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Heterologous Coproduction of Enterocin A and Pediocin PA-1 by *Lactococcus lactis*: Detection by Specific Peptide-Directed Antibodies

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Antibodies against enterocin A were obtained by immunization of rabbits with synthetic peptides PH4 and PH5 designed, respectively, on the N- and C-terminal amino acid sequences of enterocin A and conjugated to the carrier protein KLH. Anti-PH4-KLH antibodies not only recognized enterocin A but also pediocin PA-1, enterocin P, and sakacin A, three bacteriocins which share the N-terminal class IIa consensus motif (YGNGVXC) that is contained in the sequence of the peptide PH4. In contrast, anti-PH5-KLH antibodies only reacted with enterocin A because the amino acid sequences of the C-terminal parts of class IIa bacteriocins are highly variable. Enterocin A and/or pediocin PA-1 structural and immunity genes were introduced in *Lactococcus lactis* IL1403 to achieve (co)production of the bacteriocins. The level of production of the two bacteriocins was significantly lower than that obtained by the wild-type producers, a fact that suggests a low efficiency of transport and/or maturation of these bacteriocins by the chromosomally encoded bacteriocin translocation machinery of IL1403. Despite the low production levels, both bacteriocins could be specifically detected and quantified with the anti-PH5-KLH (anti-enterocin A) antibodies isolated in this study and the anti-PH2-KLH (anti-pediocin PA-1) antibodies previously generated (J. M. Martínez, M. I. Martínez, A. M. Suárez, C. Herranz, P. Casaus, L. M. Cintas, J. M. Rodríguez, and P. E. Hernández, Appl. Environ. Microbiol. 64:4536–4545, 1998). In this work, the availability of antibodies for the specific detection and quantification of enterocin A and pediocin PA-1 was crucial to demonstrate coproduction of both bacteriocins by *L. lactis* IL1403(pJM04), because indicator strains that are selectively inhibited by each bacteriocin are not available.

Bacteriocins produced by lactic acid bacteria (LAB) have received considerable research attention due to their potential application in the food industry as natural food preservatives (20, 26, 29, 42). In fact, the role of LAB and their bacteriocins as food biopreservatives may increase in the future as a result of consumer awareness of the potential risks derived not only from food-borne pathogens but also from the artificial chemical preservatives currently used to control them (28).

The application of bacteriocins in food biocontrol is mainly oriented towards two alternative directions: (i) the use of bacteriocin-producing LAB or (ii) the direct addition of bacteriocin preparations, either synthetic or purified from the culture supernatant of the producer strains. Such applications could be greatly facilitated with the development of efficient procedures for detection, quantification, and purification of bacteriocins (34). Up to now, bioassays that assess the inhibitory effect of bacteriocins on indicator microorganisms have been most commonly used for detection and quantification of bacteriocin activity. Although the importance of these biologically based methods in the bacteriocin field is undeniable, they also have some major drawbacks, such as lack of specificity (44) and low reproducibility (7).

The generation of antibodies against bacteriocins may allow the detection and quantification of bacteriocins in different substrates by the use of immunochemical assays (8, 33, 44, 45). Recently, we have reported the generation of polyclonal antibodies directed to chemically synthesized fragments deduced from the sequence of mature pediocin PA-1 (33, 34). The use of these peptide-directed antibodies combined with the choice of suitable immunoassay formats has provided specific and sensitive methods for the quantification of pediocin PA-1 and for the rapid isolation of strains producing it.

The application of bacteriocin-producing LAB in foods may have some limitations, such as narrow antimicrobial spectrum, low-level or unstable production, and inability to grow in foods in which the bacteriocin(s) would be particularly effective (1). In this context, interest in the heterologous production of LAB bacteriocins is growing rapidly (2, 6, 12, 27, 28, 50). Furthermore, the antimicrobial efficiency of a bacteriocin may be enhanced by combining it with other bacteriocins, seen for combinations of sakacin A and nisin A (41), pediocin PA-1 and nisin A (19), and pediocin PA-1 and lacticin 481, lacticin B, or lacticin F (35).

In this work, we describe the development of sensitive and specific rabbit polyclonal antibodies against two synthetic amino acid fragments of enterocin A, peptides PH4 (residues 1 to 14) and PH5 (residues 37 to 47). Additionally, we report the heterologous (co)production of enterocin A and pediocin PA-1, two bacteriocins that contain the N-terminal class IIa consensus amino acid motif (YGNGVXC) and a closely related inhibitory spectrum (4, 5, 11, 16, 18, 21, 36, 40).

