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Biochemical and Genetic Characterization of Enterocin P, a Novel *sec*-Dependent Bacteriocin from *Enterococcus faecium* P13 with a Broad Antimicrobial Spectrum

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Enterocin P is a new bacteriocin produced by *Enterococcus faecium* P13 isolated from a Spanish dry-fermented sausage. Enterocin P inhibited most of tested spoilage and food-borne gram-positive pathogenic bacteria, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens*, and *Clostridium botulinum*. Enterocin P is produced during growth in MRS broth from 16 to 45°C; it is heat resistant (60 min at 100°C; 15 min at 121°C) and can withstand exposure to pH between 2.0 and 11.0, freeze-thawing, lyophilization, and long-term storage at 4 and −20°C. The bacteriocin was purified to homogeneity by ammonium sulfate precipitation, gel filtration, cation-exchange, hydrophobic-interaction, and reverse-phase liquid chromatography. The sequence of 43 amino acids of the N terminus was obtained by Edman degradation. DNA sequencing analysis of a 755-bp region revealed the presence of two consecutive open reading frames (ORFs). The first ORF encodes a 71-amino-acid protein containing a hydrophobic N-terminal *sec*-dependent leader sequence of 27 amino acids followed by the amino acid sequence corresponding to the purified and sequenced enterocin P. The bacteriocin is apparently synthesized as a prepeptide that is cleaved immediately after the Val-Asp-Ala residues (positions −3 to −1), resulting in the mature bacteriocin consisting of 44 amino acids, and with a theoretical molecular weight of 4,493. A second ORF, encoding a putative immunity protein composed of 88 amino acids with a calculated molecular weight of 9,886, was found immediately downstream of the enterocin P structural gene. Enterocin P shows a strong antilisterial activity and has the consensus sequence found in the pediocin-like bacteriocins; however, enterocin P is processed and secreted by the *sec*-dependent pathway.

Lactic acid bacteria (LAB) are used to preserve and develop flavor in food. The preserving effect is due to lactic acid and other antagonistic compounds (53) such as bacteriocins, which have attracted increasing interest in recent years (14, 31, 33). The LAB bacteriocins which have been described and characterized to date share a number of common traits which justify their classification into at least three well-defined classes (43): class I, the lantibiotics; class II, the small heat-stable nonlantibiotics, which are divided into the subgroups IIa (pediocin-like bacteriocins with strong antilisterial effect), IIb (bacteriocins whose activity depends on the complementary activity of two peptides), and IIc *sec*-dependent secreted bacteriocins; and class III, large heat-labile bacteriocins. A fourth class of bacteriocins, claimed to consist of an undefined mixture of proteins, lipids, and carbohydrates, has been suggested. However, this group has yet to be confirmed by purification and biochemical characterization.

All bacteriocins characterized to date are ribosomally synthesized as precursor peptides with an N-terminal leader sequence, which is cleaved off concomitantly with export (31). The leader sequences of most nonlantibiotics and some lantibiotics are clearly related and contain two conserved glycine residues, which may serve as a recognition signal for protein processing and secretion (17, 24). The removal of the so called double-glycine-type leader sequences and the translocation of the bacteriocins across the cytoplasmic membrane is accomplished by dedicated ATP-binding cassette (ABC) transporters and their accessory proteins (19, 25). However, it has now been found that some class II bacteriocins (40, 58, 67) are secreted by the general secretory pathway (for a review, see reference 48). Proteins that can access the general secretory pathway contain an N-terminal leader sequence, called the signal peptide, with a positively charged N terminus, a hydrophobic core, and a cleavage region (21, 32, 64), which directs the secretory proteins to the cytoplasmic membrane and is processed by a signal peptidase during translocation across the cytoplasmic membrane (48, 65).

Bacteriocin-producing enterococci are widespread in nature, and they have been isolated from dairy products (16, 45, 59, 63), fermented sausages (5, 7), vegetables (20, 42, 62), fish (6), silage (42), and mammalian gastrointestinal tract (39, 55). In previous reports, we described the isolation of several bacteriocinogenic LAB belonging to the genera *Lactobacillus* (51, 56), *Pediococcus* (10), *Enterococcus* (7), and *Lactococcus* (52) from Spanish dry-fermented sausages. These bacteriocinogenic LAB may have a selective advantage in the ecological niches of fermented food, in which they play an important role in the sensoric properties and microbial safety of the final product. In this paper, we report the characterization of a new bacteriocin by its purification to homogeneity, amino acid sequence, DNA analysis, and functional properties. The new pediocin-like bacteriocin, termed enterocin P, is produced by *Enterococcus faecium* P13 isolated from a Spanish dry-fer-

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TABLE 1. Antimicrobial activities of *E. faecium* P13 and MICs of enterocin P against gram-positive bacteria

Indicator species	Strain	Source[a]	Inhibition by crude extracts[b]		Purified bacteriocin MIC (ng/ml)[c]
			S	CS	
Lactobacillus acidophilus	4356	ATCC	NIZD	NIZD	NID
L. bulgaricus	11842	ATCC	NIZD	10.3	NID
L. casei	334	ATCC	NIZD	NIZD	NID
L. curvatus	2739	NCFB	10.3	14.2	17
L. fermentum	9338	ATCC	14.7	19.7	1
L. helveticus	15009	ATCC	NIZD	10.0	NID
L. plantarum	1193	NCDO	NIZD	NIZD	NID
L. reuteri	20016	DSM	NIZD	NIZD	NID
L. sake	2714	NCB	10.7	15.4	144
	148	Our strain collection	NIZD	NIZD	NID
L. salivarius	2747	NCFB	NIZD	NIZD	NID
Pediococcus acidilactici	L50	Our strain collection	NIZD	11.6	69
	347	Our strain collection	NIZD	NIZD	NID
P. pentosaceus	FBB61	TNO	NIZD	9.0	136
	FBB63	TNO	9.7	15.3	18
	PC1	TNO	NIZD	NIZD	NID
Leuconostoc cremoris	DB1275	TNO	NIZD	NIZD	NID
Lactococcus cremoris	CNRZ117	INRA	NIZD	NIZD	NID
L. lactis	CNRZ148	INRA	NIZD	NIZD	NID
L. lactis	CNRZ150	INRA	NIZD	NIZD	NID
L. lactis	BB24	Our strain collection	NIZD	NIZD	22
Enterococcus faecium	T136	Our strain collection	15.9	20.8	2
E. faecalis	EF	TNO	13.0	16.0	238
Staphylococcus carnosus	MC1	TNO	13.3	16.0	139
Listeria innocua	BL86/26	TNO	12.3	16.6	395
Bacillus cereus	9139	ATCC	NIZD	NIZD	286
Clostridium sporogenes	C22/10	TNO	15.5	19.4	4
C. tyrobutyricum	3,5CT	TNO	NIZD	12.0	559
	1754	NCDO	NIZD	NIZD	412
Propionibacterium sp.	P4	TNO	12.0	15.0	37
	P6	TNO	12.0	16.0	26
C. perfringens	376	CECT	NIZD	10.0	4
C. botulinum	551	CECT	NIZD	11.5	259
Listeria monocytogenes	7973	NCTC	11.0	16.0	35
	LI5sv1/2	FVM	13.0	16.0	33
	5105	NCTC	12.8	14.6	18
	LI1sv4	FVM	11.4	15.5	39
	Scott A	FVM	12.0	15.6	125
Staphylococcus aureus	137	FRI	12.4	16.0	190
	196E	FRI	12.0	16.0	407
	349	FRI	12.3	15.9	221
	361	FRI	12.0	16.3	294
	472	FRI	12.0	15.4	269

