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Biochemical and Genetic Characterization of Enterocin A from *Enterococcus faecium*, a New Antilisterial Bacteriocin in the Pediocin Family of Bacteriocins

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A new bacteriocin has been isolated from an *Enterococcus faecium* strain. The bacteriocin, termed enterocin A, was purified to homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and mass spectrometry analysis. By combining the data obtained from amino acid and DNA sequencing, the primary structure of enterocin A was determined. It consists of 47 amino acid residues, and the molecular weight was calculated to be 4,829, assuming that the four cysteine residues form intramolecular disulfide bridges. This molecular weight was confirmed by mass spectrometry analysis. The amino acid sequence of enterocin A shared significant homology with a group of bacteriocins (now termed pediocin-like bacteriocins) isolated from a variety of lactic acid-producing bacteria, which include members of the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Carnobacterium*. Sequencing of the structural gene of enterocin A, which is located on the bacterial chromosome, revealed an N-terminal leader sequence of 18 amino acid residues, which was removed during the maturation process. The enterocin A leader belongs to the double-glycine leaders which are found among most other small nonlantibiotic bacteriocins, some lantibiotics, and colicin V. Downstream of the enterocin A gene was located a second open reading frame, encoding a putative protein of 103 amino acid residues. This gene may encode the immunity factor of enterocin A, and it shares 40% identity with a similar open reading frame in the operon of leucocin AUL 187, another pediocin-like bacteriocin.

In recent years, a number of new bacteriocins have been purified from lactic acid bacteria (LAB). These bacteriocins inhibit the growth of or kill other gram-positive bacteria. Some bacteriocins, such as lactococcin A (19), produced by *Lactococcus lactis*, kill only bacteria belonging to the same species as the producer, whereas other bacteriocins kill a broad range of gram-positive bacteria (22). Bacteriocins can be grouped according to similarities in properties such as primary sequence (32, 49), chemical modifications (45, 48), physicochemical properties (32, 35), leader sequence (15, 19, 22, 30), and number of peptides constituting the bacteriocin activity (2, 33, 52).

Of the bacteriocins produced by gram-positive bacteria, the most widely studied are the lantibiotics, which are distinguished by the presence of lanthionine and/or 2-methyl-lanthionine residues in the polypeptide (45). Another group contains the pediocin-like bacteriocins, named after the first-characterized and most widely studied bacteriocin, pediocin PA-1 (pediocin AcH) (5, 14, 16–18, 25, 27, 28, 32, 38, 49). The pediocin-like bacteriocins have a consensus amino acid sequence motif, as initially outlined in our previous studies (32, 49). Another distinctive and important feature of these bacteriocins is their ability to kill listeriae. The pediocin-like bacteriocins are consequently potential additives for inhibiting growth of *Listeria monocytogenes* in food to ensure food safety (13, 16, 24, 39, 40). The ability of several pediocin-like bacteriocins and bacteriocin-producing LAB to inhibit the growth of potential pathogens in food has been investigated (3, 4, 6, 11, 31, 37, 43, 56).

Bacteriocins within the pediocin-like family have so far been identified in the genera *Pediococcus* (17, 27, 28, 32), *Leuconostoc* (14, 16), *Lactobacillus* (18, 25, 48, 50, 51), and *Carnobacterium* (38). In the present report, we describe the isolation and characterization of a new bacteriocin from *Enterococcus faecium*, called enterocin A. Its amino acid sequence demonstrated that it is a new member in the pediocin-like family of bacteriocins.

A number of reports describing bacteriocin activity in *Enterococcus* species have appeared recently (26, 36, 42, 47, 55) but enterocin A is the first bacteriocin from *E. faecium* to be purified and characterized at the amino acid and DNA sequence levels.

MATERIALS AND METHODS

Bacterial strains and media. The bacteriocin producer, *E. faecium* CTC492, was isolated from fermented Spanish sausage, and the indicator organism used in the bacteriocin assay was *Listeria innocua* BL86/26 (obtained from The Netherlands Organization for Applied Scientific Research [TNO], Zeist, The Netherlands). They were grown at 30°C in de Man, Rogosa, Sharp broth (MRS) medium and brain heart infusion broth, respectively (Oxoid, Basingstoke, United Kingdom). *Pediococus acidilactici*, producing pediocin PA-1 (32), *Lactobacillus sake* Lb706 (18), producing curvacin A, and *Lactobacillus sake* LTH673 (49), producing sakacin P, were grown in MRS medium as described previously.

Bacterial characterization. The carbohydrate fermentation was determined by the method of Sharpe (46), using the miniaturized method described by Jayne-Williams (20, 21) and adapted by Schillinger and Lücke (44). The plates were incubated at 25°C in anaerobic jars (Oxoid gas-generating kit anaerobic system code BR38) for 72 h. The isolated strain fermented glucose, melibiose, cellobiose, sucrose, L-arabinose, maltose, ribose, mannitol, lactose, and trehalose but did not ferment melezitose, rhamnose, or D-raffinose. Gas production from

