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Characterization of the Chemical and Antimicrobial Properties of Piscicolin 126, a Bacteriocin Produced by *Carnobacterium piscicola* JG126

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A novel peptide bacteriocin produced by the lactic acid bacterium *Carnobacterium piscicola* JG126 isolated from spoiled ham was purified and characterized. This bacteriocin, designated piscicolin 126, inhibited the growth of several gram-positive bacteria, especially the food-borne pathogen *Listeria monocytogenes*, but had no effect on the growth of a number of yeasts and gram-negative bacteria. Bactericidal activity was not destroyed by exposure to elevated temperatures at low pH values; however, bactericidal activity was lost at high pH values, especially when high pH values were combined with an elevated temperature. Piscicolin 126 activity was not affected by catalase, lipase, or lysozyme but was destroyed by exposure to a range of proteolytic enzymes. Piscicolin 126 was purified to homogeneity and was found to be a peptide having a molecular weight of 4,416.6 ± 1.9. A sequence analysis revealed that this compound is a cystibiotic (class IIa) bacteriocin containing 44 amino acid residues and one intrapeptide disulfide ring. Piscicolin 126 has regions of homology with some other bacteriocins obtained from lactic acid bacteria and is most closely related to sakacin P and pediocin PA-1 (levels of identity, 75 and 55%, respectively). Addition of piscicolin 126 to a devilled ham paste test food system inhibited the growth of *L. monocytogenes* for at least 14 days. Piscicolin 126 was more effective than two commercially available bacteriocin preparations tested in the same system.

Lactic acid bacteria often produce low-molecular-weight, antibacterial peptides that are collectively referred to as bacteriocins (for a recent review, see reference 15). Since many of these compounds exhibit bactericidal activity against potentially pathogenic food-borne bacteria, such as *Listeria monocytogenes*, they have become the focus of considerable research, which is principally aimed at the development of bacteriocins as biopreservatives. Several bacteriocins have been shown to have in vitro efficacy for reducing the numbers of viable cells of *Listeria monocytogenes* and other potential pathogens in various food products (26), while nisin, from *Lactococcus lactis*, has been used extensively over the past 30 years to prevent spoilage of a variety of different foods (3, 4, 13, 15).

Bacteriocins are synthesized on ribosomes, and many of them undergo posttranslational modifications, including cleavage of a leader peptide, to form the mature, biologically active species (18, 31). The bacteriocins produced by lactic acid bacteria can be broadly divided into two subgroups on the basis of the specific posttranslational modifications that they undergo (18). Bacteriocins belonging to the first group (class I bacteriocins) are also referred to as lantibiotics because they contain the modified thioether-linked amino acids lanthionine and 3-methyl-lanthionine (33). Nisin, lacticin 481, streptococcin SA-FF22, and lactocin S are typical lantibiotics (6, 14, 23, 28). The members of the second group of bacteriocins (class II bacteriocins) do not contain posttranslationally modified amino acids apart from cystine. This group includes pediocins PA-1 and AcH (9, 21, 24), lactococcins A, B, and M (12, 34, 39, 40), lacticin F (25), curvacin A (37), sakacins A, P, and 674 (10, 11, 38), and acidocin A (17).

In the course of a survey of lactic acid bacteria for novel antimicrobial compounds, we discovered a bacteriocin produced by *Carnobacterium piscicola* JG126 which exhibits bactericidal activity against the food-borne human pathogen *Listeria monocytogenes*. Purification and structural analysis of this bacteriocin revealed that it is a class II bacteriocin that has a novel amino acid sequence. This compound, which we designated piscicolin 126, exhibited strong activity against *Listeria monocytogenes* in a test food system.

MATERIALS AND METHODS

Materials. Chemicals and other products were obtained from the following suppliers. Growth media and agar were obtained from Oxoid, Basingstoke, United Kingdom. Catalase was obtained from Boehringer, Mannheim, Germany. α-Chymotrypsin, β-chymotrypsin, lipase, lysozyme, pepsin, trypsin, protease type I, protease type XIV, protease type XXIII, and α-cyano-4-hydroxycinnamic acid were obtained from Sigma Chemical Co., St. Louis, Mo. Nisin (as Nisaplin) was obtained from Aplin and Barrett, Trowbridge, United Kingdom. ALTA 2431 was obtained from Quest International, Sydney, New South Wales, Australia. Devilled ham paste was obtained from Master Foods, Sydney, New South Wales, Australia. Plasmid pGEM-T was obtained from Promega Corp., Madison, Wis. CM-Sepharose Fast Flow was obtained from Pharmacia Biotech, Uppsala, Sweden. 2-Mercaptoethanol was obtained from BDH, Kilsyth, Victoria, Australia. 4-Vinyl pyridine was obtained from Aldrich Chemicals, Castle Hill, New South Wales, Australia. The chemicals used for amino acid and DNA sequencing were obtained from Applied Biosystems, Foster City, Calif.

Bacterial strains and culture conditions. All cultures used in this study were obtained from the culture collection of the Australian Food Industry Science Centre, Werribee, Victoria, Australia. *Carnobacterium piscicola* JG126 was propagated by overnight incubation at 22°C either in acetate-free, reduced-glucose MRS broth (1% peptone, 0.8% Lab-lemco, 0.4% yeast extract, 0.5% glucose, 0.11% Tween 80, 0.2% K_2HPO_4, 0.2% triammonium citrate, 0.02%

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$MgSO_4 \cdot 7H_2O$, 0.005% $MnSO_4 \cdot 4H_2O$; pH 6.5) or on the equivalent solid medium prepared by adding 1.2% agar. Other organisms were propagated for 16 h as described by Coventry et al. (2). The soft agar media used for overlays were prepared by adding 0.6% agar to the broth before autoclaving. Stock cultures of all strains were stored at −80°C in nutrient broth containing 10% glycerol.