[a] Abbreviations: ATCC, American Type Culture Collection (Rockville, Md.); CECT, Colección Española de Cultivos Tipo (Valencia, Spain); DSM, Deutsch Sammlung von Mikroorganismen und Zell Kulturen, GmbH (Braunschweig, Germany); INRA, Station de Recherche Laitières (Jouy-en-Josas Cedex, France); FRI, Food Research Institute Madison, Wis.); FVM, Facultad de Veterinaria (Madrid, Spain); NCDO and NCFB, National Collection of Dairy Organisms (Reading, United Kingdom); NCTC, National Collection of Type Cultures (London, United Kingdom); TNO, Nutrition and Food Research (Zeist, The Netherlands).

[b] Diameter of inhibition zone in milliliters. S, supernatant; CS, 20-fold-concentrated supernatant; NIZD, no inhibition zone detected with 50 μl of supernatant or 20-fold-concentrated supernatant.

[c] Bacteriocin concentration inhibiting growth of indicator microorganisms by 50%; NID, no inhibition detected at 1.25 μg ml^{-1}.

mented sausage; it shows a broad antimicrobial spectrum which includes not only *Listeria monocytogenes* but also a wide range of spoilage and food-borne gram-positive pathogenic bacteria. Moreover, enterocin P is to our knowledge the first *sec*-dependent pediocin-like bacteriocin characterized at the biochemical and molecular level.

MATERIALS AND METHODS

Bacterial strains and media. The microorganisms used for bacteriocin screening and evaluation of antimicrobial activities are listed in Table 1. The LAB were propagated in MRS broth (Oxoid Ltd., Basingstoke, United Kingdom) at 32 or 37°C. *Clostridium* spp. strains were propagated anaerobically (Oxoid Anaerobic System) in RCM (Oxoid) at 32 or 37°C. *Propionibacterium* spp. were grown anaerobically in GYE medium at 32°C as previously described (10). All other strains were propagated in brain heart infusion (Oxoid) at 32 or 37°C. The bacteriocinogenic LAB were isolated from chorizo, a typical Spanish dry-fermented sausage, manufactured with no added starter cultures. The isolates were screened for antimicrobial activity by a stab-on agar test, using *L. monocytogenes* Scott A as indicator (10).

Phenotypic identification. The isolate P13 was examined by phase-contrast microscopy to determine cell morphology and tested for Gram-staining reaction and catalase activity; preliminary identification was determined according to the classical tests proposed by Schleifer and Kilpper-Bälz (54). The strain was further submitted to the tests suggested by Devriese et al. (13), which included arginine dihydrolase activity, Voges-Proskauer reaction (acetoin production) (50), growth on media containing 0.04% sodium azide (Bacto EVA broth; Oxoid), urease activity, and hemolysis on 5% calf blood agar plates (Oxoid). Configuration of lactic acid produced from glucose was determined enzymatically with D- and L-lactate dehydrogenases as described by the supplier (Boehringer GmbH, Mannheim, Germany). Fermentation patterns were determined with API Rapid

CH fermentation strips (API, Biomérieux, Montallieu Vercieu, France) in CHL medium. Total proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (37), and the pattern obtained was compared with those of reference strains as described by Kersters and de Ley (34) and Pot et al. (46) by B. Pot, University of Ghent, Ghent, Belgium.

Bacteriocin assays. The bacteriocinogenic strain *E. faecium* P13 was grown in MRS broth at 32°C. The supernatant from early stationary growth phase was neutralized with 1 M NaOH and filter sterilized through 0.22 μm-pore-size filters (Millipore Corp., Bedford, Mass.). The antimicrobial activity of the supernatant was determined by the agar well diffusion assay, performed as described by Cintas et al. (10); 50-μl aliquots of supernatants were placed in wells (6-mm diameter) cut in cooled soft MRS, BHI, RCM, or GYE agar plates (20 ml) previously seeded (10^5 CFU ml^{-1}) with indicator microorganisms listed in Table 1. After 2 h at 4°C, the plates were incubated at 30 or 37°C for growth of the target organism; after 24 h, the diameters (millimeters) of the growth inhibition zones were measured. The agar well diffusion assay described above was also used to study the bacteriocin production in MRS broth at different growth temperatures and the stability of the bacteriocin.

The bacteriocin activity during the purification process was determined by a microtiter plate assay performed basically as described by Holo et al. (30). Each well of the microtiter plate contained 50 μl of twofold serial dilutions (in MRS) of the bacteriocin samples and 150 μl of a diluted (in MRS) fresh overnight culture of indicator microorganism (approximately 2×10^7 CFU ml^{-1}). Growth inhibition was measured spectrophotometrically at 620 nm with a microtiter plate reader (Titertek Multiskan Plus; Flow Laboratories, Helsinki, Finland) after 14 h of incubation at 32°C. One bacteriocin unit was defined as the reciprocal of the highest dilution of bacteriocin causing 50% growth inhibition (50% of the turbidity of the control culture without bacteriocin). The MIC of purified bacteriocin was determined by the microtiter plate assay, with adequate growth media and incubation conditions for each indicator strain, as described by Cintas et al. (10). Briefly, plates containing twofold serial dilutions of a sample with a known amount of purified bacteriocin and the indicator microorganism were prepared as described above and incubated until the cultures with no added bacteriocin had reached the stationary phase. The MIC was defined as the bacteriocin concentration (nanograms per milliliter) causing 50% growth inhibition.