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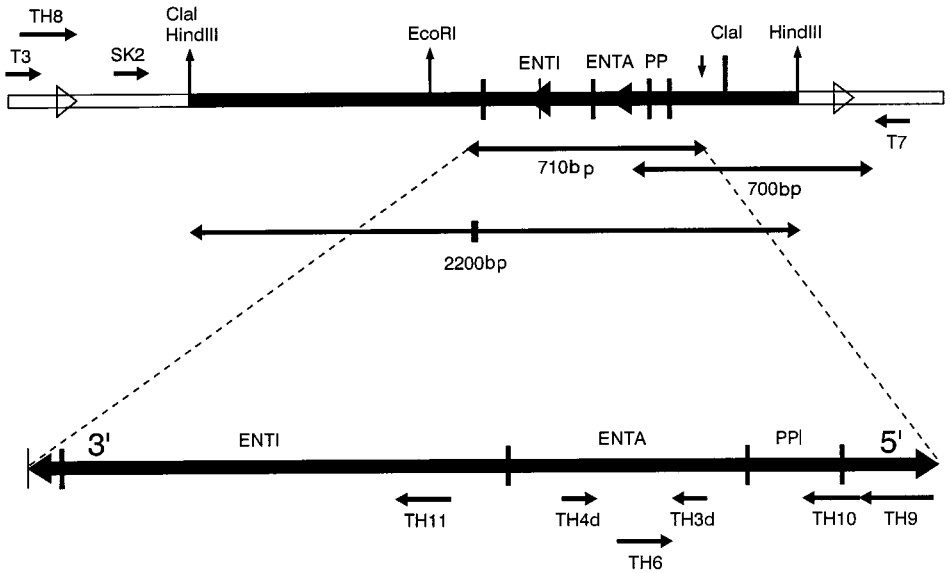

FIG. 1. DNA sequencing strategy for the enterocin A gene of *E. faecium.* ENTA, structural gene for enterocin A; EBTI, putative immunity gene; PP, putative promoter region.

glucose under anaerobic conditions was tested in bacteria grown for 72 h at 30°C in MRS medium supplemented with sodium citrate instead of ammonium citrate.

Growth at 6.5% NaCl and at pH 9.6 was determined as described previously (29). Deamination of arginine was tested anaerobically in the absence of glucose by the method of Schillinger and Lücke (44). The configuration of the produced lactic acid from glucose was determined enzymatically with D-lactate and L-lactate dehydrogenases as described by the supplier (Boehringer GmbH, Mannheim, Germany). Acetoin production was determined by the Voges-Proskauer test (41). The presence of *meso*-diaminopimelic acid in the cell wall was detected by the method of Abo-Elnaga and Kandler, using thin-layer chromatography on cellulose plates (1). The group antigen and the hydrolysis of L-pyrrolidonyl-β-naphthylamide were determined by using the STREPTEX test as specified by the producers (Wellcome Diagnostics, London, England). Growth in 0.1% thallous acetate and reduction of triphenyltetrazolium chloride were assayed in nutrient agar.

Bacteriocin assay. The bacteriocin was quantified in a microtiter plate assay system (33). Each well of the microtiter plate contained 200 μl of MRS broth, bacteriocin fractions at twofold dilutions, and the indicator organism (A_{600} = 0.1). The microtiter plate cultures were incubated for about 4 h at 30°C, and the growth inhibition of the indicator organism was measured spectrophotometrically at 600 nm by using a Dynatech microplate reader. One bacteriocin unit (BU) was defined as the amount of bacteriocin which inhibited the growth of the indicator organism by 50% (resulting in 50% of the turbidity of the control culture without bacteriocin).

Bacteriocin purification. The bacteriocin was purified from a 2-liter culture of *Enterococcus faecium* similarly to the method described for pediocin PA-1 (32). The cultures were grown to the early stationary phase. The cells were then removed by centrifugation at 4,000 × *g* for 15 min at 4°C (fraction I), and 400 g of ammonium sulfate per liter of culture supernatant was added. The protein precipitate was pelleted by centrifugation at 7,000 × *g* for 20 min and dissolved in 500 ml of 20 mM sodium phosphate buffer (pH 5.0) (buffer A) (fraction II). Fraction II was applied at a flow rate of about 10 ml/min to a 7-ml S-Sepharose Fast Flow cation-exchange column equilibrated with buffer A. The column was then washed with 20 ml of buffer A, and the bacteriocin was eluted from the column with 40 ml of 1 M NaCl in buffer A (fraction III). Ammonium sulfate was added to fraction III to a final concentration of 10% (wt/vol), after which the sample was applied at a flow rate of about 4 ml/min to a 2-ml octyl-Sepharose CL-4B column (Pharmacia Biotechnology, Uppsala, Sweden) equilibrated with 10% (wt/vol) ammonium sulfate in buffer A. The column was then washed with 8 ml of buffer A, and the bacteriocin activity was eluted with 10 ml of 70% (vol/vol) ethanol–0.1% trifluoroacetic acid (fraction IV). Fraction IV was diluted to 50 ml with H_2O and subsequently applied to a C_2/C_{18} reverse-phase column (PepRPC HR 5/5; Pharmacia Biotechnology) equilibrated with 0.1% trifluoroacetic acid in water. The bacteriocin was eluted with 30% 2-propanol containing 0.1% trifluoroacetic acid (fraction IV). This chromatographic step was repeated once to obtain electrophoretically pure bacteriocin. The bacteriocin peptides eluted from the reverse-phase column (fraction V) in a 1.1-ml fraction. Purified bacteriocin was stored at −20°C in 50 to 60% 2-propanol and/or ethanol containing 0.1% trifluoroacetic acid.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with PhastGel homogeneous 20% gels, Phast-Gel SDS buffer strips, and the PhastSystem (Pharmacia Biotechnology) as recommended by the manufacturer. The molecular weight standards (Pharmacia Biotechnology) consisted of five peptides with molecular weights between 2,512 and 16,949. The gel was stained with Coomassie blue.

N-terminal amino acid sequencing and mass spectrometry analysis. The amino acid sequencing was performed by automated Edman degradation with a model 477A sequencer/model 120A phenylthiohydantoin analyzer (Applied Biosystems, Foster City, Calif.) by K. Sletten, University of Oslo, Oslo, Norway. The mass spectrometry was performed by S. Bayne, NOVO Nordisk, Gentofte, Denmark, with a PE Sciex AP 1 electrospray mass spectrometer.