MATERIALS AND METHODS

Microbiological techniques, strains, and plasmids. The LAB strains and plasmids used in this work are listed in Table 1. Lactococcal strains were grown in M17 medium (47) supplemented with 0.5% (wt/vol) glucose (GM17 medium) at 30°C, and the rest of strains were grown in MRS broth (Oxoid Unipath, Ltd., Basingstoke, United Kingdom) at 32°C. Agar plates were made by the addition

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source and/or reference[b]
Strains		
E. faecium		
T136	(Enterocins A and B) ADT indicator	OC (11)
P21	(Enterocins A and B)	OC
P13	(Enterocin P) ADT indicator	OC (11)
AA13	(Enterocin P)	OC (22)
G16	(Enterocin P)	OC (22)
L50	(Enterocins L50A and L50B)	OC (15)
E. faecalis INIA 4	(Enterocin AS-48)	INIA (30)
P. acidilactici		
347	(Pediocin PA-1)	OC (40)
Ped$^-$	*P. acidilactici* 347 isogenic strain (Non-pediocin PA-1 producer)	OC (33)
P. pentosaceus FBB61	(Pediocin A)	TNO (37)
L. sakei		
706	(Sakacin A)	NHL (23)
LTH673	(Sakacin P)	NHL (48)
148	(Lactocin S)	OC (38)
L. lactis		
BB24	(Nisin A)	OC (39)
MG1614	(Non-bacteriocin producer)	IFR (17)
IL1403	Plasmid-free host strain	INRA (13)
Plasmids		
pMC117	Em^r, pMG36e derivative carrying the pediocin operon under control of the lactococcal promoter P32	12
pMG36c	Cm^r, pMG36e derivative, gene expression vector	RuG-MG Laboratory collection
pHB04	Cm^r, pMG36c derivative carrying *entA* and *orf2* under control of P32	RuG-MG Laboratory collection
pJM03	Cm^r, pMG36c derivative carrying *pedA* and *pedB* under control of P32	This work
pJM04	Cm^r, pHB04 derivative carrying *pedA* and *pedB* under control of P32	This work

[a] The bacteriocin(s) produced is shown in parentheses. Em, erythromycin; Cm, chloramphenicol; r, resistance.
[b] OC, our collection; IFR, Institute of Food Research, Norwich Laboratory (Norwich, United Kingdom); INIA, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (Madrid, Spain); INRA, Institut National de la Recherche Agronomique (Jouy-en-Josas, France); NHL, Laboratory of Microbial Gene Technology, Agricultural University (As, Norway); RuG-MG, Department of Molecular Genetics, University of Groningen, (Groningen, The Netherlands); TNO, Nutrition and Food Research (Zeist, The Netherlands).

of 1.5% (wt/vol) agar to broth media. Chloramphenicol (Sigma-Aldrich, St. Louis, Mo.) was added to the cultures of *Lactococcus lactis* IL1403-derived recombinant strains as a selective agent (5 μg ml^{-1}).

The supernatants were obtained by centrifugation of overnight cultures at 12,000 × *g* for 10 min at 4°C, adjusted to pH 6.2 with 1 N NaOH, filtered through 0.2-μm-pore-size filters (Whatman International, Ltd., Maidstone, United Kingdom), and stored at −20°C until use. The antimicrobial activity of the supernatants or eightfold concentrated supernatants was evaluated by an agar diffusion test (ADT). The ADT was performed as previously described (33). *Enterococcus faecium* P13 (enterocin A sensitive, pediocin PA-1 sensitive) and *E. faecium* T136 (enterocin A resistant, pediocin PA-1 sensitive) were used as indicator microorganisms.

Molecular cloning. Plasmid DNA was isolated from *L. lactis* IL1403 as described by Leenhouts et al. (31). All DNA-modifying enzymes were purchased from Roche Molecular Biochemicals (Mannheim, Germany) and were used as recommended by the supplier. All DNA manipulations were carried out according to procedures described by Maniatis et al. (32). Electroporation of *L. lactis* was performed according to the method of Holo and Nes (24) with a gene pulser (Bio-Rad Laboratories, Hercules, Calif.).

A PCR fragment (613 bp) containing the *pedA* and *pedB* genes was obtained from plasmid pMC117 with the primers PedA2 (5′-AACTGCAGAGCTCTCG GAGGAATTTTGAAATGAAAAAAATTGAAAAATTAACTG-3′) and PedA3 (5′-AACTGCAGCATGCTCTAGACTATTGGCTAGGCCACGTATTGG-3′). The PCR product was cloned as a *Pst*I/*Sph*I fragment into plasmid pMG36c or as a *Sac*I/*Xba*I fragment into plasmid pHB04, resulting in the plasmids pJM03 and pJM04, respectively. After transformation of *L. lactis* IL1403 with the ligation mixtures, the bacteriocinogenicity of the recombinant cells grown in GM17 agar was tested by overlaying plates with MRS semisolid agar seeded with the indicator strains *E. faecium* P13 and T136. The plasmid from a representative colony from each cloning experiment was extracted and analyzed by restriction enzyme analysis, and the inserted PCR fragment was checked by nucleotide sequencing.

