{32} ribosome binding site and the SP_{usp45} fused to mature *entP* and *entiP*	
F	224-bp *Bsp*HI/*Hind*III fragment containing the SP_{usp45} fused to mature *entP*	
G	484-bp *Bsp*HI/*Hind*III fragment containing the SP_{usp45} fused to mature *entP* and *entiP*	
H	110-bp *Sac*I fragment containing the P_{32} ribosome binding site and the Usp45 signal peptide (SP_{usp45})	
I	151-pb *Hind*III fragment containing mature *hirJM79*	
J	464-pb *Hind*III fragment containing mature *hirJM79* and *hiriJM79*	
K	232-pb *SacI*/*Hind*III fragment containing the P_{32} ribosome binding site and the SP_{usp45} fused to mature *hirJM79*	
L	545-pb *SacI*/*Hind*III fragment containing the P_{32} ribosome binding site and the SP_{usp45} fused to mature *hirJM79* and *hiriJM79*	
M	226-bp *Bsp*HI/*Hind*III fragment containing the SP_{usp45} fused to mature *hirJM79*	
N	539-bp *Bsp*HI/*Hind*III fragment containing the SP_{usp45} fused to mature *hirJM79* and *hiriJM79*	

[a] Cleavage site for restriction enzymes is italicized; P_{32} ribosome binding site is shown in bold

overnight (4°C) with supernatants from *E. faecium* P13, *E. hirae* DCH5, or the recombinant *L. lactis* hosts. After addition of the anti-EntP or the anti-HirJM79 antibodies and the goat antirabbit immunoglobulin G (IgG) 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.

Purification of EntP and HirJM79, mass spectrometry analysis, and Western blotting assays

EntP and HirJM79 were purified from *L. lactis* NZ9000 (pMUPE) and *L. lactis* NZ9000 (pMUHE), respectively, 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 EntP and HirJM79 were estimated using the extinction coefficient of each bacteriocin 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). Aliquots of purified EntP and HirJM79 were analyzed by Western blotting after tricine (16% *w*/*v*)-

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Schägger and von Jagow 1987) in a Mini-Protean II cell (Bio-Rad). Electrophoresis gels were blotted onto an Immun-Blot™ polyvinylidene difluoride membrane (pore size 0.2 μm) (Bio-Rad), and Western blotting was done essentially as previously described (Gutiérrez et al. 2004) using anti-EntP (Gutiérrez et al. 2004) and anti-HirJM79 (Sánchez et al. 2008) specific antibodies and the ECL Western blotting Detection Reagents (Amersham) for chemiluminescence detection with the Odissey Infrarred Imaging System (LiCor Biosciences, Lincoln, NE, USA).

Results

Construction of the recombinant *Lactococcus lactis* strains for production of EntP or HirJM79

Cloning of chimeras containing the SP_{usp45} fused to mature *entP*, with or without *entiP*, into pMG36c resulted in plasmids pMUPE (SP_{usp45}:*entP*) and pMUPEI (SP_{usp45}: *entP*+*entiP*), while cloning of the same fragments into pNZ8048 originated plasmids pNUPE (SP_{usp45}: *entP*) and pNUPEI (SP_{usp45}: *entP*+*entiP*). Similarly, cloning of PCR fragments containing the SP_{usp45} fused to mature *hirJM79*, with or without *hiriJM79*, into pMG36c resulted in plasmids pMUHE (SP_{usp45}: *hirJM79*) and pMUHEI (SP_{usp45}: *hirJM79*+*hiriJM79*), whereas cloning of the same fragments into pNZ8048 originated plasmids pNUHE (SP_{usp45}: hirJM79) and pNUHEI (SP_{usp45}: *hirJM79*+*hiriJM79*).