Determination of bacteriocin activity. Bacteriocin activity (piscicolin 126 or ALTA 2341 activity) was determined by a critical dilution method, as described previously (2), using 5 μl of a bacteriocin solution and *Listeria monocytogenes* 4A as the indicator culture. This activity, in arbitrary units (AU) per milliliter, was defined as the reciprocal of the highest dilution of the sample being assayed (corrected to 1 ml) that caused a zone of growth inhibition on the indicator culture lawn.

Preparation of crude and concentrated piscicolin 126. The crude preparations of piscicolin 126 used were the supernatant fractions obtained after centrifugation (10,000 × *g*, 10 min, 4°C) of overnight cultures of *Carnobacterium piscicola* 126. Concentrated preparations of piscicolin 126 were prepared from crude preparations by precipitation with solid $(NH_4)_2SO_4$ to a final concentration of 516 g/liter at 4°C with stirring. The precipitate was collected by centrifugation at 20,000 × *g* for 30 min at 4°C, redissolved in 50 mM potassium phosphate buffer (pH 5.8) or distilled water, and filter sterilized (pore size, 0.45 μm; type Minisart NML; Sartorius GmbH, Göttingen, Germany) before its spectrum of activity was determined.

Preparation of homogeneous piscicolin 126. Concentrated piscicolin 126 was prepared as an $(NH_4)_2SO_4$ pellet (as described above) from 2 liters of culture. The pellet was dissolved in 200 ml of 50 mM potassium phosphate buffer (pH 5.8) and sonicated with a bath type of sonicator (Sonorex) to aid dissolution. After residual insoluble material was removed by centrifugation at 20,000 × *g* for 10 min at 4°C, the preparation was applied to a CM-Sepharose Fast Flow column (1.5 by 28 cm; Pharmacia) that previously had been equilibrated with 50 mM potassium phosphate buffer (pH 5.8) at a flow rate of 5 ml min^{-1}. The column was washed with the same buffer and at the same flow rate until the A_{280} stabilized and was then developed at a flow rate of 2 ml min^{-1} with a 300-ml linear gradient of 50 to 600 mM potassium phosphate buffer (pH 5.8). Fractions (5 ml) were collected and assayed for biological activity as described above. The active fractions were pooled and further fractionated by C_{18} reversed-phase high-performance liquid chromatography (HPLC) by using a Spherisorb ODS II column (4.6 by 250 mm; particle size, 5 μm) and a linear gradient of 20 to 40% aqueous acetonitrile containing 0.1% trifluoroacetic acid for 80 min at a constant flow rate of 1 ml min^{-1}. Peak fractions were assayed for antibacterial activity, and the active fractions were stored at −20°C in the eluting buffer.

The homogeneity of the piscicolin 126 preparations recovered was confirmed by both rechromatography and electrophoretic analysis. For the chromatographic analysis, purified piscicolin 126 was separated by C_{18} reversed-phase HPLC by using a Spherisorb ODS II column (2.1 by 250 mm; particle size, 5 μm) and a linear gradient of 0 to 60% aqueous acetonitrile containing 50 mM triethylamine acetate (pH 6.8) for 60 min at a flow rate of 200 μl min^{-1}. Alternatively, samples of piscicolin 126 were analyzed by capillary electrophoresis by using a model 270A CE apparatus (Applied Biosystems) fitted with an uncoated silica capillary (50 μm by 50 cm). Electrophoresis was carried out at 20 kV in 20 mM sodium citrate buffer (pH 2.5) at 30°C, and the A_{200} of the effluent was monitored continuously.

Spectrum of activity of piscicolin 126. The activities of a concentrated preparation of piscicolin 126 (5 μl of a 204,800-AU/ml aqueous solution) against a range of bacteria and yeasts were determined by the agar diffusion method described previously (2). For each target organism the spectrum was determined in at least two separate tests.

Sensitivity of piscicolin 126 to hydrolytic enzymes, temperature, and pH. To determine the effects of enzymatic treatments on piscicolin 126 activity, samples (180 μl of a 14,200-AU/ml solution) of crude piscicolin 126 were incubated with 20-μl portions of enzyme solutions (200 μg/ml in 0.2 M sodium phosphate buffer [pH 7.0]) at 37°C for 2 h, and then the remaining piscicolin activity was measured. For each of the enzymes tested, controls were incubated without piscicolin 126. None of the controls exhibited antilisterial activity. To determine the thermal and pH stability of piscicolin 126, the pHs of samples of a concentrated preparation of piscicolin 126 (819,200 AU/ml) were adjusted to different values by adding either HCl or NaOH, and then the samples were placed in a water bath at the appropriate temperature. Aliquots (1 ml) were removed after 10, 30, 60, and 120 min, and their antilisterial activities were determined by the critical dilution method.

Effects of piscicolin 126, ALTA 2341, and nisin on the viable counts of *Listeria monocytogenes* in ham paste. Portions (260 g) of a shelf-stable devilled ham paste product (pH 6.0) that had been heated to sterility during manufacture were inoculated with 10^3 CFU of *Listeria monocytogenes* 4A per g in sterile stomacher bags. The contents of each bag were mixed by externally massaging the bag, and then bacteriocin solutions (1 ml) were added to give the following final concentrations in the ham paste: nisin, 400 IU/g; ALTA 2341, 2,048 AU/g; and piscicolin 126, 2,048 AU/g. After the bag contents were mixed a second time, triplicate samples (10 g) of the mixtures were incubated at 10°C for the times indicated below and then homogenized for 1 min with a Colworth Stomacher apparatus with 90 ml of a peptone solution. The viable count of *Listeria monocytogenes* 4A in each homogenate was determined on plate count agar.