Immunological materials. Two enterocin A fragments, peptides PH4 (residues 1 to 14, NH_2-TTHSGKYYGNGYYC-COOH; 20 mg) and PH5 (residues 37 to 47, NH_2-GFLGGAIPGKC-COOH; 20 mg) were synthesized by 9-fluorenylmethoxy carbonyl chemistry with an Applied Biosystems 431A automated solid-phase synthesizer (Perkin-Elmer, Foster City, Calif.) in the Protein Chemistry Facility at the Centro de Biología Molecular Severo Ochoa (Madrid, Spain) under the direction of J. Vázquez. Purity of the peptides (higher than 95%) was monitored by reverse-phase high-performance liquid chromatography (RP-HPLC), and peptide identity was confirmed by mass spectrometry with a MALDI-TOF mass spectrometer (Shimadzu Scientific Instruments, Inc., Columbia, Md.). Polyclonal antibodies of predetermined specificity against pediocin PA-1, anti-PH2-KLH antibodies (33), were used for detection and quantitation of pediocin PA-1. The amino acid sequence of peptide PH2 (residues 34 to 44 of pediocin PA-1) was NH_2-ATGGHQGNHKC-COOH. Ovalbumin (OA) (grade III and fraction VII), Tween 20, glutaraldehyde, Freund's adjuvants, and ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid] substrate were obtained from Sigma. The Imject Activated Immunogen Conjugation kit containing maleimide-activated keyhole limpet hemocyanin (KLH), conjugation buffer, and gel filtration columns was obtained from Pierce Chemical Co. (Rockford, Ill.). Goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase was obtained from Cappel Laboratories (West Chester, Pa.). Pure nisin A (30,000 U mg^{-1}) was purchased from NBS Biologicals (Hartfield, United Kingdom). Female New Zealand White rabbits were purchased from a local supplier (Granja San Bernardo, Navarra, Spain).

Preparation of immunoconjugates and immunization. PH4 and PH5 were conjugated to maleimide-activated KLH (peptide-KLH; 1:2 [wt/wt]), employing the Imject Activated Immunogen Conjugation kit, for use as immunogens. The PH4 and PH5 fragments were also conjugated to OA (peptide-OAG; 12:1 [mol/

TABLE 2. Purification of enterocin A and pediocin PA-1 from *L. lactis* IL1403(pHB04) and *P. acidilactici* 347 supernatants, respectively

Supernatant and purification stage	Vol (ml)	Total A_{254}[a]	Total activity (10^5 bacteriocin units)	Sp act[b]	Increase in sp act[c] (fold)	Yield (%)
L. lactis IL1403(pHB04)						
Culture supernatant	1,000	30,500	15.8	52	1	100
Fraction						
Ammonium sulfate precipitation	100	1,910	11.6	606	12	73
Gel filtration chromatography	200	980	7.4	752	14	47
Cation-exchange chromatography	50	6.9	1.2	16,726	322	7
Hydrophobic-interaction chromatography	10	2.75	6.3	2.3×10^5	4,390	40
RP-HPLC	0.75	0.06	1.2	2.1×10^6	39,956	7
P. acidilactici 347						
Culture supernatant	1,000	23,900	61.0	255	1	100
Fraction						
Ammonium sulfate precipitation	100	1,260	49.2	3,904	15	81
Gel filtration chromatography	200	390	37.0	9,487	37	61
Cation-exchange chromatography	50	16.7	30.5	1.8×10^5	706	50
Hydrophobic-interaction chromatography	10	9.3	29.1	3.1×10^5	1,216	48
RP-HPLC	1.15	0.51	500.0	98.0×10^6	384,314	820

[a] A_{254} is the absorbance at 254 nm multiplied by the volume in milliliters.
[b] Specific activity is the number of bacteriocin units divided by total A_{254}.
[c] Increase in specific activity is specific activity of a fraction divided by the specific activity of the culture supernatant.

mol]) by the glutaraldehyde method (3, 10) for use as solid-phase antigens. Rabbits were immunized with PH4-KLH and PH5-KLH according to a previously described scheme (33). Rabbits were bled via marginal ear veins on days 28 and 63, and a final bleed was performed on day 72 by cardiac puncture.

ELISAs. An indirect enzyme-linked immunosorbent assay (ELISA) and noncompetitive indirect ELISA (NCI-ELISA) were performed as described by Martínez et al. (33). Briefly the indirect ELISA was performed for antiserum titration. Flat-bottom polystyrene microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight (at 4°C) with 100 μl of PH4-OAG (5 μg ml^{-1}) or PH5-OAG (5 μg ml^{-1}) in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6) coating buffer (CB). Plates were washed three times with 300 μl of PBST washing solution (0.05% Tween 20 in 0.01 M phosphate-buffered saline [PBS] [pH 7.4]). Wells were blocked for 30 min at 37°C with 300 μl of 1% (wt/vol) OA (grade III) in PBS (OA-PBS) and then washed six times. Next, 50 μl of serially diluted serum was added to each well and incubated for 1 h at 37°C. Unbound antibody was removed by washing the wells four times, after which 100 μl of goat anti-rabbit IgG peroxidase conjugate (diluted 1:500 in OA-PBS) was added to each well. Plates were incubated for 30 min at 37°C and washed eight times, and the amount of bound peroxidase present was determined with ABTS substrate. Absorbance was read at 405 nm. The titer of each serum was arbitrarily set as the reciprocal of the maximum dilution that yielded at least twice the absorbance of the same dilution of nonimmune control serum.