Sequence analysis. The strategy for sequencing the enterocin A gene is outlined in Fig. 1. Chromosomal DNA was prepared as described previously (9). All the DNA primers used for PCR and DNA sequencing are shown in Table 1. The degenerated primer, TH3 (biotinylated), and primer TH4 were used in a PCR together with chromosomal DNA from *E. faecium* CTC492 as target DNA. The PCR conditions included a hot start at 97°C (5 min), an annealing temperature of 40°C (1 min), and polymerization at 72°C (2 min), and the reaction was repeated for 30 cycles. PCR was performed with the GeneAmp PCR kit as described by the supplier (Perkin-Elmer Cetus). The small DNA fragment was analyzed and separated by agarose gel (1.2%) electrophoreses and purified by transfer to DEAE paper. Single-stranded DNA for DNA sequencing was obtained by use of the Dynabead M-280 streptavidin kit (Dynal, Oslo, Norway). DNA sequencing (Sequenase, 2.0 System; U.S. Biochemicals, Cleveland, Ohio) allowed us to read 41 nucleotides, verifying that we had obtained the correct fragment of DNA. From the known sequence, sequence-specific primers were synthesized (TH5 and TH6) to be used as hybridization probes, PCR primers, and DNA-sequencing primers. Chromosomal DNA was restricted with *Hin*dIII, *Cla*I, *Eco*RI-*Cla*I, and *Eco*RI-*Hin*dIII, and Southern analysis with the TH5 ^{32}P-labeled probes identified 2.1-, 1.9-, 0.7-, and 1.0-kb DNA fragments, respectively. The *Hin*dIII-specific fragment was purified from an agarose gel and ligated to Bluescript II SK+/− vector (Stratagene, La Jolla, Calif.). PCR was performed on the ligated products with the vector-specific T7 primer and the bacteriocin-specific TH6 primer (Table 1). The PCR conditions were as described above, except that the annealing was performed at 45°C. An approximately 700-bp PCR DNA fragment was obtained and used for sequencing. Nested PCR was performed on the ligated *Hin*dIII fragment-Bluescript vector DNA hybrid by using the two primer sets, first the TH8 (vector primer)-TH9 (bacteriocin primer) pair and then the SK2 (vector primer)-TH10 (bacteriocin primer) pair. DNA sequencing with TH11 as the sequencing primer of the nested PCR product gave the finalized DNA sequence of the putative immunity gene. Analysis of open reading frames (ORFs) and amino acid alignments were performed with programs in the Sequence Analysis Software Package (version 7.2) licensed from the Genetics Computer Group, University of Wisconsin, Madison (7).

RESULTS

Characterization of the bacteriocin-producing strain. The isolated strain was a catalase-negative, gram-positive, faculta-

TABLE 1. Primers used for PCR and DNA sequencing

Primer	Technique	Sequence
TH3 bio[a]	PCR	5′-TAC GGI AAC GGI GTI TAC TG-3′
	SEQ[b]	T T T 3 6 9 12 15 18
TH4	PCR	5′-GTI GTI GCC TTI GCI GTG TC-3′
	SEQ	3 6 9 12 15 18
TH5	PCR	5′-CAC TAA AAA TAA ATG TAC GGT GG 3 6 9 12 15 18 21
TH6	PCR	5′-GAC CGT ACA TTT ATT TTT AGT GC-3′
	SEQ	3 6 9 12 15 18 21
TH8	PCR	5′-CTC ACT AAA GGG AAC AAA AGC TGG AG-3′ 3 6 9 12 15 18 21 24
TH9	PCR	5′-GTT AGA ATA ATA GTC TAA AAA CAT TAA TTT AGG GGT G-3′ 3 6 9 12 15 18 21 24 27 30 33 36
TH10	PCR	5′-GAT TAT GAA ACA TTT AAA AAT TTT GTC-3′
	SEQ	3 6 9 12 15 18 21 24 27
TH11	SEQ	5′-CCT AAA TAT TCT GAT ATT CTT G-3′ 3 6 9 12 15 18 21
SK2	PCR	5′-CCG CTC TAG AAC TAG TGG ATC-3′
	SEQ	3 6 9 12 15 18 21
T3 bio	SEQ	5′-AAA TTA ACC CTC ACT AAA GG-3′ 3 6 9 12 15 18
T7 bio	PCR	5′-GTA ATA CGA CTC ACT ATA GGG-3′
	SEQ	3 6 9 12 15 18 21

[a] Biotinylated.
[b] SEQ, used for sequencing.

tively anaerobic coccus which possessed the Lancefield group D antigen. It did not produce gas from glucose, and it tested positive in the Voges-Proskauer and pyrrolidonylarylamidase tests. Furthermore, it was resistant to bile-esculin and was able to grow at 10 and 45°C, at pH 9.6, and in the presence of 6.5% NaCl. These features, as well as its ability to grow on arabinose and its inability to reduce triphenyl tetrazolium chloride, identified the strain as *Enterococcus faecium* (8).

Purification and characterization of enterocin A. The bacteriocin was secreted into the growth medium, and maximum bacteriocin activity was obtained in the early stationary growth phase (results not shown). The first step in the purification protocol was to concentrate the activity from the growth medium by ammonium sulfate precipitation. The next steps in the purification were cation-exchange chromatography followed by hydrophobic interaction chromatography. The final step in the purification was reverse-phase chromatography, which was repeated twice to obtain an electrophoretically pure bacteriocin (Fig. 2 and 3). The overall purification procedure is summarized in Table 2. Surprisingly, the last reverse-phase chromatographic step resulted in a significant increase in total activity. An increased amount of biological activity has also been reported during purification of other bacteriocins in the pediocin family and may be due to the presence of some inhibitory compound at an earlier stage of the purification (17, 33, 49).

The purification resulted in an electrophoretically pure protein with a molecular weight of approximately 5,000 (Fig. 3). The molecular weight as determined by ionic spray spectrometry was 4,828; in addition, a minor peak of 4,845, which may be an oxidized form of enterocin A, was detected (data not shown).