Heterologous production and functional expression of EntP and HirJM79 by different *L. lactis* strains

When supernatants of recombinant *L. lactis* subsp. *cremoris* NZ9000 strains, transformed with derivatives of vectors pMG36c or pNZ8048, were assayed against *E. faecium* T136 and *L. monocytogenes* 4032, it was noted that the *L. lactis* NZ9000 producers showed a higher antimicrobial activity than the original EntP and HirJM79 enterococcal producer strains. Interestingly, *L. lactis* NZ9000 (pNUPE) and *L. lactis* NZ9000 (pNUPEI) showed larger halos of activity against *L. monocytogenes* CECT4032 than against *E. faecium* T136, while the opposite occurred for *L. lactis* NZ9000 (pNUHE) and *L. lactis* NZ9000 (pNUHEI) (Fig. 1). The secretion and functional expression of EntP and HirJM79 by the various recombinant *L. lactis* strains was further quantified. Transformation of competent *L. lactis* subsp. *lactis* IL1403, *L. lactis* subsp. *lactis* DPC5598, and *L. lactis* subsp. *cremoris* NZ9000 hosts with the recombinant plasmids showed that while the supernatants of the control strains *L. lactis* IL1403 (pMG36c), *L. lactis* NZ9000 (pMG36c), and *L. lactis* NZ9000 (pNZ8048) did not show any antagonistic effect, the supernatants of all recombinant *L. lactis* hosts showed a measurable production of EntP (Table 3) and HirJM79 (Table 4), as well as a measurable antimicrobial activity and specific antimicrobial activity against *E. faecium* T136 (Tables 3 and 4). *L. lactis* DPC5598 (pMG36c) showed a weak antimicrobial effect against *E. faecium* T136 because of the production of NisA.

As shown in Table 3, the production of EntP by *L. lactis* IL1403 (pMUPE) and *L. lactis* (pMUPEI) was 3.2- to 3.7-fold higher, respectively, than that of *E. faecium* P13, while the production of EntP in the supernatants of *L. lactis* NZ9000 (pMUPE) and *L. lactis* NZ9000 (pMUEI) was 1.5- to 1.6-fold higher, respectively, than that of *E. faecium* P13. Similarly, the antimicrobial activity of the supernatants of *L. lactis* IL1403 (pMUPE) and *L. lactis* (pMUPEI) was 1.7- to 1.8-fold higher, respectively, than that in the supernatant of *L. lactis* NZ9000 (pMUPE) and *L. lactis* NZ9000 (pMUEI). The production of EntP by *L. lactis* DPC5598 (pMUPE) and *L. lactis* DPC5598 (pMUPEI) was 13% of that of *E. faecium* P13, and the specific antimicrobial activity of the EntP produced by these derivatives was lower than that calculated for the *L. lactis* IL1403 and *L. lactis* NZ9000 strains carrying the same recombinant plasmids. Furthermore, the content of EntP in the supernatants of *L. lactis* NZ9000 (pNUPE) and *L. lactis* NZ9000 (pNUPEI) was 2.5- to 3.1-fold higher, respectively, than in *E. faecium* P13, while their antimicrobial activity was 9.8- to 19.4-fold higher than that observed in the *E. faecium* P13 supernatant.

The heterologous production and functional expression of the HirJM79 produced by the various *L. lactis* derivatives is shown in Table 4. Although production of HirJM79 is 34% higher in *L. lactis* IL1403, transformed with pMUHE and pMUHEI than in *L. lactis* NZ9000, transformed with the same plasmids, the latter show a 10% to 19% higher antimicrobial activity than the formers. The production of HirJM79 in the supernatants of *L. lactis* DPC5598 was up to 2.0-fold higher than that of *E. hirae* DCH5 but lower than that calculated for the *L. lactis* IL1403 and *L. lactis* NZ9000 strains carrying either pMUHE or pMUHEI. However, the *L. lactis* NZ9000 (pNUHE) and the *L. lactis* (pNUHEI) transformants showed a 2.7- to 3.6-fold higher production, respectively, of HirJM79 than *E. hirae* DCH5, while their antimicrobial activity was 2.1- to 3.2-fold higher than that by *E. hirae* DCH5.

Purification of recombinant EntP and HirJM79, mass spectrometry analysis, and Western blotting

Results of the purification of EntP and HirJM79 from the *L. lactis* NZ9000 (pMUPE) and *L. lactis* NZ9000 (pMUHE) supernatants, respectively, are summarized in Table 5.