For NCI-ELISA, wells of microtiter plates were coated with 100 μl (each) of different concentrations of different controls (enterocin A, pediocin PA-1, nisin A, PH4-OAG, PH5-OAG, and OA) in CB or 100 μl (each) of the supernatants of the LAB strains to be tested diluted in CB 1:1 (vol/vol). The plates were maintained for 3 h at 40°C and then blocked and washed as described for the antiserum titration procedure. Next, 50 μl of antiserum, diluted 1:200 in PBS for anti-PH4-KLH serum and 1:300 in PBS for anti-PH5-KLH serum, was added, and the plates were incubated for 1 h at 37°C. After the washing step and addition of the goat anti-rabbit IgG peroxidase conjugate (diluted 1:500 in OA-PBS), the amount of bound peroxidase was determined with ABTS substrate as described above.

For quantification of enterocin A and pediocin PA-1 in the supernatants diluted in CB 1:1 (vol/vol), NCI-ELISA was used as described above. Different concentrations of both bacteriocins in MRS or GM17 with chloramphenicol (5 μg ml^{-1}) were employed to construct standard curves. The standard concentrations (in MRS when employing supernatants from *Enterococcus* and *Pediococcus* strains or in GM17 with 5 μg of chloramphenicol ml^{-1} when employing supernatants from *Lactococcus*) were diluted in CB 1:1 (vol/vol) before coating. For quantification of enterocin A, anti-PH5-KLH serum was diluted 1:300 in PBS, while for quantification of pediocin PA-1, anti-PH2-KLH serum (33) was diluted 1:1,000 in PBS.

Purification of enterocin A and pediocin PA-1. The bacteriocins produced by *L. lactis* IL1403(pHB04) (Ent A^+) and *Pediococcus acidilactici* 347 (Ped $PA\text{-}1^+$), were purified to homogeneity as previously described (14, 27, 33). Final concentrations of the purified bacteriocins were estimated by using the extinction coefficient of enterocin A (A_{280} of 2.1 corresponds to 1 mg ml^{-1}) and pediocin PA-1 (A_{280} of 3.1 corresponds to 1 mg ml^{-1}).

RESULTS

Purification of enterocin A and pediocin PA-1. The results of the purification of enterocin A and pediocin PA-1 are summarized in Table 2. The final specific activities of enterocin A and pediocin PA-1 were approximately 40,000- and 385,000-fold higher than those in the culture supernatants of *L. lactis* IL1403(pHB04) and *P. acidilactici* 347, respectively. The recovery of bacteriocin activity was approximately 7% of the initial activity for enterocin A and 820% for pediocin PA-1. The final amounts of enterocin A and pediocin PA-1, each purified from 1 liter of culture, were 27 and 406 μg, respectively.

Sensitivity of the rabbit anti-peptide antibodies for enterocin A. Two regions of the enterocin A linear sequence were chosen for the production of synthetic peptides. Synthetic peptides PH4 (amino acid residues 1 to 14) and PH5 (amino acid residues 37 to 47) were conjugated to KLH and used to immunize rabbits. On day 72 of the immunization process after six doses of the immunogen were administered, the animals had apparent titers in serum ranging from 12,800 to 102,400. The highest serum immunogen titers for fragments PH4 and PH5, respectively, were used throughout this work. The sensitivity of the anti-PH4-KLH and anti-PH5-KLH antibodies for enterocin A was initially determined by NCI-ELISA (Fig. 1). The anti-PH4-KLH antibodies showed a high recognition of PH4-OAG and enterocin A, with absorbance values higher than 2 with 4 μg of antigen ml^{-1}. More importantly, the antibodies recognized the pediocin PA-1 present in the wells of the microtiter plates (absorbance value of 2.7 with 4 μg of pediocin ml^{-1}. These results suggest that a large number of antibodies recognized the consensus sequence of the class IIa bacteriocins. In the case of the anti-PH5-KLH antibodies, serum titers showed a high recognition of PH5-OAG and enterocin A, with absorbance values higher than 2 with 4 μg of antigen ml^{-1} but with weak or no recognition of pediocin PA-1 (absorbance values from 0.5 to 0.6 with 4 to 10 μg of pediocin ml^{-1}). Both antibodies could not detect the presence of equivalent concentrations of OA or pure nisin A in the wells of the microtiter