The first 43 amino acid residues were identified by N-terminal automatic amino acid sequencing of the isolated bacteriocin (Fig. 4A). The sequencing was incomplete, but it was not possible to distinguish the various phenylthiohydantoin amino acid derivatives in the last sequencing cycles of enterocin A. The molecular weight obtained by mass spectrometry indicated that four or five amino acids remained unidentified in the C terminus of the molecule. An amino acid sequencing homology search revealed that enterocin A shared significant homology with other pediocin-like bacteriocins (Fig. 4B): the identities between enterocin A and pediocin PA-1, sakacin P, sakacin A, leucocin AUL183, carnobacteriocin BM1, and carnobacteriocin B2 were calculated to be 54.6, 44.2, 43.9, 46, 44.2, and 38.3%, respectively.

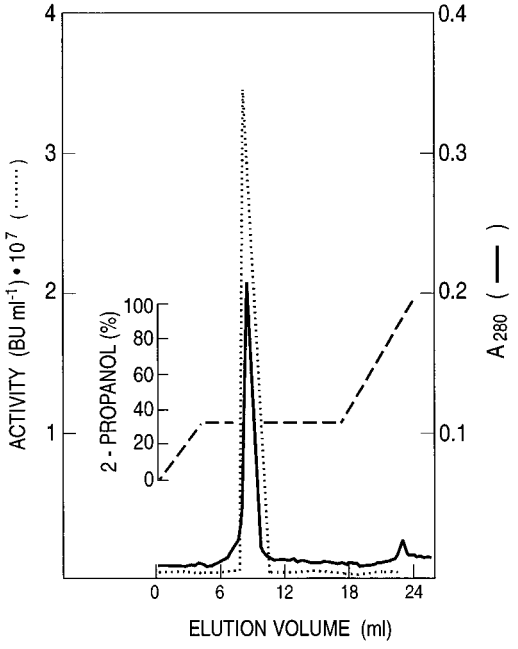

FIG. 2. Reverse-phase chromatographic analysis of fraction IV. The amount applied to the column was obtained from approximately 1 liter of culture.

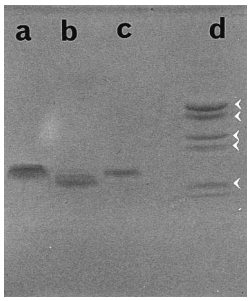

FIG. 3. SDS-PAGE analysis of purified enterocin A. Lanes: a, pediocin PA-1 (molecular weight, 4,628); b, plantaricin A (molecular weight, 2,986); c, enterocin A; d, molecular weight standards (from top to bottom, 16,900, 14,400, 8,200, 6,200, and 2,500) (white arrowheads).

Genetic analysis by PCR and DNA sequencing. Southern analysis revealed that the gene was on the bacterial chromosome (data not shown). After proper DNA primers deduced from the amino acid sequence of enterocin A had been designed, PCRs were carried out on the chromosomal DNA from *E. faecium* CTC492. By using the strategy described in Materials and Methods, various PCR products were sequenced and 712 contiguous nucleotides were obtained (Fig. 5).

ORF analysis revealed the presence of two putative ORFs. ORF 1, termed *entA*, encoded the amino acid sequence of enterocin A, including a presequence of 18 amino acid residues in the putative N terminus. The presequence is a typical double-glycine leader sequence found in most nonlantibiotic bacteriocins and a few lantibiotics, as well as colicin V (10, 12, 15, 19, 26, 32). The sequenced portion of enterocin A was confirmed, and the last five C-terminal residues were identified as Ile Pro Gly Lys Cys. By assuming that the four cysteines exist in two disulfide bridges, the molecular weight of enterocin A was calculated to 4,829. This value is different by only 1 from the mass spectrometry value obtained (data not shown), which strongly suggests that no other posttranslational modification is present in enterocin A. The extinction coefficient of enterocin A was calculated as $1 \times 10^4\ M^{-1}\ cm^{-1}$.

A second ORF, ORF 2, encoding a tentative protein of 103 amino acids with a calculated molecular weight of 12,217, was found 2 nucleotides downstream of the stop codon of *entA*.

Activity spectrum. The inhibitory activity of *E. faecium* CTC492 was tested and compared with the inhibitory activity of three different pediocin-like bacteriocin producers on a number of gram-positive bacteria (Table 3). All the bacteriocin-producing strains inhibited the six *Listeria* strains tested. The only qualitative differences between the four bacteriocin producers was that the pediocin PA-1 and the enterocin A producers were the only strains which inhibited the growth of the pediococcus strains. This finding has also been confirmed with the use of purified pediocin PA-1. None of the four bacteriocin producers inhibited the growth of lactococci (data not presented).

DISCUSSION

A group of related bacteriocins are produced by quite different LAB. They have been found in *Pediococcus*, *Lactobacillus*, *Leuconostoc*, and *Carnobacterium* species, and now we have characterized such a bacteriocin from the genus *Enterococcus*. It has been suggested that this group of bacteriocins should be termed the pediocin-like bacteriocins, because the most widely studied and extensively characterized member is pediocin PA-1 (pediocin AcH). The most striking difference between enterocin A and the other pediocin-like bacteriocins was found in the very N-terminal part of the polypeptide. The first five N-terminal amino acid residues of enterocin A are not found in the other pediocin-like bacteriocins, although sakacin A (identical to curvacin A) contains one additional N-terminal alanine residue (18, 50).

The pediocin-like bacteriocins are apparently very effective in preventing the growth of listeriae. Screening several *Listeria* strains revealed that enterocin A inhibited listeria growth and was similar to the other members of the pediocin-like bacteriocin family in this respect. The most extensive antilisteria study has been performed with the bavaricin A (identical to sakacin P) producer, which inhibited the growth of 242 of 245 *Listeria* strains tested (25).