Effect of enterocin P on growing cells of *L. monocytogenes*. The effect of the addition of supernatant of *E. faecium* P13 on the growth of *L. monocytogenes* Scott A was determined by the method described by Schillinger and Lücke (53), with minor modifications. Briefly, 1.0 ml of 20-fold-concentrated supernatant was added to 4.0 ml of a fresh culture of *L. monocytogenes* Scott A (approximately 10^5 CFU ml^{-1}) in BHI broth and then incubated at 32°C. At appropriate intervals, the number of viable bacteria was determined by plate counting. In the control tube, the sensitive bacteria were tested for the effect of 1.0 ml of a 20-fold-concentrated culture supernatant of *Pediococcus acidilactici* 144, a strain which does not display antimicrobial activity.

Bacteriocin purification. The bacteriocin was purified from a 1-liter *E. faecium* P13 culture which was grown in MRS at 32°C until the late logarithmic phase (A_{620} = 0.9). The cells were removed by centrifugation at 10,000 × *g* for 12 min at 4°C, and ammonium sulfate was gradually added to achieve 75% saturation. The sample was kept at 4°C with stirring for 30 min. After centrifugation at 12,000 × *g* for 30 min, the pellet and floating materials were mixed and solubilized in 120 ml of 10 mM sodium phosphate buffer, pH 5.8 (buffer A). To remove any trace of ammonium sulfate, the bacteriocin preparation was applied to gel filtration G-25 PD-10 columns (Pharmacia-LKB, Uppsala, Sweden) previously equilibrated with buffer A. The gel-filtered fraction was subjected to cation-exchange (SP-Sepharose Fast Flow) and hydrophobic-interaction (Octyl-Sepharose CL-4B) chromatographies followed by reverse-phase chromatography in a C_2 to C_{18} column (PepRPC HR5/5) (fast-performance liquid chromatography system; Pharmacia-LKB) (10). The bacteriocin was eluted from the reverse-phase column with a 55-min linear gradient of 20 to 40% 2-propanol in aqueous 0.1% (vol/vol) trifluoroacetic acid at a flow rate of 0.5 ml min^{-1}. Fractions with high bacteriocin activity were mixed and rechromatographed on the reverse-phase column to obtain chromatographically pure bacteriocin. Purified bacteriocin was stored in 50 to 60% 2-propanol or ethanol containing 0.1% trifluoroacetic acid at −20°C. The protein concentration of purified bacteriocin was determined by the Coomassie protein assay reagent as described by the manufacturer (Pierce, Rockford, Ill.).

Amino acid sequence analysis. The N-terminal amino acid sequence of the purified bacteriocin was determined by Edman degradation with an Applied Biosystems (Foster City, Calif.) model 477A automatic sequence analyzer with an on-line 120A phenylthiohydantoin amino acid analyzer, as described by Cornwell et al. (11).

DNA sequence analysis. Total DNA from *E. faecium* P13 was obtained by the alkaline lysis method (3). Different samples of this DNA were digested with the blunt-end cutter *Dra*I, *Rsa*I, or *Ssp*I, and ligated to *Hinc*II-digested, dephosphorylated pBluescript II SK+ (Stratagene, La Jolla, Calif.). The three ligation reactions, which together represent libraries of overlapping fragments from the total DNA of *E. faecium* P13, were subsequently used as templates to amplify DNA segments near the bacteriocin gene. A specific PCR product consisting of approximately 500 bp was obtained with the *Rsa*I ligation reaction when the biotinylated polylinker primer SK2 (5′-CCGCTCTAGAACTAGTGGATC-3′) was used in combination with the degenerated primer ENTP-1 (5′-TG(TC)TGGGTIAA(TC)TGGGGIGA(AG)GC-3′). The sequence of the degenerated primer was derived from the amino acid sequence of enterocin P obtained by Edman degradation. Oligonucleotides used for PCR and DNA sequencing were obtained from Kebo-Lab (Spanga, Sweden). The PCR conditions used included a hot start at 97°C (2 min), annealing at 55°C (30 s), polymerization at 72°C (2.5 min), and denaturation at 94°C (1 min). Amplification reactions (35 cycles) were carried out with the Gen Amp PCR kit in a DNA-Thermal Cycler according to the supplier's instructions (Perkin-Elmer Cetus, Norwalk, Conn.). The 500-bp PCR fragment was eluted from an agarose gel with a QIAEX II agarose gel extraction kit (Qiagen GmbH, Hilden, Germany) and further purified by using a QIAquick PCR purification kit (Qiagen). The strands of the DNA fragment were separated with Dynabeads M-280 coupled to streptavidin as prescribed by the supplier (Dynal, Oslo, Norway), and sequencing was performed on the single-stranded template by using the Sequenase system (U.S. Biochemical, Cleveland, Ohio). Based on the sequence of this DNA fragment, new specific primers were synthesized and the procedure described above was repeated until the complete sequence shown in this paper was determined.

Analysis of open reading frames (ORFs) and amino acid alignments were performed with the Sequence analysis software package (version 7.2) licensed from the Genetics Computer Group, University of Wisconsin, Madison (12).

Nucleotide sequence accession number. The DNA and deduced amino acid sequences presented in this paper have been deposited in GenBank under accession no. AF005726.

RESULTS

Isolation and identification of bacteriocinogenic isolate P13. A total of 300 lactic acid bacteria isolated from three different kinds of chorizo sausage were screened for antibacterial activity by a stab-on agar test. Twenty-five strains obtained from the three sausage samples showed inhibitory activity against *L. monocytogenes* Scott A. Isolate P13 was found to produce the largest inhibition zones against this microorganism (results not shown), and this isolate was selected for further studies.

As shown in Table 2, isolate P13 is a catalase-negative, gram-positive, facultatively anaerobic coccus with the ability to grow at 4, 10, and 45°C, at pH 9.6, in 6.5% NaCl broth, and in the presence of both 40% (vol/vol) bile and 0.04% sodium azide. It fermented glucose to L-lactic acid but did not produce gas, gave a positive Voges-Proskauer reaction, and produced ammonia from the hydrolysis of arginine. The final pH in glucose broth was 4.6, and acid was produced from ribose and L-arabinose but not from glycogen, D-arabitol, D-tagatose, sorbitol, or gluconate. The strain did not show urease activity, and it was nonhemolytic on calf blood. By comparison of its sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein pattern (37) with a database of protein patterns from other LAB (34, 46), and considering all of the features cited above, isolate P13 was identified as *E. faecium.*

Antimicrobial spectrum and production kinetics of the bacteriocin. Table 1 summarizes the antimicrobial activities of culture supernatants and 20-fold-concentrated culture supernatants of *E. faecium* P13 in the agar well diffusion assay, using 43 different gram-positive bacteria as indicators. Culture supernatants were inhibitory to 5 of 11 *Lactobacillus* spp. and to 3 of 5 *Pediococcus* spp. strains tested. No inhibition zones were observed when *Leuconostoc* and *Lactococcus* strains were used as indicators. Nevertheless, antimicrobial activity against a broad range of spoilage and food-borne gram-positive pathogenic bacteria was found. All the tested *Listeria* and *Staphylococcus* strains were inhibited by either supernatants or 20-fold-concentrated supernatants of *E. faecium* P13. None of the gram-negative bacteria tested (*Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas fluorescens*, *Yersinia enterocolitica*, *Enterobacter aerogenes*, and *Aeromonas hydrophila*) were inhibited by supernatants or 20-fold-concentrated supernatants of *E. faecium* P13 (results not shown).