Alkylation of piscicolin 126. Purified bacteriocin was lyophilized and redissolved in 150 μl of H_2O-acetonitrile (2:1) containing 20 mM Tris-HCl (pH 8.0) and 1 μl of 2-mercaptoethanol. The resulting mixture was incubated at 25°C for 1 h to ensure that the cystine residues were reduced, 1 μl of 4-vinylpyridine was added, and the reaction mixture was incubated for 18 h at 4°C. The resulting peptides were separated from other components of the reaction mixture by C_8 reversed-phase HPLC with a Spherisorb RP5 column (2.1 by 50 mm), and the peak fraction corresponding to alkylated piscicolin 126 was identified by mass spectrometry and was further characterized by N-terminal amino acid sequencing.

To establish whether piscicolin 126 contained cysteine or cystine, duplicate samples of the purified peptide (400 pmol) were treated as described above except that the reducing agent 2-mercaptoethanol was added to one of the two samples. Following alkylation, the reaction products were immediately separated by C_8 reversed-phase HPLC with an Aquapore RP-300 column (2.1 by 100 mm), and peak fractions were identified and characterized by mass spectrometry.

Determination of peptide and DNA sequences. Peptides were applied to a Biobrene-pretreated glass fiber disc, and the N-terminal amino acid sequences of these peptides were determined by using the manufacturer's protocols recommended for an Applied Biosystems model 477A (pulsed-liquid gas phase) protein microsequencer equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer. Cysteine was detected as its PTH-pyridethyl cysteine derivative following alkylation with 4-vinylpyridine (as described above) prior to sequencing. The DNA sequences of PCR products were determined by using a PRISM Ready Reaction Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems) and the protocol recommended by the supplier. Sequencing reaction products were analyzed with a model 373A automated DNA sequencer (Applied Biosystems).

Mass spectrometry. An analysis of purified piscicolin 126 by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry was performed with a LaserMAT instrument (FiniganMAT). Peptides (1 to 10 pmol/μl) were mixed with an equal volume of 10-mg/ml α-cyano-4-hydroxycinnamic acid solution in 70% aqueous acetonitrile (containing 0.1% trifluoroacetic acid). A 1-μl sample was applied to an instrument sample slide and dried at 25°C, and spectra were recorded and analyzed by using the positive collection mode, as recommended by the manufacturer. Peptides having known molecular masses were included in the sample-matrix mixture as internal standards. The average masses ± standard deviations were calculated by determining the mean values from 35 independent 50-shot-averaged spectra.

Amino acid sequence accession number. The amino acid sequence of piscicolin 126 determined in this study has been deposited in the SWISS-PROT database under accession number P80569.

RESULTS

Isolation and characterization of the bacteriocin-producing organism. A bacteriocin-producing organism was isolated during the course of a program in which lactic acid bacteria were screened for the ability to produce antibacterial compounds (1). This organism was a gram-positive, catalase-negative bacterium which was isolated from the surface of ham. To identify this bacterium, chromosomal DNA was prepared (36) and used as the template for PCR performed with primers 5′GAGTTTGATCCTGGCTCAG and 5′TACAAGGCCCGGGAACG; these primers correspond to nucleotides 9 to 27 and 1394 to 1378, respectively, of 16S ribosomal DNA (*Escherichia coli* numbering system [41]). The nucleotide sequence of the PCR product was determined directly by using primer 9-27 (data not shown). When a FastA comparison of 403 bp of this nucleotide sequence with all of the 16S rRNA sequences in the ribosomal database (27) was performed, the highest levels of similarity were with the sequences of *Carnobacterium piscicola* (accession numbers M58812 [100% identity] and X54268 [1 mismatch]), *Carnobacterium maltoromicus* (accession number M58825; 3 mismatches), *Carnobacterium gallinarum* (accession number X52269; 8 mismatches), and *Carnobacterium divergens* (accession number X54270; 28 mismatches). We concluded from these data that the bacteriocin-producing organism was a strain of *Carnobacterium piscicola*; which we designated *Carnobacterium piscicola* JG126 (= AFISC 4008).

Spectrum of activity of piscicolin 126. Agar diffusion assays indicated that piscicolin 126 (5 μl of a 204,800-AU/ml solution) prevented the growth of the following gram-positive bacteria: *Brocothrix thermosphacta* AFISC 602, *Carnobacterium* sp.