A few recent reports deal with bacteriocin activity in enterococci, including *E. faecium* (26, 36, 42, 47, 55). However, these bacteriocins have not been purified sufficiently to enable sequencing or to perform any genetic characterization. Enterocin 1146, produced by an *E. faecium* strain, strongly inhibits the growth of listeriae, suggesting that it may belong to the pediocin family (36). Purification and amino acid or DNA sequencing will be necessary to conclusively confirm this hypothesis.

All the sequenced genes of LAB bacteriocins have shown that these bacteriocins are synthesized as precursor peptides and that the N-terminal extension is cleaved off to give the mature bacteriocin. The DNA sequencing revealed that the EntA leader peptide was 18 amino acids long and contained all the consensus elements of double-glycine-type leader peptides (15, 22).

By computer analysis, putative transmembrane sequences have been identified in a number of bacteriocins (22, 23). Analysis of enterocin A also predicts a transmembrane helix

TABLE 2. Purification of the enterocin A from *E. faecium* CTC492

Fraction	Vol (ml)	Total A_{280}[a]	Total activity (10^6 BU)[b]	Sp act (10^3)[c]	Fold increase in sp act	Yield (%)[d]
I. Supernatant	2,000	44,400	82	1.85	1	100
II. Precipitation $(NH_4)_2SO_4$	200	1,590	167	105	57	204
III. Binding to cationic exchanger	40	21.6	20.5	950	514	25
IV. Binding to octyl-Sepharose	10	2.2	34.3	15,600	8,430	42
V. Reverse-phase chromatography						
First run	0.55	0.25	11.3	45,200	24,400	14
Second run	1.17	0.15	42.1	281,000	152,000	51

[a] Total optical density is the A_{280} multiplied by the volume in milliliters.
[b] BU, bacteriocin activity units (quantity of bacteriocin that inhibits 50% of the indicator culture).
[c] Specific activity represents the bacteriocin units divided by the A_{280}.
[d] 100% yield is defined as total bacteriocin units measured in the cell-free growth medium.

A

Thr Thr His Ser Gly Lys Tyr Tyr Gly Asn Gly Val Tyr Cys Thr Lys
Asn Lys Cys Thr Val Gln Trp Ala Lys Ala Thr Thr Cys Ile Ala Gly
Met Ser Ile Gly Gly Phe Lys Gly Gly Ala Ile Pro Gly Lys Cys

B

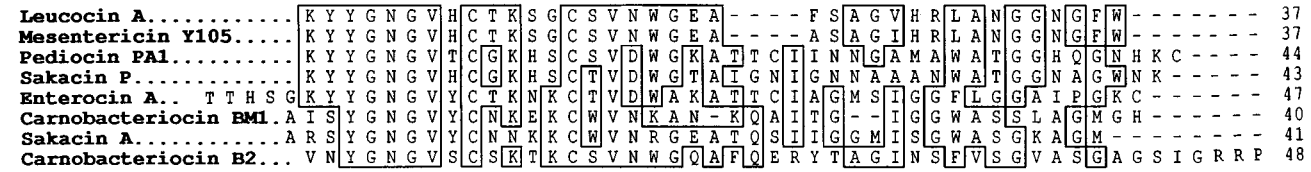

FIG. 4. (A) Amino acid sequence of enterocin A. The residues in boldface type were obtained by both amino acid and DNA sequencing. The other residues were deduced from the DNA sequence. (B) Alignment of the pediocin-like bacteriocins. The sequence was obtained from references 14, 16 to 18, 32, 38, 39, and 49 to 51.

between residues 26 and 43. This putative transmembrane region is not part of the conserved region of the pediocin-like bacteriocins; however, the membrane-permeabilizing mode of action found for pediocin PA-1 (53) may suggest that the most hydrophobic region in these bacteriocins is involved. One may also speculate that the most highly conserved region found in the N-terminal part of the mature pediocin-like bacteriocins is devoted to the recognition function required for dedicated receptors.

Bacteriocin producers are immune to their own bacteriocin. Several immunity genes to LAB nonlantibiotic bacteriocins have been cloned and shown to confer immunity to the respective bacteriocin (5, 19, 39, 52), and immunity proteins have been characterized (34, 39). In all these cases, the bacteriocin gene precedes its corresponding immunity gene in an operon. Such a gene organization has been found for all bacteriocins of the pediocin family, and it is likely that the second gene in each bacteriocin operon encodes an immunity protein in all these cases. Considering the similarity between the pediocin-like bacteriocins, it is surprising that there is much less similarity in the amino acid sequences in their putative immunity proteins. Homology searches for ORF2 and other putative immunity proteins of pediocin-like bacteriocins revealed, however, significant similarity to the putative immunity protein of leucocin AUL187 (Fig. 6). This is the first significant similarity (44.5% identity) reported between two putative immunity proteins of LAB bacteriocins. The similarity is most pronounced in the carboxyl end of the molecules. In view of the similarity between the two putative immunity proteins, it is surprising that their respective bacteriocins are not the most closely related bacteriocins. Enterocin has 46% identity with leucocin AUL183 compared with 56% to pediocin PA-1. This may indicate that the immunity proteins do not interact directly with their respective bacteriocins but interact indirectly through some kind of common target or receptor, whose existence has been suggested in work with lactococcin A and its immunity protein (23, 54). In addition to the size of the immunity factor, another apparently common property of these proteins is the presence of slightly hydrophobic regions, which also are found in the putative enterocin A immunity protein. It has been shown that the immunity protein of lactococcin A is loosely associated with the membrane, which may be the target for the immunity proteins (34, 54). The hydrophobic region of the immunity protein may serve as anchor to the membrane. Also, a smaller