Bacteriocin production in MRS was studied at several growth temperatures. No bacteriocin activity was found in cultures grown at 4 or 8°C. However, bacteriocin production was

TABLE 2. Phenotypic characteristics of isolate P13

Test	Reaction or characteristic	Test	Reaction or characteristic
Morphology	Cocci	Rhamnose	−
Gram reaction	+	Dulcitol	−
Calf blood hemolysis	−	Inositol	−
Catalase	−	Mannitol	+
Growth		Sorbitol	−
At 4°C	+	α-Methyl-D-mannoside	−
At 10°C	+	α-Methyl-D-glucoside	−
At 45°C	+	*N*-Acetylglucosamine	+
At 50°C	−	Amygdalin	+
At pH 4.5–9.6	+	Arbutin	+
In 40% bile	+	Esculin	+
In 6.5% NaCl	+	Salicin	+
In 10% NaCl	−	Cellobiose	+
In 0.04% sodium azide	+	Maltose	+
Urease	−	Lactose	+
Arginine hydrolysis	+	Melibiose	+
CO_2 production	−	Saccharose	+
H_2S production	−	Trehalose	+
Voges-Proskauer	+	Inulin	−
Lactic acid[a]	L	Melezitose	−
Glycerol	+	D-Raffinose	−
Erythritol	−	Starch	−
D-Arabinose	−	Glycogen	−
L-Arabinose	+	Xylitol	−
Ribose	+	β-Gentibiose	−
D-Xylose	−	D-Turanose	−
L-Xylose	−	D-Lyxose	−
Adonitol	−	D-Tagatose	−
β-Methylxyloside	−	D-Fucose	−
Galactose	+	L-Fucose	−
D-Glucose	+	D-Arabitol	−
D-Fructose	+	L-Arabitol	−
D-Mannose	+	Gluconate	−
L-Sorbose	−	2-Ketogluconate	−

[a] Configuration of lactic acid produced from glucose.

observed at 16, 24, 32, 37, and 45°C. At all of these temperatures, the maximum antimicrobial activity in the growth medium was obtained in the late logarithmic phase of growth (Fig. 1). The amounts of bacteriocin produced at 16, 24, and 37°C were similar (~1,350 mm^2 ml^{-1}) but only 80% of that detected at 32°C (1,700 mm^2 ml^{-1}). The bacteriocin activity in the supernatant was very stable, and no decrease in activity was detected after 5 days at 16°C. Nevertheless, at 24, 32, and 37°C, a drop in antimicrobial activity was observed during the stationary phase. The bacteriocin produced at 45°C (1,200 mm^2 ml^{-1}) was 90% of that at 37°C, and a rapid decrease in activity was detected at the beginning of the stationary phase at 45°C.

Partial characterization of the bacteriocin. The antimicrobial activity of the supernatant of *E. faecium* P13 was abolished by proteolytic treatment but withstood heating (i.e., 60 min at 100°C or 15 min at 121°C) and exposure to pH between 2 and 11 for 24 h at 25°C. The antimicrobial activity was not lost by freezing and thawing, lyophilization, and long-term storage at 4 and −20°C (results not shown).

The bactericidal effect of the antimicrobial activity on growing cells of *L. monocytogenes* Scott A is shown in Fig. 2. Within 45 min after addition of 1 ml of 20-fold-concentrated supernatant of *E. faecium* P13, the viable colony counts dropped rapidly to approximately 20% (from 3.7×10^5 to 7.0×10^4 CFU ml^{-1}), and after 4 h the viable count (7.7×10^3 CFU ml^{-1}) represented only 2% of the initial viable count. After incubation for 8 h, the viable counts of cultures with and without bacteriocin were 1.4×10^4 and 2.0×10^8 CFU ml^{-1}, respectively.

Purification of enterocin P. Results of bacteriocin purification obtained from a late-logarithmic-growth phase culture of *E. faecium* P13 grown at 32°C in MRS broth are summarized in Table 3. Ammonium sulfate precipitation allowed an eightfold increase in specific antimicrobial activity and 99% recovery of bacteriocin activity. The 10-ml fraction eluted from the hydrophobic interaction column contained 90% of the initial antimicrobial activity. The reverse-phase chromatography was repeated three times, which resulted in a single absorbance peak, coinciding with the antimicrobial activity peak (Fig. 3). The final specific activity of the pure enterocin P was approximately 115,000-fold greater than that in the culture supernatant. The protein concentration in this fraction was calculated to be 10 μg ml^{-1}.

MICs of enterocin P. The MICs of pure enterocin P estimated by a microtiter plate assay against selected gram-positive microorganisms are shown in Table 1. Most LAB were not inhibited by pure enterocin P. However, all of the spoilage and food-borne pathogenic bacteria tested were inhibited by pure enterocin P at MICs ranging from 4 to 559 ng ml^{-1}. The growth of *Bacillus cereus* ATCC 9139 and *Clostridium tyrobutyricum* NCDO1754 was inhibited by purified enterocin P, although these strains were not sensitive to the crude bacteriocin preparations. No test was performed to determine whether enterocin P could inhibit the outgrowth of spores.