strain AFISC 4001, *Enterococcus faecalis* AFISC 3901, *Enterococcus faecium* AFISC 3902, *Lactobacillus curvatus* AFISC 2103, *Lactobacillus sake* AFISC 2101, *Leuconostoc dextranicus* AFISC 2208, *Leuconostoc mesenteroides* subsp. *cremoris* AFISC 2201, *Listeria grayi* AFISC 2303, *Listeria innocua* AFISC 2305, *Listeria ivanovii* AFISC 2306, *Listeria monocytogenes* 4A (= AFISC 2310) and 4B (= AFISC 2311), *Listeria seeligeri* AFISC 2320, *Pediococcus pentosaceus* AFISC 2704, and *Streptococcus thermophilus* TS1 and TS2. Growth of the following microorganisms was not inhibited by the presence of piscicolin 126: *Aeromonas hydrophila* AFISC 110, *Bacillus cereus* AFISC 303, *Bacillus polymyxa* AFISC 312, *Bacillus stearothermophilus* AFISC 315, *Bacillus stearothermophilus* subsp. *calidolactis* AFISC 316, *Bacillus subtilis* AFISC 320, *Clostridium botulinum* type B strain AFISC 901, *Clostridium sporogenes* AFISC 914, *Corynebacterium* sp. strain AFISC 1001, *Debaryomyces hansei* AFISC 1105, *Enterobacter aerogenes* NCTC 10006, *Escherichia coli* NCTC 8196, *Lactobacillus plantarum* AFISC 2102, *Lactococcus lactis* subsp. *cremoris* AFISC 2002, *Lactococcus lactis* subsp. *lactis* biovar diacetylactis AFISC 2001, *Lactococcus lactis* subsp. *lactis* AFISC 2011, *Leuconostoc cremoris* AFISC 2206, *Listeria denitrificans* ATCC 14870, *Micrococcus luteus* AFISC 2405, *Micrococcus varians* AFISC 2415, *Pediococcus acidilactici* AFISC 2702, *Proteus vulgaris* AFISC 3020, *Pseudomonas aeruginosa* AFISC 3101, *Pseudomonas fluorescens* AFISC 3105, *Saccharomyces cerevisiae* AFISC 3301, *Salmonella salford* AFISC 3410, *Salmonella typhimurium* AFISC 3412, *Serratia marcescens* AFISC 3501, *Staphylococcus aureus* NCTC 6571, *Staphylococcus carnosus* AFISC 3605, *Staphylococcus epidermidis* NCTC 6513, and *Yersinia enterocolitica* AFISC 3801. The bacteriocin-insensitive microorganisms included all of the gram-negative bacteria and yeasts examined and some of the gram-positive bacteria, including three strains of *Lactococcus lactis*, an organism commonly used as a cheese starter.

Sensitivity of piscicolin 126 to various enzymes, pH, and temperature. Treatment of crude preparations of piscicolin 126 with catalase, lipase, or lysozyme had no effect on the antibacterial activity of this compound against *Listeria monocytogenes*. In contrast, each of the proteolytic enzymes tested (α-chymotrypsin, β-chymotrypsin, protease type I, protease type XIV, protease type XXIII, and trypsin) inactivated the antibacterial activity by 94 to 100%. Piscicolin 126 solutions (pH 2.0) exhibited no loss in activity after 2 months of storage at 4°C. Measurements of antibacterial activity at 100°C over the pH range from 2 to 10 (Fig. 1) indicated that piscicolin 126 was more stable at low pH values at this elevated temperature.

Isolation and characterization of piscicolin 126. Piscicolin 126 was purified from supernatant fractions of *Carnobacterium piscicola* JG126 cultures (51,000 AU/ml) by $(NH_4)_2SO_4$ fractionation, cation ion-exchange chromatography, and reversed-phase chromatography. The piscicolin 126 activity eluted as a discrete peak at A_{210} from the reversed-phase HPLC column (Fig. 2A). Rechromatography of the isolated bacteriocin by reversed-phase HPLC with a different ion pairing agent (Fig. 2B), as well as electrophoretic separation by capillary electrophoresis (Fig. 2C), yielded data consistent with the conclusion that the antibacterial activity coeluted with a single peptide.

A typical positive-mode matrix-assisted laser desorption ionization–time-of-flight mass spectrum of purified piscicolin 126 and spectra for porcine insulin and lantibiotic SA-FF22 (14), which were included as internal standards, are shown in Fig. 3. The average mass of piscicolin 126 determined from the 35 separate spectra recorded was 4,416.6 ± 1.9 Da.

Purified piscicolin 126 was reduced and then alkylated with 4-vinyl pyridine and subjected to N-terminal amino acid sequencing. A single unambiguous sequence of 40 amino acid residues that included 2 pyridethylated cysteinyl residues at positions 9 and 14 was obtained (Fig. 4). No residue was detected at position 41, but additional residues were found at positions 42 (PTH-asparagine) and 43 (PTH-lysine) (trace only). The calculated mass of the sequence containing the 42 amino acid residues detected (4,174.9 Da) was 241.7 Da less than the experimentally determined mass of piscicolin 126 (4,416.6 ± 1.9 Da). The difference in the values was attributed to the unidentified residue at position 41 and an additional unsequenced amino acid at the C terminus of the peptide. These data are consistent with the hypothesis that residue 41 is tryptophan, which would not have been detected in the low-picomolar sequence analysis and is present in the closely related bacteriocin sakacin P (Fig. 4), and the hypothesis that the C-terminal residue is glycine (mass of tryptophan + mass of glycine = 243.3 Da). A search of the Brookhaven Protein Data Bank, EMBL, GenBank, GenPept, PIR, and SWISS-PROT databases indicated that the sequence determined in this study was unique, although there was appreciable similarity to the sequences of other bacteriocins, particularly pediocin PA-1 (21) and sakacin P (38) (Fig. 4).

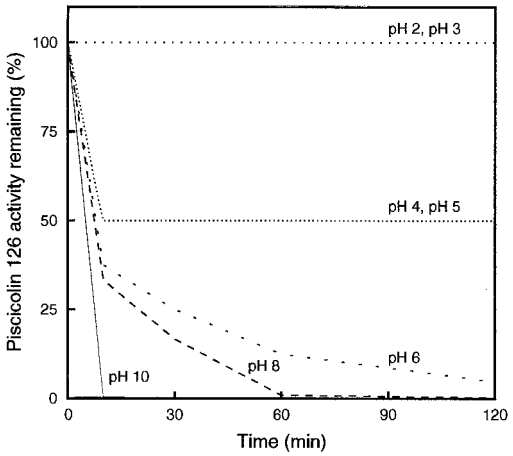

FIG. 1. Effect of pH on the bactericidal activity of piscicolin 126 at 100°C. See Materials and Methods for details.