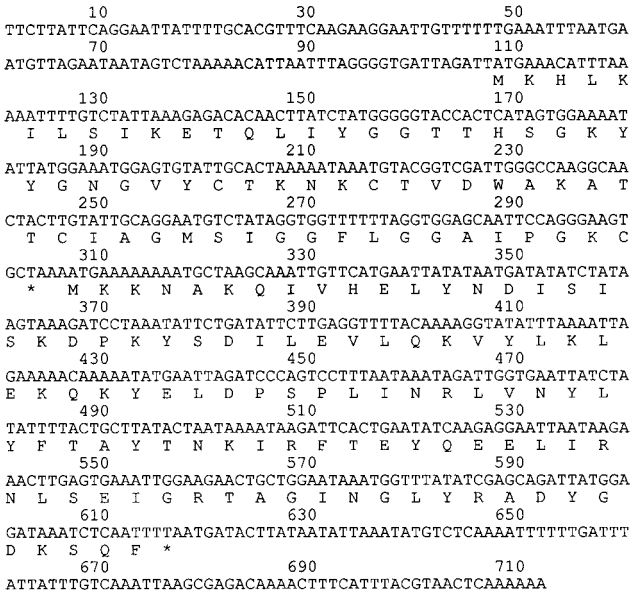

FIG. 5. DNA sequence of the enterocin A gene and ORF2 (encoding the putative immunity protein). The translation products of both genes are also shown.

TABLE 3. Comparison of antibacterial activity of four different pediocin-like bacteriocins

Indicator	Inhibitory zone (mm) produced by:			
	Pediocin PA-1	Sakacin A	Sakacin P	Enterocin A
Lactobacillus sake NCDO 2714	25	20	20	26
P. acidilactici NCDO 1859	19	0	0	16
P. pentosaceus FBB61.1	25	0	0	15
Lactobacillus plantarum 965	26	11	14	19
Listeria inocua 10	21	16	22	19
L. inocua 54	19	13	19	19
Listeria monocytogenes 50	21	13	22	16
L. monocytogenes 51	21	15	23	17
L. monocytogenes 52	19	14	23	19
L. monocytogenes 53	21	14	21	17
E. faecium TS	10	8	5(?)[a]	5(?)
E. faecalis TS (D)	18	16	20	14
E. faecalis BT2	9	14	16	5
E. faecalis	20	13	16	18

[a] (?) Diffuse inhibitory zones.

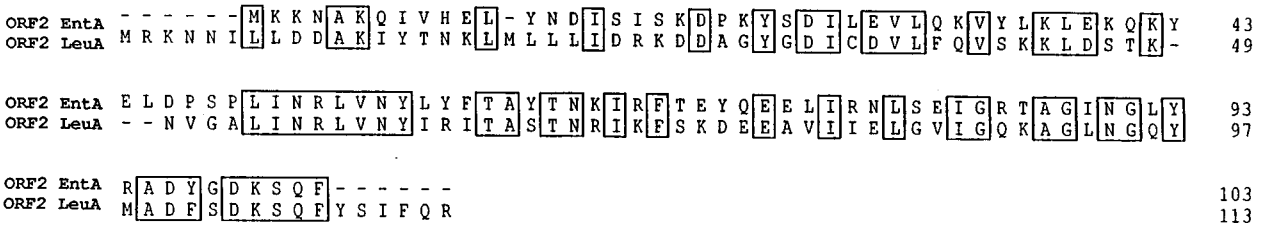

FIG. 6. Alignment of the putative immunity proteins of enterocin A (ORF2 EntA) and leucocin A (ORF2 LeuA). The sequences were aligned with the BESTFIT program (7). Conserved amino acids are boxed.

but significant proportion of the immunity protein of carnobacteriocin B2 is found in the membrane fraction (39).

The present work extends our knowledge about bacteriocins in general and the pediocin-like bacteriocins in particular. The variety of pediocin-like bacteriocins will make it easier to elucidate which structural features determine the specificity of a bacteriocin and which determine the biological activity. This information may facilitate future work on protein engineering of new and more efficient bacteriocins directed specifically against problem organisms such as *Listeria monocytogenes.*