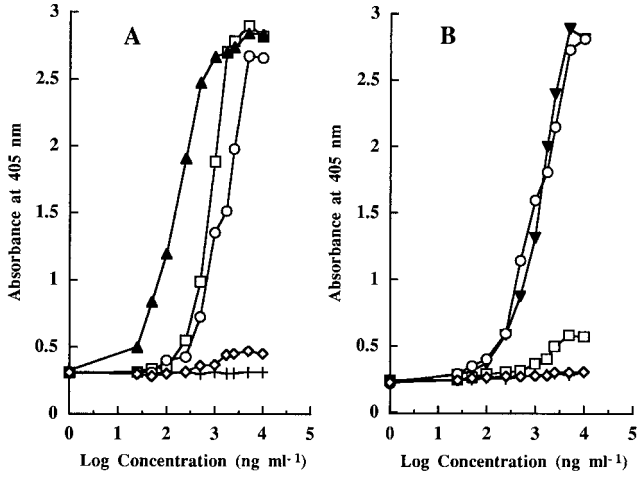

FIG. 1. NCI-ELISA detection with anti-PH4 (A) or anti-PH5 (B) antibodies of enterocin A (○), pediocin PA-1 (□), nisin A (◇), PH4-OAG (▲), PH5-OAG (▼), and pure OA (+) in CB.

plates, because absorbance values smaller than 0.5 were obtained with 10 μg of antigen ml^{-1}.

Immunoreactivity of the rabbit anti-peptide antibodies to different bacteriocins. The specificities of the serum polyclonal antibodies in neutralized and filter-sterilized supernatants of 16-h cultures of representative LAB strains were evaluated by NCI-ELISA (Table 3). The anti-PH4-KLH antibodies showed a high cross-reactivity (>75%) with the supernatants of the *E. faecium* enterocin A producers T136 and P21. The cross-reactivity was medium to low (5 to 25%) with the supernatants of the strains producing pediocin PA-1, enterocin P, and sakacin A, three class IIa bacteriocins with the N-terminal consensus amino acid motif YGNGVXC. A negligible to no reaction was observed with the supernatants of *Lactobacillus sakei* LTH673, a producer of sakacin P, another bacteriocin of the pediocin family, *P. acidilactici* Ped$^-$ (non-bacteriocin producer), *Pediococcus pentosaceus* FBB61 (pediocin A), *E. faecium* L50 (enterocin L50A and L50B), *Enterococcus faecalis* I4 (enterocin AS-48), *L. sakei* 148 (lactocin S), *L. lactis* BB24 (nisin A) and *L. lactis* MG1614 (non-bacteriocin producer). The anti-PH5-KLH antibodies showed a high cross-reactivity (>75%) with the supernatants of the enterocin A producers *E. faecium* T136 and P21, but did not react with the supernatants of the other LAB strains tested.

TABLE 3. Reactivities of anti-PH4 and anti-PH5 serum polyclonal antibodies against culture supernatants of LAB as determined by NCI-ELISA

Microorganism (bacteriocin)	Cross-reactivity (%)[a]	
	Anti-PH4	Anti-PH5
E. faecium T136 (enterocins A and B)	100	100
E. faecium P21 (enterocins A and B)	78.8	76.4
P. acidilactici 347 (pediocin PA-1)	22.9	NR
P. acidilactici Ped$^-$ (non-pediocin PA-1 producer)	NR	1.1
E. faecium P13 (enterocin P)	8.2	NR
E. faecium AA13 (enterocin P)	9.5	NR
E. faecium G16 (enterocin P)	13.9	1.5
L. sakei 706 (sakacin A)	6.9	NR
L. sakei LTH673 (sakacin P)	NR	NR
E. faecium L50 (enterocins L50A and L50B)	NR	NR
P. pentosaceus FBB61 (pediocin A)	NR	NR
E. faecalis INIA 4 (enterocin AS-48)	NR	NR
L. sakei 148 (lactocin S)	NR	NR
L. lactis BB24 (nisin A)	NR	NR
L. lactis MG1614	NR	NR

[a] Cross-reactivity is calculated as follows: [(absorbance reading produced by a culture supernatant above the absorbance reading produced by MRS/absorbance reading produced by the supernatant of *E. faecium* T136 above the absorbance reading produced by MRS) × 100]. NR, no reaction.