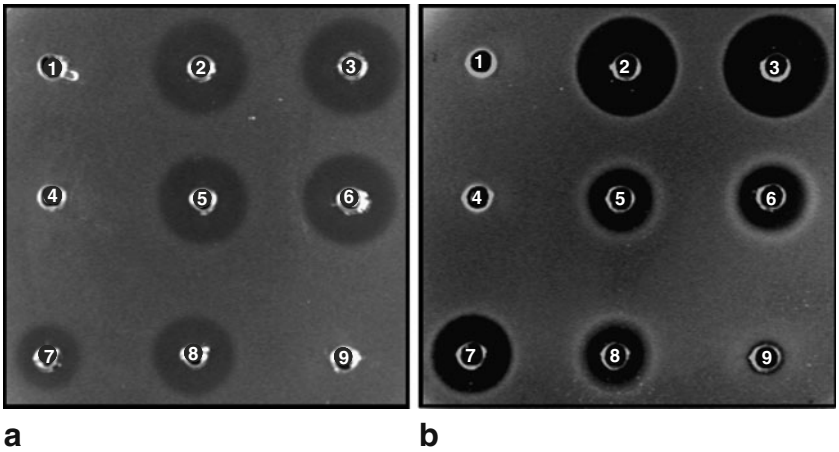

Fig. 1 Antimicrobial activity of supernatants of *L. lactis* NZ9000 as determined by an agar well difussion test (ADT) with *E. faecium* T136 (**a**) and *L. monocytogenes* 4032 (**b**) as the indicator microorganisms. Supernatants of: (*1*) *L. lactis* NZ9000 (pNZ8048); (*2*) *L. lactis* NZ9000 (pNUPE); (*3*) *L. lactis* NZ9000 (pNUPEI); (*4*) *L. lactis* NZ9000 (pNZ8048); (*5*) *L. lactis* NZ9000 (pNUHE); (*6*) *L. lactis* NZ9000 (pNUHEI); (*7*) *E. faecium* P13; (*8*) *E. hirae* DCH5; (*9*) *L. lactis* BB24

Purification of the antimicrobial activity of *L. lactis* NZ9000 (pMUPE) permitted a 61,111-fold increase of its specific activity and a 14% recovery of the initial antimicrobial activity. MALDI-TOF mass spectrometry analysis of the purified antagonistic activity showed a major peak of 4,629.12 Da, suggesting that EntP was purified to homogeneity (Fig. 2a). Purification of the antimicrobial activity of *L. lactis* NZ9000 (pMUHE) also permitted the recovery of a major peak of antimicrobial activity, with an 87,500-fold increase of its specific activity

Table 3 Bacteriocin production and antimicrobial activity of supernatants from recombinant *L. lactis* strains producing enterocin P (EntP)

Strain	Bacteriocin production (μg mg^{-1} cell dry weight)[a]		Antimicrobial activity (BU mg^{-1} cell dry weight)[b]		Specific antimicrobial activity
	Enterocin P	Nisin A	*E. faecium* T136	*L. lactis* MG1363	(BU μg^{-1} EntP)[c]
L. lactis subsp. *lactis*					
IL1403 (pMG36c)	NP		NA		NE
IL1403 (pMUPE)	23.45		2,241		96
IL1403 (pMUPEI)	26.73		2,498		94
DPC5598 (pMG36c)	NP	1.33	42	562	NE
DPC5598 (pMUPE)	0.96	1.41	55	591	57
DPC5598 (pMUPEI)	0.99	1.62	49	659	50
BB24[d]	NP	2.64	73	1,052	NE
L. lactis subsp. *cremoris*					
NZ9000 (pMG36c)	NP		NA		NE
NZ9000 (pMUPE)	11.09		1,440		130
NZ9000 (pMUPEI)	11.75		1,540		131
NZ9000 (pNZ8048)	NP		NA		NE
NZ9000 (pNUPE)	18.04		5,778		320
NZ9000 (pNUPEI)	22.44		11,458		511
E. faecium[d]					
P13	7.20		590		82