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REFERENCES

1. **Abo-Elnaga, I. G., and O. Kandler.** 1965. Zur Taxonomie der Gattung *Lactobacillus beijerinck*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I orig. **119:**1–36 and 117–129.
2. **Allison, G. E., C. Fremaux, and T. R. Klaenhammer.** 1994. Expansion of bacteriocin activity and host range upon complementation of two peptides encoded within the lactacin F operon. J. Bacteriol. **176:**2235–2241.
3. **Berry, E. D., R. W. Hutkins, and R. W. Mandigo.** 1991. The use of bacteriocin-producing *Pediococcus acidilactici* to control postprocessing *Listeria monocytogenes* contamination of frankfurters. J. Food Prot. **53:**681–688.
4. **Berry, E. D., M. B. Liewen, R. W. Mandigo, and R. W. Hutkins.** 1990. Inhibition of *Listeria monocytogenes* by bacteriocin-producing *Pediococcus* during the manufacture of fermented semidry sausage. J. Food Prot. **52:** 194–197.
5. **Chikindas, M. J., M. J. Garcia Garcera, A. M. Driessen, A. M. Lederboer, J. Nissen-Meyer, I. F. Nes, T. Abee, W. N. Konings, and G. Venema.** 1993. Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0, forms hydrophillic pores in the cytoplasmatic membrane of target cells. Appl. Environ. Microbiol. **59:**3577–3584.
6. **Degnan, A. K., A. E. Yousef, and J. B. Luchansky.** 1992. Use of *Pediococcus acidilactici* to control *Listeria monocytogenes* in temperature abused, vacuum-packed wieners. J. Food Prot. **55:**98–103.
7. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
8. **Devriese, L. A., B. Pot, and M. D. Collins.** 1993. Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. J. Appl. Bacteriol. **75:**399–408.
9. **Diep, D. B., L. S. Håvarstein, J. Nissen-Meyer, and I. F. Nes.** 1994. The gene encoding the PlnA bacteriocin from *Lactobacillus plantarum* C11 is located on the same transcription unit as *agr*-like regulatory system. Appl. Environ. Microbiol. **60:**160–166.
10. **Faith, M. J., L. H. Zhang, J. Rush, and R. Kolter.** 1994. Purification and characterization of colicin V from *Escherichia coli* culture supernatants. Biochemistry **33:**6911–6917.
11. **Foegeding, P. M., A. B. Thomas, D. H. Pilkington, and T. R. Klaenhammer.** 1992. Enhanced control of *Listeria monocytogenes* by in situ-produced pediocin during dry fermented sausage production. Appl. Environ. Microbiol. **58:** 884–890.
12. **Fremaux, C., C. Ahn, and T. R. Klaenhammer.** 1993. Molecular analysis of the lacticin F operon. Appl. Environ. Microbiol. **59:**3906–3915.
13. **Harris, L. J., M. A. Daeschel, M. E. Stiles, and T. R. Klaenhammer.** 1989. Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*. J. Food Prot. **52:**384–387.
14. **Hastings, J. W., M. Sailer, K. Johnson, K. L. Roy, J. C. Vederas, and M. E. Stiles.** 1991. Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. J. Bacteriol. **173:**7491–7500.
15. **Håvarstein, L. S., H. Holo, and I. F. Nes.** 1994. The leader peptide of colicin V shares consensus sequences with leader peptides that are common amongst peptide bacteriocins produced by Gram-positive bacteria. Microbiology **140:**2383–2389.
16. **Hechard, Y., D. B. Derijard, F. Letellier, and Y. Cenatiempo.** 1992. Characterization and purification of mesentericin Y105, an anti-*Listeria* bacteriocin from *Leuconostoc mesenteroides*. J. Gen. Microbiol. **138:**2725–2731.
17. **Henderson, J. T., A. L. Chopko, and P. D. van Wasserman.** 1992. Purification and primary structure of pediocin PA-1 produced by *Pediococcus acidilactici* PAC1.0. Arch. Biochem. Biophys. **295:**5–12.
18. **Holck, A., L. Axelsson, S. E. Birkeland, T. Aukrust, and H. Blom.** 1992. Purification and amino acid sequence of sakacin A, a bacteriocin from *Lactobacillus sake* Lb 706. J. Gen. Microbiol. **138:**2715–2720.
19. **Holo, H., Ø. Nilssen, and I. F. Nes.** 1991. Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. J. Bacteriol. **173:**3879–3887.
20. **Jayne-Williams, D. J.** 1975. Miniaturized methods for the characterization of bacterial isolates. J. Appl. Bacteriol. **38:**305–309.
21. **Jayne-Williams, D. J.** 1976. The application of miniaturized method for characterization of various organisms isolated from animal gut. J. Appl. Bacteriol. **40:**189–200.
22. **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12:**39–86.
23. **Kok, J., H. Holo, M. van Belkum, and A. Haandrikman, and I. F. Nes.** 1993. Non-nisin bacteriocins in lactococci: biochemistry, genetics and mode of action, p. 121–151. *In* D. Hoover and L. Steensen (ed.), Bacteriocins in lactic acid bacteria. Academic Press, Inc., New York.
24. **Larsen, A. G., and B. Nørrung.** 1993. Inhibition of *Listeria monocytogenes* by bavaricin A, a bacteriocin produced by *Lactobacillus bavaricus* MI401. Lett. Appl. Microbiol. **17:**132–134.
25. **Larsen, A. G., F. K. Vogensen, and J. Josephsen.** 1993. Antimicrobial activity of lactic acid bacteria isolated from sour doughs: purification and characterization of bavaricin A, a bacteriocin produced by *Lactobacillus bavaricus* MI201. J. Appl. Bacteriol. **75:**113–122.
26. **López-Lara, I., A. Gálvez, M. Martínez-Bueno, M. Maqueda, and E. Valdivia.** 1991. Purification, characterization and biological effects of a second bacteriocin from *Enterococcus faecalis* ssp. *liquefaciens* S-48 and its mutant strain B-48-28. Can. J. Microbiol. **37:**768–774.
27. **Marugg, J. D., C. F. Gonzales, B. S. Kunka, A. M. Ledeboer, M. J. Pucci, M. Y. Toonen, S. A. Walker, L. C. M. Zoetmulder, and P. A. Vandenbergh.** 1992. Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0. Appl. Environ. Microbiol. **58:**2360–2367.
28. **Motlagh, A. M., A. K. Buhunia, F. Szostek, T. R. Hansen, M. C. Johnson, and B. Ray.** 1992. Nucleotide and amino acid sequence of *pap* gene (pediocin AcH) produced in *Pediococcus acidilactici* H. Lett. Appl. Microbiol. **15:**45–48.
29. **Mundt, J. O.** 1986. Streptococci, p. 1063–1065. *In* P. H. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
30. **Muriana, P. M., and T. R. Klaenhammer.** 1991. Cloning, phenotypic expression, and DNA sequence of the gene for lactacin F, an antimicrobial peptide produced by *Lactobacillus* spp. J. Bacteriol. **173:**1779–1788.
31. **Nielsen, J. W., J. S. Dickson, and J. D. Crouse.** 1990. Use of bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. Appl. Environ. Microbiol. **56:**2142–2145.
32. **Nieto Lozano, J. C., J. Nissen-Meyer, K. Sletten, C. Peláz, and I. F. Nes.** 1992. Purification and amino acid sequence of a bacteriocin produced by *Pediococcus acidilactici*. J. Gen. Microbiol. **138:**1985–1990.
33. **Nissen-Meyer, J., H. Holo, L. S. Håvarstein, K. Sletten, and I. F. Nes.** 1992. A novel lactococcal bacteriocin whose activity depends on the complemen-