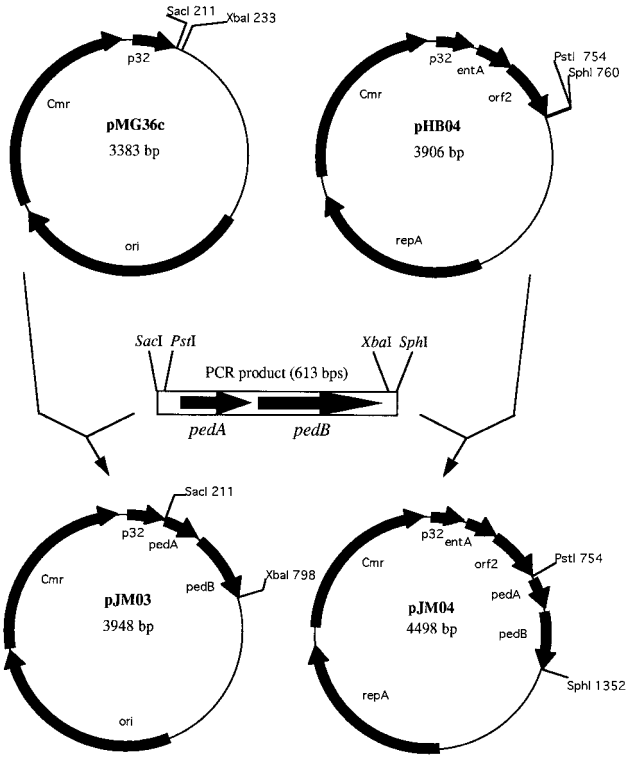

FIG. 2. Construction of plasmids pJM03 and pJM04. Sizes of plasmids are given in base pairs. Only relevant restriction enzyme sites are given. p32, strong lactococcal promoter (51); ori, origin of replication; Cmr, chloramphenicol resistance marker; repA, gene encoding plasmid pWV01 replication protein; entA, enterocin A structural gene; orf2, enterocin A immunity gene; pedA, pediocin PA-1 structural gene; pedB, pediocin PA-1 immunity gene.

Quantification of the homologous and heterologous (co)-production of enterocin A and pediocin PA-1. A PCR fragment carrying the *pedA* and *pedB* genes was cloned in plasmids pMG36c and pHB04. After transformation of *L. lactis* IL1403 with the ligation mixtures, eight colonies produced bacteriocin, as evidenced by halos on the indicators. The plasmid from a representative colony from each cloning experiment was examined by restriction enzyme analysis, and the correctness of the inserted PCR fragment was confirmed by nucleotide sequencing. Maps of the two resulting plasmids, pJM03 and pJM04, are given in Fig. 2.

The concentration of enterocin A and pediocin PA-1 in the supernatants of 16-h cultures of *E. faecium* T136 and P21, *P. acidilactici* 347, and *L. lactis* IL1403 carrying either pHB04, pJM03, or pJM04 was evaluated by NCI-ELISA employing the PH2-KLH- and the PH5-KLH-generated antibodies (Table 4). The detection limits of enterocin A and pediocin PA-1 in the supernatants were below 50 ng ml^{-1}. The concentrations of enterocin A and pediocin PA-1 in the supernatants of *L. lactis* IL1403(pHB04 (Ent A$^+$) and *L. lactis* IL1403(pJM03) (Ped

TABLE 4. Pediocin PA-1 and enterocin A concentrations in culture supernatants as determined by NCI-ELISA with anti-PH2 and anti-PH5 antibodies

Microorganism (bacteriocin)	Concn in supernatant with:			
	Anti-PH2		Anti-PH5	
	Pediocin PA-1 (ng/ml)	Cross-reactivity[a] (%)	Enterocin A (ng/ml)	Cross-reactivity[b] (%)
E. faecium T136 (enterocin A and enterocin B)		NR[c]	2,483	100
E. faecium P21 (enterocin A and enterocin B)		NR	1,896	76.4
P. acidilactici 347 (pediocin PA-1)	1,724	100		NR
L. lactis IL1403(pHB04) (enterocin A)		NR	187	7.5
L. lactis IL1403(pJM03) (pediocin PA-1)	115	6.7		NR
L. lactis IL1403(pJM04) (pediocin PA-1 and enterocin A)	87	5.1	93	3.8
L. lactis IL1403		NR		NR

[a] Cross-reactivity is calculated as follows: [(concentration of pediocin PA-1 in a culture supernatant/concentration of pediocin PA-1 in the supernatant of *P. acidilactici* 347) × 100].
[b] Cross-reactivity is calculated as follows: [(concentration of enterocin A in a culture supernatant/concentration of enterocin A in the supernatant of *E. faecium* T136) × 100].
[c] NR, no reaction.

PA-1$^+$), were determined to be around 7% of those found in the supernatants of *E. faecium* T136 (2,483 ng of Ent A ml^{-1}) and *P. acidilactici* 347 (1,724 ng of Ped PA-1 ml^{-1}), respectively. The concentration of bacteriocins in the supernatant of *L. lactis* IL1403(pJM04) (Ent A$^+$ and Ped PA-1$^+$) was 93 ng of enterocin A ml^{-1} and 87 ng of pediocin PA-1 ml^{-1}, which is approximately 4 and 5%, respectively, of the concentrations found in the wild-type bacteriocin producers *E. faecium* T136 and *P. acidilactici* 347.