In order to determine whether the two observed cysteinyl residues were present in a disulfide ring in native piscicolin 126, a sample of the purified bacteriocin was treated with the alkylating agent 4-vinylpyridine both with and without prior incubation with the reducing agent 2-mercaptoethanol. The products from each reaction were separated by reversed-phase HPLC (Fig. 5A and B) and characterized by mass spectrometry (Fig. 5C and D). For the nonreduced sample, the mass of the product was identical to the mass of unmodified piscicolin 126 (4,416.6 Da) (Fig. 5C), whereas the reduced sample yielded an adduct having a greater mass (4,627.2 Da) (Fig. 5D). The additional mass corresponded to the mass of the two pyridethyl groups detected as PTH-pyridethyl cysteine during the sequence analysis. These data indicated that the two cysteinyl residues in piscicolin 126 are present in a disulfide ring.

Effect of piscicolin 126, ALTA 2341, and nisin on the growth of *Listeria monocytogenes* 4A in ham paste. The viable counts of *Listeria monocytogenes* in challenged ham paste with and without added bacteriocins were determined after incubation at 10°C for up to 14 days (Table 1). Piscicolin 126 reduced the viable count of *Listeria monocytogenes* to below the detection limit (<100 CFU/g) immediately after it was added, and this

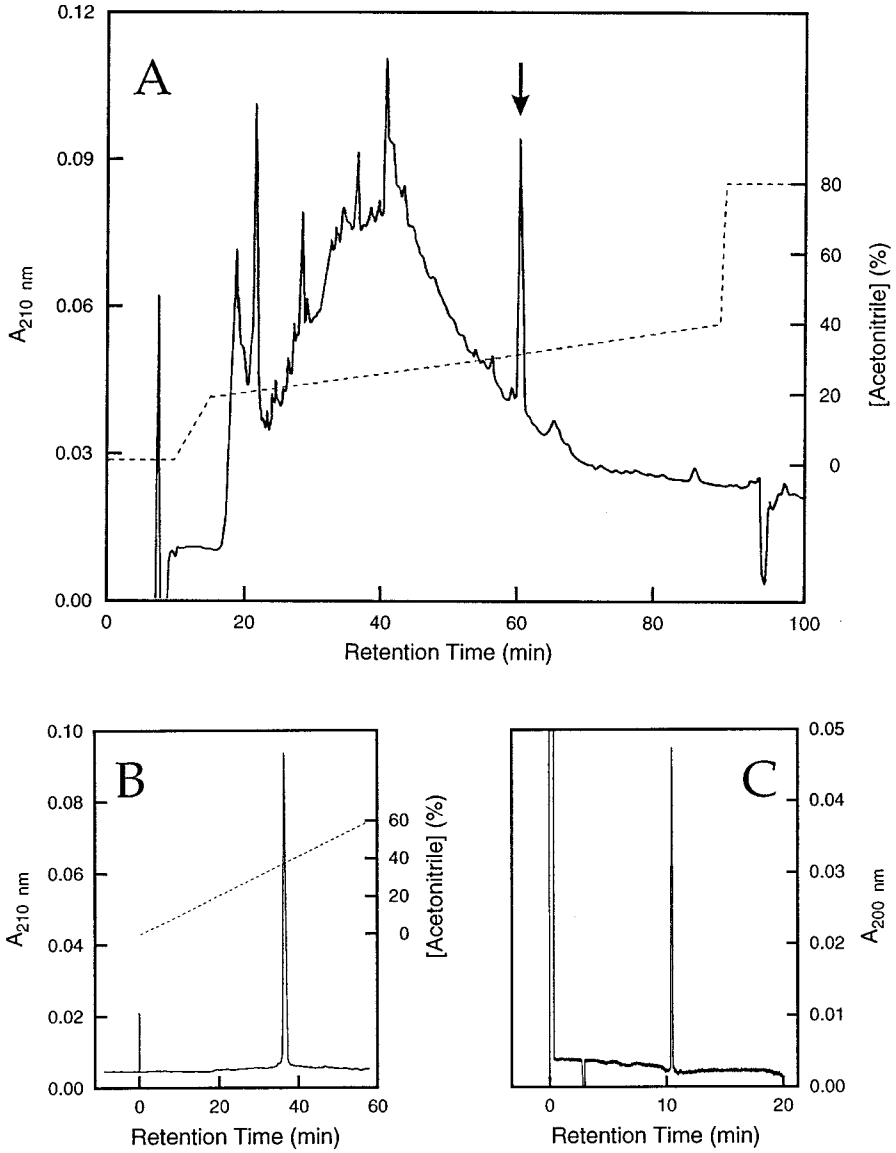

FIG. 2. Isolation of piscicolin 126. (A) Reversed-phase HPLC separation in aqueous trifluoroacetic acid of partially purified piscicolin 126 recovered from cation-exchange chromatography. Individual column fractions were assayed for piscicolin 126 activity; activity was detected only in the fraction containing the peak indicated by the arrow. The acetonitrile gradient used to elute piscicolin 126 is indicated by the dashed line. (B) Rechromatography of the peak isolated in panel A by reversed-phase HPLC in aqueous triethylamine, using the acetonitrile gradient indicated by the dashed line. (C) Electropherogram of purified piscicolin 126 separated by capillary electrophoresis. See Materials and Methods for experimental details.

level of preservation was maintained throughout the 14 days of the trial. ALTA 2341, a food-grade commercially available shelf life extender with antilisterial activity (32), initially exhibited preservative activity similar to that of piscicolin 126 when it was used at the manufacturer's recommended dose of 1% (wt/wt), but limited growth of *Listeria monocytogenes* was observed by day 14. In contrast, nisin did not inhibit the growth of *Listeria monocytogenes* during the 14-day incubation period, even though it was added at a level that is moderately high for food application (3).