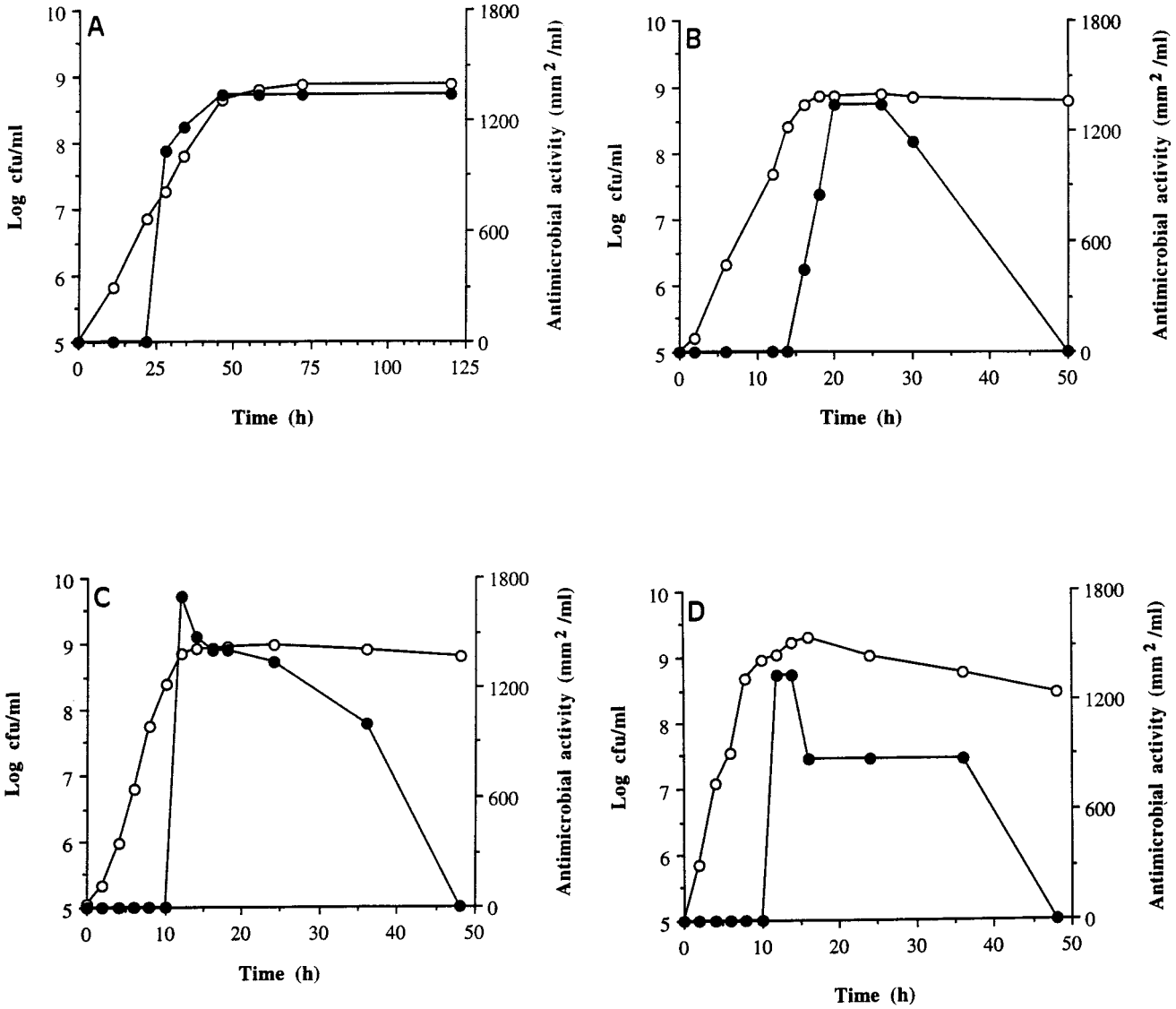

FIG. 1. Growth kinetics of *E. faecium* P13 (○) and production of enterocin P (●) in MRS broth at 16°C (A), 24°C (B), 32°C (C), 37°C (D), and 45°C (E). Data presented are the means of two experiments for which differences were small and not significant.

Amino acid sequence analysis. The first 43 amino acid residues of the N terminus of enterocin P were determined by Edman degradation. The following sequence was obtained: ATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWA SGLAGMG. The partial sequence includes the pediocin-like bacteriocin consensus amino acid sequence YGNGV in positions 5 to 9, the two conserved cysteine residues in positions 11 and 16, and the conserved valine residue in position 18 (5). The N-terminal part of the molecule (from residues 1 to 25) contains a high percentage of polar amino acids, with 14 residues of noncharged polar amino acids, 3 residues of positively charged amino acids, and 2 residues of the negatively charged glutamic acid. The C-terminal region is more hydrophobic, containing 10 nonpolar residues and 8 neutral amino acids. No charged residues are present at the C-terminal region.

Nucleotide sequence of the enterocin P structural and immunity genes. By DNA sequencing a number of PCR products,

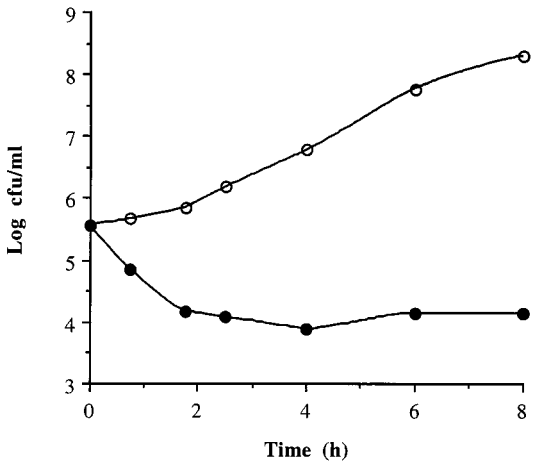

FIG. 2. Effect of bacteriocin extract (20-fold-concentrated supernatant) from *E. faecium* P13 on the viable count of *L. monocytogenes* Scott A (●). The viable count of *L. monocytogenes* Scott A (○) with the addition of a 20-fold-concentrated supernatant of *P. acidilactici* 144, a nonbacteriocinogenic strain, is shown as a control.

755 contiguous nucleotides were obtained. Analysis of this sequence revealed the presence of two consecutive ORFs (Fig. 4). The first ORF, termed *ent*P, encodes a 71-amino-acid protein containing a 27-amino-acid leader sequence followed by the amino acid sequence corresponding to the purified and sequenced enterocin P. The amino acid sequence of the mature bacteriocin deduced from the nucleotide sequence (Fig. 4) matches that obtained from Edman degradation except for the presence of a residue of His at the C-terminal position, which was not detected in the amino acid sequencing. The bacteriocin is apparently synthesized as a prepeptide that is cleaved immediately after the Val-Asp-Ala residues (positions −3 to −1), resulting in the mature bacteriocin consisting of 44 amino acids and a theoretical molecular weight of 4,493. In the DNA sequence, an ATG start codon is preceded 8 bp upstream by a potential ribosome binding site (AAAGGAGGT). A likely −10 consensus promoter region (Pribnow box) (TATAAT) is located at position 140, and a sequence (TTATCA) showing resemblance to σ^{70} promoter −35 region is located at the optimal distance of 19 nucleotides upstream the −10 region. Two sets of direct repeats were seen in the promoter region. In one case, the repeat consisted of 9 bp with the sequence TTA TG-T-T. The two repeats were spaced by an AT-rich region of 12 bp. The repeat close (2 bp) to the −35 site was termed right (*DR1R*), whereas the second repeat further upstream (12 bp) was termed left (*DR1L*). In the other pair of direct repeats (DR2), the repeat consisted of 9 bp with the same sequence (TATAATTAT). The two repeats were spaced by an AT-rich region of 21 bp. *DR2R* and *DR2L* were partially overlapped the putative −10 and −35 promoter regions, respectively. Two 10-bp dyad symmetry sequences (inverted repeats) rich in AT were also present in the promoter region. One repeat (*IRR*) was immediately upstream of the −10 site, whereas the second one (*IRL*) was located 55 bp upstream of the *IRR*.