Most of the data are mean from two independent determinations in triplicate

NP no production, *NA* no activity, *NE* not evaluable

[a] Production of EntP was calculated by using a NCI-ELISA with polyclonal antibodies specific for EntP. Production of NisA by *L. lactis* DPC5598 was estimated according to its antimicrobial activity and related to the production of NisA and the antimicrobial activity *of L. lactis* BB24 (Suárez et al. 1996)

[b] Antimicrobial activity was calculated against *E. faecium* T136 ($EntP^S$, $NisA^S$) and *L. lactis* MG1363 ($NisA^S$, $EntP^R$)

[c] Specific antimicrobial activity refers to the antimicrobial activity against *E. faecium* T136 divided by the EntP produced

[d] Cultures of *L. lactis* BB24 and *E. faecium* P13 were used as controls for NisA and EntP production, respectively

Table 4 Bacteriocin production and antimicrobial activity of supernatants from recombinant *L. lactis* strains producing hiracin JM79 (HirJM79)

Strain	Bacteriocin production (μg mg^{-1} cell dry weight)[a]		Antimicrobial activity (BU mg^{-1} cell dry weight)[b]		Specific antimicrobial activity
	Hiracin JM79	Nisin A	*E. faecium* T136	*L. lactis* MG1363	(BU μg^{-1} EntP)[c]
L. lactis subsp. *lactis*					
IL1403 (pMG36c)	NA		NA		NE
IL1403 (pMUHE)	3.65		7,297		1,999
IL1403 (pMUHEI)	3.71		10,290		2,773
DPC5598 (pMG36c)	NA	1.38	NA	562	NE
DPC5598 (pMUHE)	2.76	1.97	5,263	798	1,907
DPC5598 (pMUHEI)	1.41	1.42	3,272	575	2,321
BB24[d]	NA	2.60	157	1,052	NE
L. lactis subsp. *cremoris*					
NZ9000 (pMG36c)	NA		NA		NE
NZ9000 (pMUHE)	2.41		8,911		3,697
NZ9000 (pMUHEI)	2.58		11,350		4,399
NZ9000 (pNZ8048)	NA		NA		NE
NZ9000 (pNUHE)	3.70		14,228		3,845
NZ9000 (pNUHEI)	4.89		21,541		4,405
E. hirae[d]					
DCH5	1.35		6,623		4,906

Most of the data are mean from two independent determinations in triplicate

NP no production, *NA* no activity, *NE* not evaluable

[a] Production of HirJM79 was calculated by using a NCI-ELISA with polyclonal antibodies specific for EntP. Production of NisA by *L. lactis* DPC5598 was estimated according to its antimicrobial activity and related to the production of NisA and the antimicrobial activity *of L. lactis* BB24 (Suárez et al. 1996)

[b] Antimicrobial activity was calculated against *E. faecium* T136 ($HirJM79^S$, $NisA^S$) and *L. lactis* MG1363 ($NisA^S$, $HirJM79^R$)

[c] Specific antimicrobial activity refers to the antimicrobial activity against *E. faecium* T136 divided by the HirJM79 produced

[d] Cultures of *L. lactis* BB24 and *E. hirae* DCH5 were used as controls for NisA and HirJM79 production, respectively

and a final recovery of 6% of the initial antimicrobial activity. MALDI-TOF mass spectrometry analysis of the purified activity showed a major peak of 5,091.68 Da (Fig. 2b), suggesting that HirJM79 was also purified to homogeneity. Further characterization of the purified recombinant EntP and HirJM79 by Western blotting confirmed that the anti-EntP and anti-HirJM79 antibodies displayed full specificity against each bacteriocin, and that both purified bacteriocins but, mostly EntP, showed a strong tendency to aggregate (Fig. 3).

Antimicrobial activity of supernatants from recombinant *L. lactis* strains against *Listeria* spp.