DISCUSSION

In the present study we isolated and characterized a peptide bacteriocin, designated piscicolin 126, from the lactic acid bacterium *Carnobacterium piscicola* JG126. Molecular characterization of this bacteriocin revealed that it has a novel amino acid sequence and mass (4,416.6 ± 1.9 Da). The spectrum of activity of piscicolin 126, the presence of the sequence YGNG VXC near the N terminus, and the absence of the modified amino acids lanthionine and 3-methyl-lanthionine established that piscicolin 126 is a member of *Listeria*-active class IIa of bacteriocins as defined by Klaenhammer (18). It has previously been noted that the amino acid sequences of the N-terminal regions of members of this family of bacteriocins exhibit appreciable sequence conservation, while the C-terminal regions vary significantly (20, 30). The amino acid sequence of piscicolin 126 is consistent with this general picture (Fig. 4). Interestingly, piscicolin 126 differs from all of the other bacteriocins except carnobacteriocin BM1 by not having a glycine at posi-

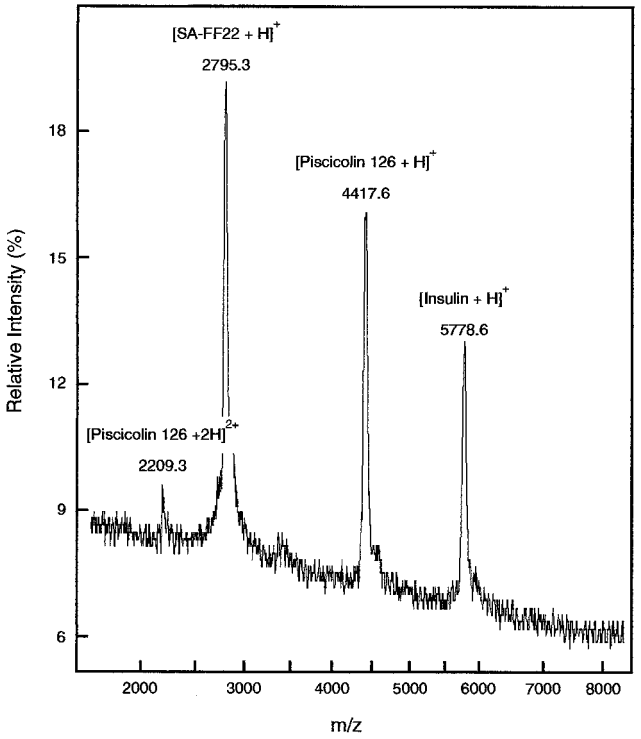

FIG. 3. Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry averaged mass spectrum of piscicolin 126. In order to externally calibrate the instrument, two additional peptides (the bacteriocin SA-FF22 and porcine insulin; MH^+ ion *m/z* values as indicated on the figure) were included with the piscicolin sample. Following the recording of 35 such averaged spectra, a mass of 4,416.6 ± 1.9 Da was calculated for piscicolin 126.

tion 19. Whether the conserved regions are essential for bactericidal activity and/or specificity of action remains to be determined.

The isolation of one lantibiotic (carnocin) and three class II bacteriocins (carnobacteriocins A, BM1, and B2) from *Carnobacterium piscicola* has been reported previously (30, 35, 42).

Piscicolin 126:	KYYGNGVSCNKNGCTVDWSKAIGIIGNNAAANLTTGGAAGWNKG	100%
Sakacin P:	KYYGNGVHCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK	75%
Pediocin PA-1:	KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC	55%
Leucocin A:	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW	34%
Mesentericin Y105	KYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGF	34%
Carnobacteriocin B2:	VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP	34%
Sakacin A, Curvacin A:	ARSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	30%
Carnobacteriocin BM1:	AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH	27%
Carnobacteriocin A:	DQMSDGVNYGKGSSLSKGGAKCGLGIVGGLATIPSGPLGWLAGAAGVINSCMK	25%

FIG. 4. Primary sequence of piscicolin 126 aligned with the sequences of other class II bacteriocins, including those previously isolated from *Carnobacterium piscicola*. Disulfide rings are indicated by solid lines if they were experimentally determined or by dashed lines; shading indicates amino acids common to piscicolin 126 and other bacteriocins. The percentages of identity with piscicolin 126 are shown on the right. The piscicolin 126 sequence was determined by a combination of direct N-terminal sequence and mass spectrophotometric analyses (see the text). Other sequences were obtained from the following sources: sakacin P, reference 38; curvacin A, reference 37; pediocin PA-1, reference 21; leucocin A, reference 7; mesentericin Y105, reference 8; carnobacteriocins B2 and BM1, reference 30; sakacin A, reference 10; and carnobacteriocin A, reference 42.