A second ORF, *orf*2, potentially encoding a protein composed of 88 amino acids with a calculated molecular weight of 9,886, was found four nucleotides downstream of the stop codon of *ent*P. The ATG start codon is preceded 10 bp upstream by a potential ribosome binding site (GGGA). There was no obvious promoter sequence upstream of the start codon. The second ORF may encode the immunity protein which is usually encoded by a gene located next to and downstream of the bacteriocin structural gene (43).

DISCUSSION

Eight percent of the LAB isolates from Spanish dry-fermented sausages exerted anti-*L. monocytogenes* activity on solid media by a stab-on agar test. Similar frequencies have been previously reported (10, 52). The isolate identified as *E. faecium* P13 shows many interesting properties related to food preservation, such as ability to grow at refrigeration temperatures, at a wide range of pH, and in presence of a 6.5% NaCl. In addition, *E. faecium* P13 produces enterocin P, a bacteriocin with a broad antimicrobial spectrum that inhibits spoilage bacteria such as *Enterococcus faecalis*, *Staphylococcus carnosus*, *Clostridium sporogenes*, *C. tyrobutyricum*, and *Propionibacterium* spp. and food-borne pathogens such as *L. monocytogenes*, *Clostridium perfringens*, *C. botulinum*, and *S. aureus*. The antilisterial activity is a common characteristic among enterococcal bacteriocins (22), but to our knowledge enterocin P is the first well-characterized enterocin with such a wide inhibitory spectrum. Apart from its antimicrobial activity, enterocin P shows other properties that make it a promising food preservative. These properties include protease sensitivity, thermal stability, activity over a wide range of pH values, and the maintenance of antimicrobial activity after freeze-thawing, lyophilization, and storage.

Several reports dealing with bacteriocins produced by enterococci of food origin have recently been published (5, 6, 8, 16, 20, 45, 59). However, only enterocins A and B, produced by *E. faecium* CTC492 and *E. faecium* T136, respectively, isolated from Spanish dry-fermented sausages, have been characterized at the biochemical and genetic levels (5, 8). The purification procedure of enterocin P resulted in the recovery of about 28% of the total antimicrobial activity of the cell-free culture super-

TABLE 3. Purification of enterocin P13

Purification stage	Vol (ml)	Total A_{254}[a]	Total activity (10^3 BU)	Sp act[b]	Increase in sp act (fold)	Yield (%)
Culture supernatant	1,000	27,500	455	16	1	100
Fraction						
Ammonium sulfate precipitation	120	3,300	452	137	8	99
Gel filtration chromatography	230	2,700	352	130	8	77
Cation-exchange chromatography	50	160	276	1,700	106	61
Hydrophobic-interaction chromatography	10	7	410	58,600	3,700	90
Reverse-phase chromatography	1	0.07	129	1,850,000	115,600	28

[a] Optical density at 254 nm multiplied by the volume in milliliters.
[b] Bacteriocin units (BU) per milliliter divided by optical density at 254 nm.

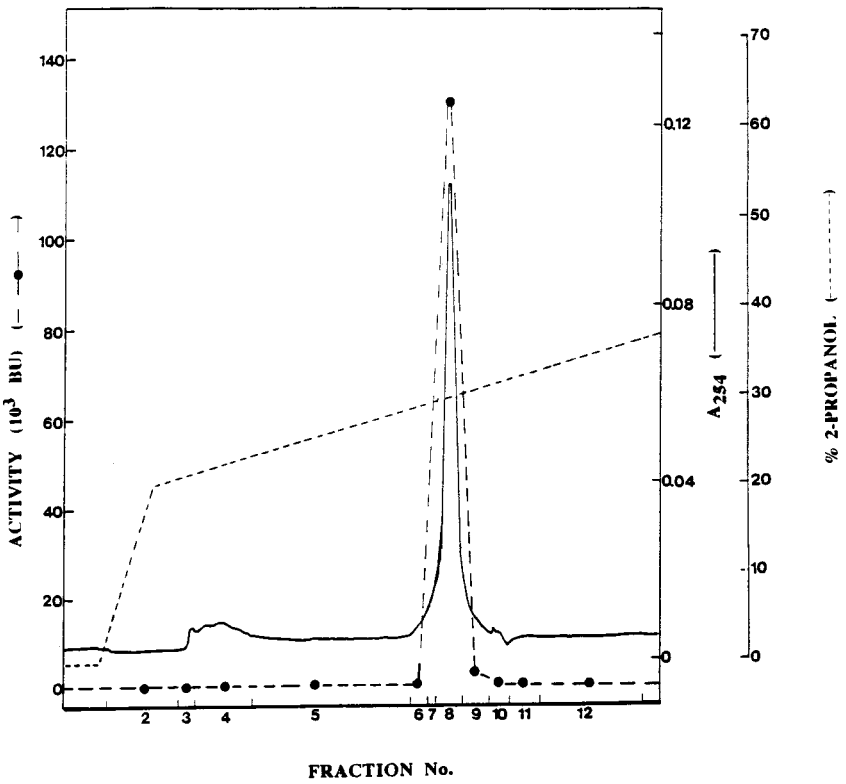

FIG. 3. Reverse-phase chromatography of enterocin P. The amount applied to the column was obtained from approximately 1 liter of culture. BU, bacteriocin units.

natant. An increase in bacteriocin activity was observed after the hydrophobic-interaction chromatography, which may be due to the removal of inhibitors of bacteriocin activity during the purification or to a conformational change of the molecule to a more active form in the hydrophobic solvent. This increase in biological activity has also been reported for pediocin L50 (10) and some pediocin-like bacteriocins, such as pediocin PA-1 (27), enterocin A (5), curvacin A (sakacin A), and sakacin P (57). The antimicrobial spectrum of purified enterocin P resembled that of culture supernatants, which may indicate that this bacteriocin is the only one produced by *E. faecium* P13. It should be mentioned that it is common for a single bacterial strain to produce more than one bacteriocin (8, 43). It is noteworthy that *B. cereus* ATCC 9139 and *C. tyrobutyricum* NCDO1754 were sensitive to the purified bacteriocin, while crude preparations were inactive against these strains.