The antimicrobial activity of supernatants from *L. lactis* NZ9000 (pNUPE) and *L. lactis* NZ9000 (pNUPEI), producers of EntP, and that of *L. lactis* NZ9000 (pNUHE) and *L. lactis* (pNUHEI), producers of HirJM79, was evaluated against *L. monocytogenes*, *Listeria ivanovii*, *Listeria grayii*, *Listeria welshimeri*, *Listeria seeligeri*, and *Listeria innocua* strains (Table 6). The supernatants of the recombinant *L. lactis* strains producing EntP showed a 3.0- to 18-fold higher antimicrobial activity than that of *E. faecium* P13 against the tested *Listeria* spp. The supernatants of the recombinant *L. lactis* strains producing HirJM79 showed up to a 2.8-fold higher antimicrobial activity than that of *E. hirae* DCH5 against the evaluated *Listeria* spp. The antilisterial activity of the recombinant *L. lactis* strains, producers of EntP, is higher than that of the *L. lactis* derivatives, producers of HirJM79, as it is also observed with the supernatants of *E. faecium* P13 and *E. hirae* DCH5 natural producers of EntP and HirJM79, respectively (Fig. 1).

Discussion

The production of bacteriocins relies on the use of expression vectors able to replicate in Gram-negative bacteria, Gram-positive bacteria, and yeasts. Similarly, the

Table 5 Purification of enterocin P from *L. lactis* NZ9000 (pMUPE) and hiracin JM79 from *L. lactis* NZ9000 (pMUHE)

Supernatant and purification stage	Volume (ml)	Total A_{254}[a]	Total activity (10^3 BU)[b]	Specific activity[c]	Increase in specific activity[d] (fold)	Yield (%)
L. lactis NZ9000 (pMUPE)						
Culture supernatant	1,000	36,800	307	8	1	100
Fraction:						
Ammonium sulfate precipitation	100	2,540	398	157	20	130
Gel filtration chromatography	200	1,260	109	87	11	36
Cation-exchange chromatography	50	15	12	800	100	4
Hydrophobic-interaction chromatography	10	2	7	3,500	438	2
Reverse phase-FPLC	1.2	0.09	44	488,889	61,111	14
L. lactis NZ9000 (pMUHE)						
Culture supernatant	1,000	30,000	227	8	1	100
Fraction:						
Ammonium sulfate precipitation	100	1,850	69	37	5	30
Gel filtration chromatography	200	1,140	47	41	5	21
Cation-exchange chromatography	50	16	32	2,000	250	14
Hydrophobic-interaction chromatography	10	3	28	9,333	1,167	12
Reverse phase-FPLC	0.7	0.02	14	700,000	87,500	6

[a] Absorbance at 254 nm multiplied by the volume in milliliters

[b] Antimicrobial activity in bacteriocin units per milliliter (BU/ml) and multiplied by the total volume

[c] Specific activity is the number of bacteriocins units divided by the total A_{254}

[d] The specific activity of a fraction divided by the specific activity of the culture supernatant

production of bacteriocins in heterologous LAB hosts may be essentially based on the expression of native biosynthetic genes, by exchanging or replacing 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). Previous studies have reported the production and secretion of EntP and HirJM79 from the structural *entP* and *hirJM79* genes expressed by recombinant *L. lactis* hosts (Herranz and Driessen 2005; Gutiérrez et al. 2006; Sánchez et al. 2008). However, of interest was to evaluate the replacement of the enterococcal signal peptides by the *usp45* lactococcal secretion signal sequence (SP_{usp45}) and to determine its effect on the production, secretion, and functional expression of EntP and HirJM79 by *L. lactis*.

In contrast to many studies that report the heterologous production of antimicrobial peptides by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis, the use of anti-EntP and anti-HirJM79 antibodies and a NCI-ELISA allowed us to detect and quantify both bacteriocins in the supernatants of the recombinant LAB strains and to determine the