The enterocin A and/or pediocin PA-1 production by the recombinants strains was confirmed by ADT (Fig. 3). The concentrations of enterocin A and pediocin PA-1 found in the eightfold-concentrated supernatants of the recombinant strains were in accordance with those determined by NCI-ELISA. However, the production of enterocin A by *L. lactis* IL1403(pJM04) could only be demonstrated immunologically with the anti-PH5-KLH antibodies, since no enterocin A-sensitive, pediocin PA-1-resistant strain was available.

DISCUSSION

With the polyclonal anti-PH4-KLH and anti-PH5-KLH antibodies, it was possible to detect not only the peptides PH4 and PH5 but also enterocin A, either purified to homogeneity or in the supernatant of the wild-type *E. faecium* producer strains T136 and P21. Both antibodies displayed maximum cross-reactivity (100%) with the supernatant of *E. faecium* T136 and a reaction of around 75 to 80% with the supernatant

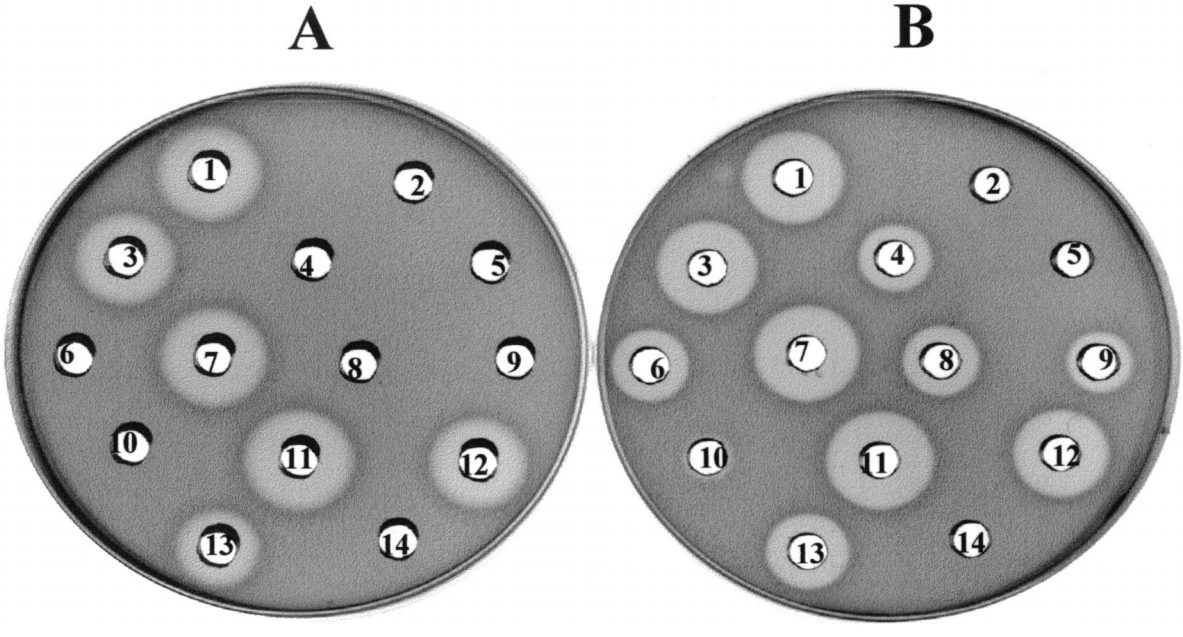

FIG. 3. ADT with *E. faecium* T136 (Ent A^r Ped PA-1^s) (A) or *E. faecium* P13 (Ent A^s Ped PA-1^s) (B) as indicator microorganisms to test the bacteriocin activity of supernatants (*Enterococcus* and *Pediococcus*), eightfold-concentrated supernatants (*L. lactis* IL1403), or pure bacteriocin in GM17 broth with 5 μg of chloramphenicol ml^{-1}. Spots: 1, *L. lactis* IL1403(pJM03); 2, *L. lactis* IL1403; 3, *L. lactis* IL1403(pJM04); 4, *L. lactis* IL1403(pHB04); 5, *P. acidilactici* Ped$^-$; 6, *E. faecium* T136; 7, *P. acidilactici* 347; 8, 2.5 μg of enterocin A ml^{-1}; 9, 0.5 μg of enterocin A ml^{-1}; 10, 0.1 μg of enterocin A ml^{-1}; 11, 2.5 μg of pediocin PA-1 ml^{-1}; 12, 0.5 of μg pediocin PA-1 ml^{-1}; 13, 0.1 μg of pediocin PA-1 ml^{-1}; 14, GM17 broth with 5 μg of chloramphenicol ml^{-1}.