The first 43 residues of enterocin P were determined, and the theoretical molecular weight was calculated to be 4,493. The bacteriocin is a small, heat-stable, cationic, and hydrophobic peptide that contains the consensus amino acid sequence Tyr-Gly-Asn-Gly-Val, which is a common motif within the pediocin-like bacteriocins (5, 44, 57). The complete amino acid sequence of the mature enterocin P deduced from the nucleotide sequence was compared with those of other bacteriocins from class II (Fig. 5B). The highest scores (80.5% identity) were obtained with sakacin A (curvacin A), produced by *Lactobacillus sake* Lb706 (28) and *L. curvatus* LTH1174 (57), respectively, and with carnobacteriocin BM1 (74.5% identity), produced by *Carnobacterium piscicola* LV17B (49). Identity of enterocin P with other bacteriocins from enterococci was not so strong: 46.5 and 44.2% identity with enterocin A (5) and bacteriocin 31 (58), respectively. Enterocin P also shows significant homology with other pediocin-like bacteriocins: the identities between enterocin P and leucocin A-UAL187 (23), sakacin P (bavaricin A) (38, 57), mesentericin Y105 (26), carnobacteriocin B2 (49), and pediocin PA-1 (27) were determined to be 48.7, 41.9, 40.5, 38.6, and 36.4%, respectively. These observations clearly show that there is no significant correlation between the homology in the primary structure of pediocin-like bacteriocins and the phylogenetic relatedness of the bacteriocinogenic bacteria. Two residues of cysteine, which may establish sulfur bridges, are present at conserved positions in these pediocin-like bacteriocins. Pediocin-like bacteriocins show strong homology at the N-terminal part of the molecule (5), but they possess differences in their antimicrobial spectra, which could be due to the more variable C-terminal region of the peptides (18). Computer analysis of enterocin P and the pediocin-like bacteriocins cited above predicted a putative transmembrane helix for enterocin P, sakacin A, carnobacteriocin BM1, and enterocin A, between residues 27 and 43, 25 and 40, 25 and 42, and 26 and 43, respectively (not shown). Transmembrane sequences have also been identified in a number of bacteriocins of LAB (29, 35, 36). Enterocin P has a bactericidal effect on *L. monocytogenes*, but the mechanism of action is not yet known. Nevertheless, the presence of this putative transmembrane helix led us to speculate that the bacteriocin may cause an ionic imbalance in the cell, by a membrane-permeabilizing mode of action, which has been already described for other LAB bacteriocins, including pediocin-like bacteriocins such as pediocin PA-1 (9). Moreover, the location of these putative transmembrane helices at the more heterogeneous C-terminal region of enterocin P, sakacin A, carnobacteriocin BM1, and enterocin A suggests the significance

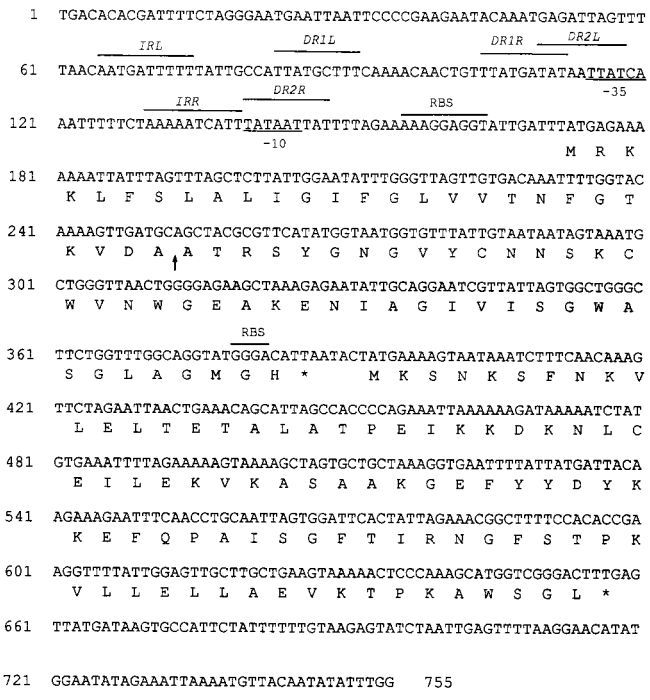

FIG. 4. Nucleotide sequence of the 755-bp fragment containing the enterocin P structural gene (*ent*P) and the putative immunity gene (*orf2*). The deduced amino acid sequences of enterocin P and ORF2 are shown below the DNA sequence. The cleavage site of the prebacteriocin is indicated by a vertical arrow. The putative −10 and −35 promoter sequences are underlined; ribosome binding sites (RBS), direct repeats (*DR1* and *DR2*) within the conserved regulatory-like boxes, and inverted repeat sequences (*IRR* and *IRL*) are overlined.

of this part of the molecules in the mode of action of these bacteriocins.

Following the N-terminal methionine, the deduced leader sequence of enterocin P contains three positively charged amino acids, a span of hydrophobic amino acids, and two small and hydrophobic amino acids at positions −1 and −3 relative to the cleavage site (Fig. 4). This sequence conforms to a typical signal peptide, as described by von Heijne (64) for proteins processed and secreted by the *sec*-dependent pathway. In contrast, most class II bacteriocins possess a double-glycine-type leader sequence with two conserved glycine residues at positions −1 and −2 (43). Signal peptides target proteins for secretion (48), and it has been shown that the double-glycine-type leaders play the same biological role (61). Despite the large number of LAB bacteriocins described to date, only three have been reported to be secreted by the *sec*-dependent pathway. Divergicin A (67) and acidocin B (40) are small, hydrophobic, thermostable bacteriocins produced by *Carnobacterium divergens* and *Lactobacillus acidophilus*, respectively. Bacteriocin 31 is a pediocin-like bacteriocin encoded on the *E. faecalis* pheromone-responsive conjugative plasmid pYI17 (58). The signal peptides of these *sec*-dependent bacteriocins were compared to that of enterocin P. The identity between the signal peptide of enterocin P and that of bacteriocin 31 (58) was 50%, whereas only about 25% identity with those of the non-pediocin-like divergicin A (67) and acidocin B (40) was observed. The signal peptides of the two *sec*-dependent pediocin-like bacteriocins includes (i) three basic residues (Arg/Lys-Lys-Lys) near the N terminus and (ii) the Val-X-Ala (positions −3 to −1) cleavage site, which contains an acidic residue (Asp or Glu) at position −2 (Fig. 5A). Recently, it has been reported that bacteriocins containing a double-glycine-type leader sequence can be secreted by heterologous bacteriocin ABC transporters (1, 2, 60, 61). In addition, it has been demonstrated that it is possible to exchange signal peptides for double-glycine-type leaders, and vice versa, and still maintain secretion of bacteriocins by the appropriate secretion apparatus (41, 61).