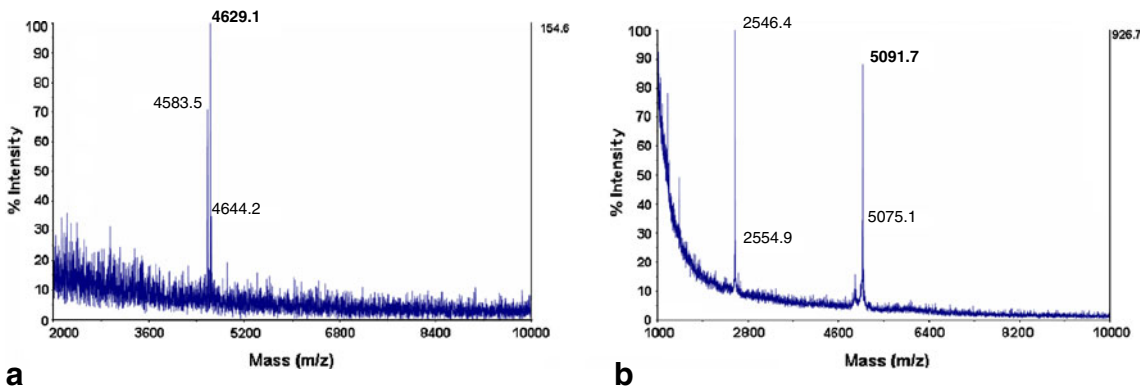

Fig. 2 Mass spectrometry analysis of (**a**) purified enterocin P from *L. lactis* NZ9000 (pMUPE) and (**b**) purified hiracin JM79 from *L. lactis* NZ9000 (pMUHE)

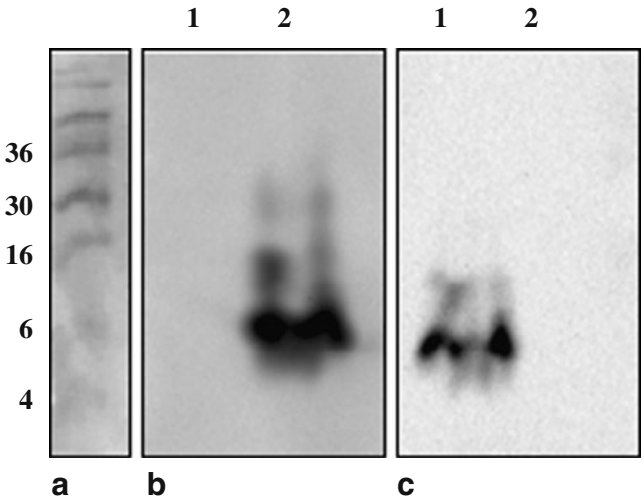

Fig. 3 Western blotting of purified enterocin P from *L. lactis* NZ9000 (pMUPE), and purified hiracin JM79, from *L. lactis* NZ9000 (pMUHE). **a** Protein molecular weight marker with sizes in kilodalton indicated in the *left margin*. **b** Western blotting with the anti-EntP antibodies. **c** Western blotting with the anti-HirJM79 antibodies. *Lane 1* shows 1 μg of hiracin JM79 and *lane 2* shows 1 μg of enterocin P

specific antimicrobial activity of the bacteriocins produced. The production of EntP by *L. lactis* IL1403 and *L. lactis* NZ9000, transformed with pMUPE and pMUPEI, was higher than by the natural EntP producer *E. faecium* P13 and higher in *L. lactis* IL1403 than in the *L. lactis* NZ9000 derivatives (Table 3). However, the specific antimicrobial activity of the EntP produced by the *L. lactis* derivatives was higher than that of the EntP produced by *E. faecium* P13, suggesting that chimeras of the SP_{usp45} fused to mature *entP* not only increase the production of EntP but also improve its antimicrobial activity. The production of HirJM79 by *L. lactis* IL1403 and *L. lactis* NZ9000, transformed with pMUHE and pMUHEI, was also higher than by *E. hirae* DCH5, with the *L. lactis* IL1403 derivatives producing higher amounts of HirJM79 (Table 4). However, the production of HirJM79 by the *L. lactis* strains was 36% to 60% lower than that produced by the same strains, transformed with pMG36c containing the native *hirJM79* gene, with or without the *hiriJM79* gene (Sánchez et al. 2008). Furthermore, the specific antimicrobial activity of the HirJM79 produced by the *L. lactis* derivatives reported in this work was 40% to 89% lower than that produced by *E. hirae* DCH5, suggesting that chimeras of the SP_{usp45} fused to mature *hirJM79* do not increase the production nor the functional expression of HirJM79 by *L. lactis*.