of *E. faecium* P21. This difference could be attributed to higher production of enterocin A by *E. faecium* T136. This is not rare, because considerable differences in bacteriocin production have, for instance, also been found among different wild-type pediocin PA-1 producers (34). Anti-PH5-KLH antibodies could not detect the other class IIa bacteriocins tested, namely pediocin PA-1, enterocin P, sakacin A, and sakacin P. This result is concordant with the fact that these antibodies were raised against amino acid residues 37 to 47 in the C-terminal part of the enterocin A molecule, a region with high sequence diversity among class IIa bacteriocins. In contrast, the anti-PH4-KLH antibodies displayed medium to low cross-reactivity (5 to 25%) with supernatants containing pediocin PA-1, enterocin P, and sakacin A. This fact suggests that a number of the antibodies were able to recognize the consensus sequence of the class IIa bacteriocins, which is present in the peptide PH4 (covering residues 1 to 14 of enterocin A). However, they were not able to detect sakacin P in the supernatant of *L. sakei* LTH673. This fact may be explained by differences in the structural conformation preferentially adopted by this part of the sakacin P molecule, causing poor access of the antibodies to the bacteriocin and/or by low bacteriocin production by this particular strain. Further analysis with purified bacteriocins and elucidation of the relation between the amount of each bacteriocin present in the supernatants and its immunodetection could facilitate a better understanding of how anti-peptide antibodies recognize the sequence against which they have been raised.

L. lactis IL1403, a plasmid-free strain that does not produce bacteriocin but harbors chromosomal genes analogous to those encoding the lactococcin A secretion apparatus, *IcnC* and *IcnD* (43, 52), was selected as the host for the heterologous (co)-production of enterocin A and pediocin PA-1. Introduction of pHB04 or pJM03 into IL1403 led to heterologous production and secretion of enterocin A or pediocin PA-1 in the respective transformants, while transformation with pJM04 resulted in coproduction of both bacteriocins.

Detection and quantification of enterocin A and pediocin PA-1 production by the *L. lactis* recombinant strains were done using the ADT and NCI-ELISA. *E. faecium* T136 (Ent A^r Ped PA-1^s) and *E. faecium* P13 (Ent A^s Ped PA-1^s) were employed as indicator strains in the ADT, while anti-PH2-KLH antibodies (33) and anti-PH5-KLH antibodies were used for the recognition of pediocin PA-1 and enterocin A by NCI-ELISA, respectively. The production of enterocin A and pediocin PA-1 by the wild-type and the recombinant single bacteriocin producers could be carried out using either the ADT or the NCI-ELISA. Both assays showed a similar sensitivity, detecting concentrations of bacteriocin as low as 90 ng ml^{-1} in the supernatant of the recombinant strains. However, the immunological method proved to be critical for detection of coproduction of enterocin A and pediocin PA-1 by *L. lactis* IL1403(pJM04), because no enterocin A-sensitive, pediocin PA-1-resistant strain is available.

The low level of bacteriocin production by the recombinant strains, around 7% for single bacteriocin production and 4 to 5% for coproduction, compared with the levels achieved by the wild types, is in accordance with the yields previously obtained for lactococcin A (25, 49) and pediocin PA-1 (27) when expressed in *L. lactis* IL1403. This reduction in bacteriocin activity may be due to the low copy number of the chromosomal *IcnC* and *IcnD* analogs in IL1403 and/or to the fact that their gene products are not identical to the equivalent lactococcin A translocation apparatus (25, 27, 43, 52), which may result in a less-efficient secretion process. Considerable increases in the production of bacteriocins containing the lactococcin A leader have been described after the introduction of plasmid copies of the *IcnC* and *IcnD* genes in IL1403 (28, 50, 52). Moreover, in IL1403, lactococcin A-leader-directed secretion of pediocin PA-1 by *IcnC* and *IcnD* is more efficient than the equivalent process directed by the pediocin PA-1 translocation machinery (12, 28). If the same were true for enterocin A, an alternative to increase enterocin A and pediocin PA-1 (co)production in IL1403 would be the introduction of the *IcnC* and *IcnD* genes together with chimeric genes encoding fusions between the lactococcin A leader and the mature part of enterocin A or pediocin PA-1.

In this study, heterologous production of enterocin A and/or pediocin PA-1 in *L. lactis* IL1403 was achieved. Although both bacteriocins were (co)produced at low levels, the heterologous system developed was very useful in demonstrating the specificity and sensitivity of anti-peptide antibodies of predetermined specificity against enterocin A (anti-PH5-KLH) and pediocin PA-1 (anti-PH2-KLH), despite the low concentration of the bacteriocins and their high sequence similarity. The use of peptide-directed antibodies to detect and quantify homologous or heterologous bacteriocin coproduction avoids dependence on the availability of indicator strains selectively inhibited by each of the bacteriocins. The antibodies also offer potential alternative methods for the (industrial-scale) purification of bacteriocins to homogeneity by the use of immunoaffinity chromatography strategies (46). Finally, these antibodies could be employed for immunolocalization of bacteriocins in bacterial strains and in those foods in which bacteriocins have been naturally produced or added (9).

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