The gene encoding the putative immunity protein of enterocin P is located immediately downstream of the structural gene of enterocin P, which is a common feature of LAB bacteriocins (43). However, exceptions to this rule are observed, as structural genes of carnobacteriocin A (66) and enterocin B (8) are both followed by a putative *rho*-independent terminator. Homology searches for the deduced amino acid sequence of *orf2* revealed a significant homology, most pronounced at the C-terminal part of the molecules, with putative immunity proteins of other pediocin-like bacteriocins (Fig. 6). The highest scores were obtained with the putative immunity proteins of sakacin A (4) (78% identity), carnobacteriocin BM1 (49) (73% identity), and bacteriocin 31 (58) (44% identity). Significant similarities between putative immunity proteins of LAB bacteriocins have been reported: 44.5% identity between those of enterocin A and leucocin A-UAL187 (5) and 49% identity between those of sakacin A and carnobacteriocin BM1 (4). Nevertheless, such a strong homology between the putative

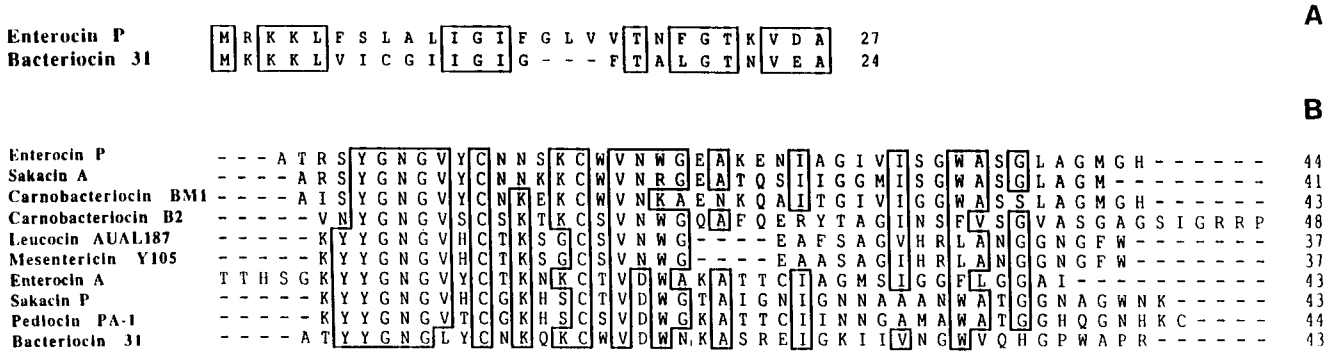

FIG. 5. (A) Comparison of the predicted signal peptide of enterocin P with that of bacteriocin 31 (58); (B) alignment of the mature enterocin P with other pediocin-like bacteriocins. The sequence of sakacin A (curvacin A) is from Tichaczek et al. (57) and from Holck et al. (28), those for carnobacteriocins BM1 and B2 are from Quadri et al. (49), that for leucocin A-UAL187 is from Hastings et al. (23), that for mesentericin Y105 is from Héchard et al. (26), that for enterocin A is from Aymerich et al. (5), that for sakacin P (bavaricin A) is from Tichaczek et al. (57) and from Larsen et al. (38), that for pediocin PA-1 is from Henderson et al. (27), and that for bacteriocin 31 is from Tomita et al. (58). Conserved amino acid residues are boxed.

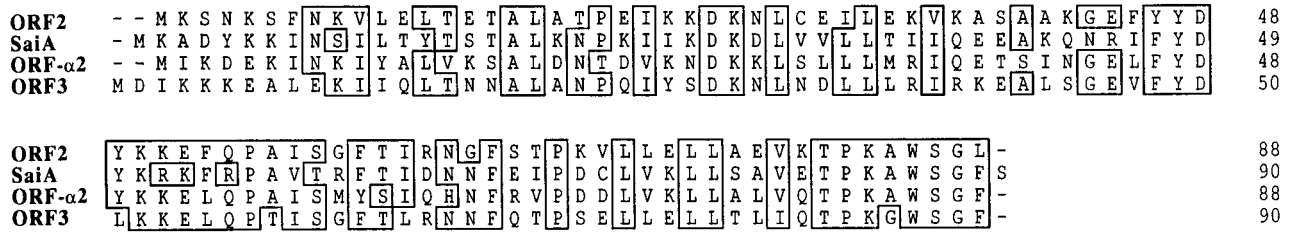

FIG. 6. Alignment of the putative immunity proteins of enterocin P (ORF2), sakacin A (SaiA) (4), carnobacteriocin BM1 (ORF-α2) (49), and bacteriocin 31 (ORF3) (58).

immunity protein of a bacteriocin and those of the most closely related bacteriocins has not been previously reported. From these observations, it is possible to speculate that the host immunity protein may protect the bacterial cell not only against its own bacteriocin but also against close related bacteriocins.

No studies on the regulation of any enterocin have been carried out yet, but there are some molecular features in the promoter region of the structural gene of enterocin P that suggest that the enterocin P operon is regulated. The presence of two 9-bp direct repeats spanned by a 12-bp region resembles the conserved regulatory-like boxes previously found in the bacteriocin promoters of *Lactobacillus plantarum* C11, which may serve as regulatory elements for gene expression (15). The other 9-bp direct repeats found in the promoter region of *ent*P are spanned by 21 bp, which indicates that they are located in the same side of the DNA double helix but separated by two helix turns. This spatial conformation would achieve optimal contacts with the DNA binding domains of the regulators (15). In addition, the inverted repeats found in the promoter region of the structural gene of enterocin P may serve as binding sites for transcription regulators (e.g., in the λ switch [47]), and they were also found in the promoter region of the structural gene of enterocin P. These findings are the first indication that transcriptional regulation may have some role in pediocin-like bacteriocins.

The present work increases our knowledge about *sec*-dependent bacteriocins and suggests that they are closely related to the ABC transporter-secreted bacteriocins, since pediocin-like bacteriocins are now shown to be processed and secreted by both mechanisms. Moreover, this novel bacteriocin represents a new and interesting model with which to study the *sec*-dependent secretion and the regulation of bacteriocin production in LAB.

Industrial use of LAB genetically modified to produce enterocin P and other broad-spectrum bacteriocins with different modes of action may overcome the bacterial resistance problem, as well as be very effective against spoilage and foodborne pathogenic bacteria, such as *L. monocytogenes*, *C. perfringens*, *C. botulinum*, and *S. aureus*. In this respect, the fact that the secretion of enterocin P does not require a dedicated ABC transporter may represent an advantage for its heterologous expression in other bacteria.

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