Interestingly, the coexpression of the *entP* and *entiP* genes or the *hirJM79* and *hiriJM79* genes increased the production of EntP (Table 3) or HirJM79 (Table 4), respectively, by the recombinant *L. lactis* hosts. This increased bacteriocin production may be explained by assuming that *L. lactis* is relatively resistant to EntP and HirJM79 but tolerates more of the bacteriocins when it expresses the EntiP or the HiriJM79 immunity proteins. Bacteriocin producers are protected from their own bacteriocins by the concomitant expression of a cognate immunity protein, and their cotranscription play an essential role for the bacteria to be able to produce and withstand the bacteriocin. LAB producing bacteriocins of the class II use components of the mannose phosphotransferase system (Man-PTS) of the susceptible cells as the target/receptor, and the immunity proteins form a strong complex with the receptor proteins and the bacteriocin, thereby preventing producer cells from being killed (Diep et al. 2007; Kjos et al. 2009). The recombinant *L. lactis* DPC5598 hosts, plasmid-free derivatives of an industrial cheesemaking strain (Trotter et al. 2002), produced EntP and NisA (Table 3) and HirJM79 and NisA (Table 4). The lower

Table 6 Antimicrobial activity of supernatants from recombinant *L. lactis* strains against *Listeria* spp.[a]

Strain	*L. ivanovii* 913	*L. grayii* 931	*L. welshimeri* 919	*L. seeligeri* 917	*L. innocua* 910	*L. monocytogenes*					
						911	935	936	939	4031	4032
L. lactis subsp. *cremoris*											
NZ9000 (pNUPE)	6,844	2,179	1,018	6,275	4,086	2,465	1,123	4,980	3,297	1,706	4,694
NZ9000 (pNUPEI)	14,735	3,719	2,315	14,088	8,966	5,553	1,923	8,196	5,294	4,566	8,153
NZ9000 (pNUHE)	1,523	4,162	440	1,115	434	1,699	410	798	298	1,758	672
NZ9000 (pNUHEI)	2,565	7,340	938	1,209	822	3,788	464	1,711	563	3,894	1,250
E. faecium P13[b]	1,706	631	334	1,240	484	607	360	892	771	572	910
E. hirae DCH5[b]	1,370	3,970	415	1,050	410	1,315	373	690	215	1,620	704

Most of the data are mean from two independent determinations in triplicate

[a] Antimicrobial activity expressed in BU per milligram cell dry weight

[b] Cultures of *E. faecium* P13 and *E. hirae* DCH5 were used as controls for production of EntP and HirJM79, respectively

production of EntP or HirJM79 and the lower specific antimicrobial activities of the *L. lactis* DPC5598 derivatives, as compared to the other *L. lactis* recombinant strains, may be related to genomic and metabolic differences among the strains or, most probably, to the higher extracellular proteinase activity of *L. lactis* DPC5598. However, since NisA is particularly active against vegetative and sporulated bacteria and EntP and HirJM79 are wide spectrum bacteriocins with antilisterial activity, these recombinant strains with synergistic antimicrobial activity would be of interest for their biotechnological evaluation in food preservation.

The production of EntP and HirJM79 also depended on the lactococcal vector used. The enhanced production of EntP and HirJM79 by the recombinant *L. lactis* strains with the nisin-inducible constructs might be due to the promoter used to drive gene expression. In this work, the functionality of the expression vectors in terms of production levels and antimicrobial activity of the target bacteriocins depended on the combination of host strains, genes of interest, promoter, and vector copy number, and these results enhance the relevance of testing various vectors for expression of the genes of interest. Interestingly, the specific antimicrobial activity of the EntP produced by *L. lactis* NZ9000 (pNUPE) and *L. lactis* NZ9000 (pNUPEI) was higher than that observed for any other *L. lactis* host or *E. faecium* P13. The short induction time for bacteriocin production most probably prevents EntP from attaching to cell walls, forming aggregates, and/or undergoing protease degradation. However, the specific antimicrobial activity of the HirJM79 produced by *L. lactis* NZ9000 (pNUHE) and *L. lactis* NZ9000 (pNUHEI) was lower than expected from their extracellular production (Tables 3 and 4). Nevertheless, MALDI-TOF MS analysis revealed that EntP, purified from *L. lactis* (pMUPE), and that HirJM79, purified from *L. lactis* (pMUHE) had a molecular mass identical to their expected theoretical molecular mass (Fig. 2), suggesting that EntP and HirJM79 are adequately processed and secreted out of the recombinant *L. lactis* cells.