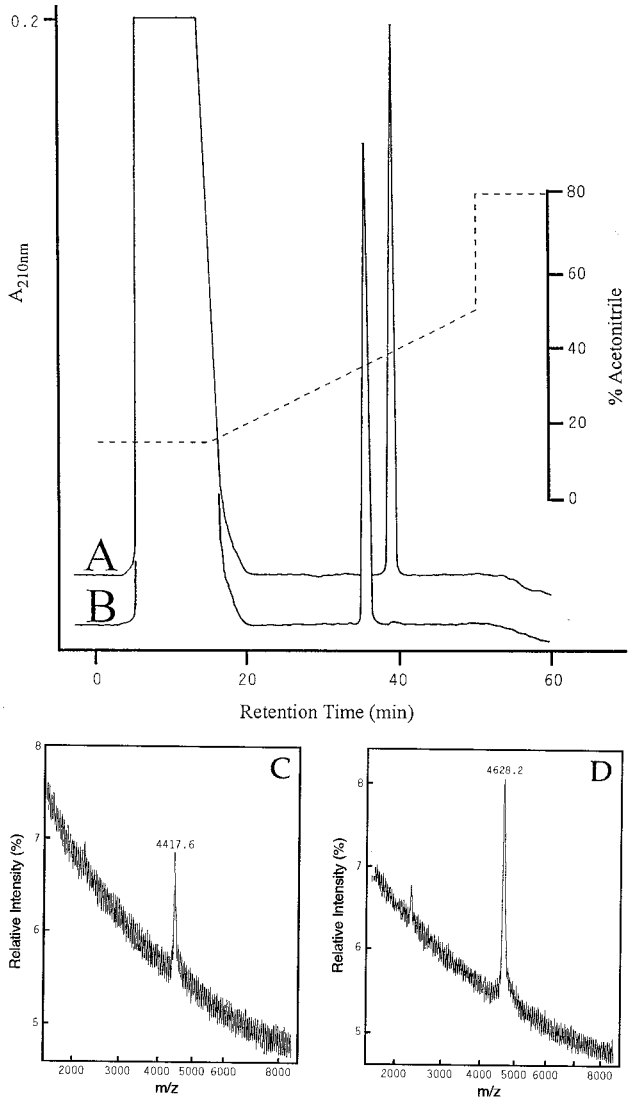

FIG. 5. Analysis of the alkylation products of piscicolin 126. Purified bacteriocin was alkylated with or without prior reduction, and the products were separated as described in Materials and Methods. The solid lines show the A_{210} for the products of alkylation without (profile A) and with (profile B) prior reduction; the dashed line shows the acetonitrile gradient. The peak from each separation was analyzed by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry to give the data shown in panels C and D.

The amino acid sequences of the three class II bacteriocins exhibit 25 to 34% identity with the sequence of piscicolin 126 (Fig. 4). Recently, Pilet et al. (29) reported the properties of another bacteriocin isolated from *Carnobacterium piscicola*, piscicocin VI. The spectra of activity of piscicocin VI and piscicolin 126 are similar, but not identical. Knowledge of the amino acid sequence of piscicocin VI should assist in determining the features of piscicolin 126 and other class II bacteriocins responsible for function and specificity.

Piscicolin 126 proved to be stable to high temperatures at acidic pH values; however, it was inactivated at neutral or alkaline pH values. The temperature and pH stability data suggest that this bacteriocin could prove to be useful in preservation of acidic foodstuffs, such as fermented products, and that it should survive heat treatments, such as pasteurization.

There have been many previous reports suggesting that bac-

TABLE 1. Effects of piscicolin 126, ALTA 2341, and nisin on the survival and growth of *Listeria monocytogenes* 4A in ham paste stored at 10°C

Treatment	Viable counts ($\log_{10}$ CFU/g) of *Listeria monocytogenes*[a]					
	At zero time	After storage for:				
		1 day	4 days	6 days	7 days	14 days
Control	3.2	3.3	5.4	6.0	6.9	9.5
Piscicolin 126[b]	<2	<2	<2	<2	<2	<2
ALTA 2341[c]	<2	<2	<2	<2	<2	4.5
Nisin[d]	3.1	3.3	5.3	6.0	6.7	9.6

[a] The values are the means of at least three replicates; the minimum threshold for detection was estimated to be 100 CFU/g.
[b] Final concentration, 2,048 AU/g.
[c] Final concentration, 1.0% (2,048 AU/g).
[d] Final concentration, 400 IU/g.

teriocins from lactic acid bacteria might prove to be useful in biopreservation of foodstuffs, and nisin, a lantibiotic-type bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, has been used in this way for more than 30 years (4, 13, 15). The use of nisin as a meat product preservative has met with variable success, depending mainly on the target bacteria and the type of meat product (3–5, 19, 22). In the present study, inclusion of piscicolin 126 in a commercial ham paste preparation inhibited the growth of *Listeria monocytogenes* for up to 14 days of storage at the abuse temperature of 10°C, even with a microbial load which reflected the maximum level of *Listeria monocytogenes* contamination generally reported for commercial meat products (16). In contrast, nisin (added as Nisaplin) failed to inhibit *Listeria monocytogenes* proliferation, while ALTA 2341, a shelf life extender with antilisterial activity derived from *Pediococcus acidilactici*, exhibited less inhibitory action against *Listeria monocytogenes* than piscicolin 126. Thus, piscicolin 126 appears to offer some advantages over existing, commercially available, bacteriocin-based food preservatives for application to meat products when *Listeria monocytogenes* contamination is a concern. However, more detailed meat product application studies will be required to enable quantitative comparisons over more extensive ranges of storage conditions and levels of *Listeria monocytogenes* contamination to be made. The relative efficacies of the different bacteriocins against a number of different wild-type *Listeria monocytogenes* strains also must be determined.