tary action of two peptides. J. Bacteriol. **174:**5686–5692.
34. **Nissen-Meyer, J., H. Holo, K. Sletten, and I. F. Nes.** 1993. Association of lactococcin A immunity factor with the cell membrane: purification and characterization of the immunity factor. J. Gen. Microbiol. **139:**1503–1509.
35. **Nissen-Meyer, J., A. G. Larsen, K. Sletten, M. Daeschel, and I. F. Nes.** 1993. Purification and characterization of plantaricin A, a *Lactobacillus plantarum* bacteriocin whose activity depends on the action of two peptides. J. Gen. Microbiol. **139:**1973–1978.
36. **Parente, E., and C. Hill.** 1992. Characterization of enterocin 1146, a bacteriocin from *Enterococcus faecium* inhibitory to *Listeria monocytogenes*. J. Food Prot. **55:**497–502.
37. **Pucci, M. J., E. R. Vedamuthu, B. S. Kunka, and P. A. Vandenberg.** 1988. Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici* Pac 1.0. Appl. Environ. Microbiol. **54:**2349–2353.
38. **Quadri, L. E. N., M. Sailer, K. L. Roy, J. C. Vederas, and M. E. Stiles.** 1994. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. J. Biol. Chem. **269:**12204–12211.
39. **Quadri, L. E. N., M. Sailer, M. R. Terebiznik, K. L. Roy, J. C. Vederas, and M. E. Stiles.** 1995. Characterization of the protein conferring immunity to the antimicrobial peptide carnobacteriocin B2 and expression of carnobacteriocins B2 and BM1. J. Bacteriol. **177:**1144–1151.
40. **Ray, B.** 1992. Pediocin(s) of *Pediococcus acidilactici* as a food biopreservative, p. 265–322. *In* B. Ray and M. Daeschel (ed.), Food biopreservatives of microbial origin. CRC Press, Inc., Boca Raton, Fla.
41. **Reuter, G.** 1970. Laktobazillen und engverwandte Mikroorganismen in Fleisch und Fleischerzeugnissen. 2. Mitteilung: die Charakterisierung der isolierten Lactobazillen-Stämme. Fleischwirtschaft **47:**397–402.
42. **Salzano, G., F. Villani, O. Pepe, E. Sorrentino, and S. Coppola.** 1992. Conjugal transfer of plasmid-borne bacteriocin production in *Enterococcus faecalis* 226 NWC. FEMS Microbiol. Lett. **99:**1–6.
43. **Schillinger, U., M. Kaya, and F.-K. Lücke.** 1991. Behaviour of *Listeria monocytogenes* in meat and its control by bacteriocin-producing strains of *Lactobacillus sake*. J. Appl. Bacteriol. **70:**473–478.
44. **Schillinger, U., and F. K. Lücke.** 1987. Identification of lactobacilli from meat and meat products. Food Microbiol. **4:**199–208.
45. **Schnell, N., K.-D. Entian, U. Schneider, F. Götz, H. Zähner, R. Kellner, and G. Jung.** 1988. Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulphide-rings. Nature (London) **333:**276–278.
46. **Sharpe, M. E.** 1962. Taxonomy of the lactobacilli. Dairy Sci. **24:**109–118.
47. **Siragusa, G. R.** 1992. Production of bacteriocin inhibitory to *Listeria* species by *Enterococcus hirae*. Appl. Environ. Microbiol. **58:**3508–3513.
48. **Skaugen, M., J. Nissen-Meyer, G. Jung, S. Stevanovic, K. Sletten, C. I. Mørtvedt Abildgaard, and I. F. Nes.** 1994. Introduction of D-Ala by modification of L-Ser. J. Biol. Chem. **269:**27183–27185.
49. **Tichaczek, P. S., J. Nissen-Meyer, I. F. Nes, R. F. Vogel, and W. P. Hammes.** 1992. Characterization of the bacteriocins curvacin A from *Lactobacillus curvatus* LTH1174 and sakacin P from *L. sake* LTH673. Syst. Appl. Microbiol. **15:**460–468.
50. **Tichaczek, P. S., R. F. Vogel, and W. P. Hammes.** 1993. Cloning and sequencing of *cur*A encoding curvacin A, the bacteriocin produced by *Lactobacillus curvatus* LTH1174. Arch. Microbiol. **160:**279–283.
51. **Tichaczek, P. S., R. F. Vogel, and W. P. Hammes.** 1994. Cloning and sequencing of *sakP* encoding sakacin P, the bacteriocin produced by *Lactobacillus sake* LTH 673. Microbiology **140:**361–367.
52. **van Belkum, M. J., B. J. Hayema, R. E. Jeeninga, J. Kok, and G. Venema.** 1991. Organization and nucleotide sequences of two lactococcal bacteriocin operons. Appl. Environ. Microbiol. **57:**492–498.
53. **van Belkum, M., J. Kok, G. Venema, H. Holo, I. F. Nes, W. N. Konings, and T. Abee.** 1991. The bacteriocin lactococcin A specifically increases the permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein-mediated manner. J. Bacteriol. **173:**7934–7941.
54. **Venema, K., R. E. Haverkort, T. Abee, A. J. Haandrikman, K. J. Leenhouts, L. deLeij, G. Venema, and J. Kok.** 1994. Mode of action of LciA, the lactococcin A immunity protein. Mol. Microbiol. **14:**521–533.
55. **Villani, F., G. Salzano, E. Sorrentio, O. Pepe, P. Marino, and S. Coppola.** 1993. Enterocin 226NWC, a bacteriocin produced by *Enterococcus faecalis* 226, active against *Listeria monocytogenes*. J. Appl. Microbiol. **74:**380–387.
56. **Yousef, A. E., J. B. Luchansky, A. J. Degnan, and M. P. Doyle.** 1991. Behavior of *Listeria monocytogenes* in wiener exudates in the presence of *Pediococcus acidilactici* H and pediocin AcH during storage at 4 and 25°C. Appl. Environ. Microbiol. **57:**1461–1467.