The lower specific antimicrobial activity of the HirJM79 produced by the *L. lactis* hosts would be ascribed to a number of factors, which are difficult to determine. In this context, the so-called secretion stress response to overexpression of secreted proteins may activate protein quality control networks involving folding factors and housekeeping proteases (Darmon et al. 2002). It is also possible to speculate that disulfide bond formation (DSB), a universally conserved mechanism for stabilizing extracytoplasmic proteins, carried out by thiol-disulfide-oxidoreductases (TDORs), is not performed adequately for HirJM79. Disulfide bonds play pivotal roles in the folding, structural integrity, and activity of numerous proteins. The enzyme-dependent formation of disulfide bonds is a prime reason why proteins containing such bonds are still difficult to produce in bacterial cell factories. Alternatively, the mature bacteriocin could undergo conformational modifications, rendering less active extracellular HirJM79 forms (Sarvas et al. 2004). It may also occur that the post-translational processing of pro-HirJM79 to active-HirJM79 is less efficient in *Lactococcus* than in *Enterococcus*, as seems to happen in bacteriocins produced by *Lactobacillus* and *Pediococcus* (Ennahar et al. 1996; Horn et al. 1998). The lower specific antimicrobial activity of the extracellular HirJM79 may be also ascribed to differences in protein folding efficiency and turnover and to bacteriocin self-aggregation (Sánchez et al. 2008; Diep et al. 2009).

The secretory production of proteins provides advantages compared to cytosolic production, and protein secretion is a preferred means of protein expression in the development of LAB as cell factories for production of biologically active molecules (Mathiesen et al. 2008). The results of this work indicate that chimeras of the SP_{usp45} fused to the mature bacteriocins EntP or HirJM79 drive a high production and secretion of these bacteriocins in *L. lactis*, even in the absence of specific immunity and dedicated secretion proteins. The successful production of Sec-dependent bacteriocins by heterologous microbial and LAB hosts, including *L. lactis*, raises the question of why most LAB bacteriocins have a dedicated processing and transport system when they can access the GSP if provided with an adequate SP (Biet et al. 1998; Gutiérrez et al. 2005a, b, 2006; Sánchez et al. 2008).

Furthermore, the supernatants of the recombinant *L. lactis* NZ9000 (pNUPE) and *L. lactis* NZ9000 (pNUPEI) strains, producers of EntP, showed a much higher antimicrobial activity against different *Listeria* spp. than that of the *L. lactis* NZ9000 (pNUHE) and *L. lactis* NZ9000 (pNUHEI) derivatives, producers of HirJM79. The production of EntP and HirJM79 in the supernatants of *L. lactis*, a microorganism considered as Generally Recognized As Safe and with a Qualified Presumption of Safety (Casalta and Montel 2008), may facilitate the biotechnological use of these bacteriocins, partially purified or purified to homogeneity, as natural antimicrobial peptides in food, pharmaceutical, veterinary, and medical applications. The heterologous production of bacterocins in lactococcal strains which could be used as food starters with antilisterial activity is also desirable, as it is the development of secretion systems with a view to potential biotechnological applications. In-frame fusions of different SP to mature bacteriocins permits the heterologous secretion of bacteriocins by the producer microbial hosts (McCormick et al. 1996; Biet et al. 1998; Moon et al. 2005). However, the low specific antimicrobial activity of the HirJM79 produced in this work and that of the pediocin PA-1 (Martín et al. 2007a), enterocin A (Martín et al. 2007b), and divercin

RV42 (Bermúdez-Humarán et al. 2007) produced by other recombinant *L. lactis*, suggest that further efforts to maximize the production and antimicrobial activity of bacteriocins driven by fusion of different SP to mature bacteriocins are desirable.

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