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REFERENCES

1. **Coventry, M. J., J. B. Gordon, A. Wilcock, K. Harmark, B. E. Davidson, M. W. Hickey, A. J. Hillier, and J. Wan.** Detection of bacteriocins of lactic acid bacteria isolated from foods and comparison with pediocin and nisin. Int. J. Food Microbiol., in press.
2. **Coventry, M. J., J. Wan, J. B. Gordon, R. F. Mawson, and M. W. Hickey.** 1996. Production of brevicin 286 by *Lactobacillus brevis* VB286 and partial characterization. J. Appl. Bacteriol. **80:**91–98.
3. **Delves-Broughton, J.** 1990. Nisin and its use as a food preservative. Food Technol. **44:**100–117.
4. **De Vuyst, L., and E. J. Vandamme.** 1994. Nisin, a lantibiotic produced by *Lactococcus lactis* subsp. *lactis*: properties, biosynthesis, fermentation and applications, p. 151–221. *In* L. De Vuyst and E. J. Vandamme (ed.), Bacteriocins of lactic acid bacteria: microbiology, genetics and applications. Chapman & Hall, London.
5. **Fowler, G. G., and M. J. Gasson.** 1991. Antibiotics—nisin, p. 133–152. *In* N. J. Russell and G. W. Gould (ed.), Food preservatives. Blackie and Sons, Ltd., Glasgow, United Kingdom.
6. **Gross, E., and J. L. Morrell.** 1971. The structure of nisin. J. Am. Chem. Soc. **93:**4634–4635.
7. **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.
8. **Héchard, Y., B. Dérijard, 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.
9. **Henderson, J. T., A. L. Chopko, and P. D. van Wassenaar.** 1992. Purification and primary structure of pediocin PA-1 produced by *Pediococcus acidilactici* PAC-1.0. Arch. Biochem. Biophys. **295:**5–12.
10. **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* Lb706. J. Gen. Microbiol. **138:**2715–2720.
11. **Holck, A., L. Axelsson, K. Hühne, and L. Kröckel.** 1994. Purification and cloning of sakacin 674, a bacteriocin from *Lactobacillus sake* Lb674. FEMS Microbiol. Lett. **115:**143–150.
12. **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.
13. **Hurst, A.** 1981. Nisin. Adv. Appl. Microbiol. **27:**85–123.
14. **Jack, R. W., A. Carne, J. Metzger, S. Stevanović, H.-G. Sahl, G. Jung, and J. Tagg.** 1994. Elucidation of the structure of SA-FF22, a lanthionine-containing antibacterial peptide produced by *Streptococcus pyogenes* strain FF22. Eur. J. Biochem. **220:**455–462.
15. **Jack, R. W., J. R. Tagg, and B. Ray.** 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. **59:**171–200.
16. **Johnson, J. L., M. P. Doyle, and R. G. Cassens.** 1990. *Listeria monocytogenes* and other *Listeria* spp. in meat and meat products, a review. J. Food Prot. **53:**81–91.
17. **Kanatani, K., M. Oshimura, and K. Sano.** 1995. Isolation and characterization of acidocin A and cloning of the bacteriocin gene from *Lactobacillus acidophilus*. Appl. Environ. Microbiol. **61:**1061–1067.
18. **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12:**39–86.
19. **Lipinska, E.** 1977. Nisin and its applications, p. 103–130. *In* M. Woodbine (ed.), Antibiotics and antibiosis in agriculture. Butterworths, London.
20. **Lozano, J. C. N., J. N. 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.
21. **Marugg, J. D., C. F. Gonzalez, 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.
22. **Molitor, E., and H.-G. Sahl.** 1991. Applications of nisin: a literature survey, p. 434–439. *In* G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. ESCOM, Leiden, The Netherlands.
23. **Mørtvedt, C. I., J. Nissen-Meyer, K. Sletten, and I. F. Nes.** 1991. Purification and amino acid sequence of lactocin S, a bacteriocin produced by *Lactobacillus sake* L45. Appl. Environ. Microbiol. **57:**1829–1834.
24. **Motlagh, A. M., A. K. Bhunia, F. Szostek, T. R. Hansen, M. C. Johnson, and B. Ray.** 1992. Nucleotide and amino acid sequence of *pap*-gene (pediocin AcH production) in *Pediococcus acidilactici H*. Lett. Appl. Microbiol. **15:**45–48.
25. **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.
26. **Nilsen, J. W., J. S. Dickson, and J. D. Crouse.** 1990. Use of a bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. Appl. Environ. Microbiol. **56:**2142–2145.
27. **Olsen, G. J., R. Overbeek, N. Larsen, T. L. Marsh, J. McCaughey, M. A. Maciukenas, W.-M. Kuan, T. J. Macke, Y. Xing, and C. R. Woese.** 1992. The Ribosomal Database Project. Nucleic Acids Res. **20:**2199–2200.
28. **Piard, J.-C., P. M. Muriana, M. J. Desmazeaud, and T. R. Klaenhammer.** 1992. Purification and partial characterization of lacticin 481, a lanthionine-containing bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CNRZ 481. Appl. Environ. Microbiol. **58:**279–284.
29. **Pilet, M.-F., X. Dousset, R. Barré, G. Novel, M. Desmazeaud, and J.-C. Piard.** 1995. Evidence for two bacteriocins produced by *Carnobacterium piscicola* and *Carnobacterium divergens* isolated from fish and active against *Listeria monocytogenes*. J. Food Prot. **58:**256–262.
30. **Quadri, L. E., 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.
31. **Sahl, H.-G., R. W. Jack, and G. Bierbaum.** 1995. Biosynthesis and biological activities of antibiotics with unique post-translational modifications. Eur. J. Biochem. **230:**827–853.
32. **Schlyter, J. H., A. J. Degnan, J. Loeffelholz, K. A. Glass, and J. B. Luchansky.** 1993. Evaluation of sodium diacetate and ALTA 2341 on viability of *Listeria monocytogenes* in turkey slurries. J. Food Prot. **56:**808–810.
33. **Schnell, N., K.-D. Entain, 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.
34. **Stoddard, G. W., J. P. Petzel, M. J. van Belkum, J. Kok, and L. L. McKay.** 1992. Molecular analyses of the lactococcin A gene cluster from *Lactococcus lactis* subsp. *lactis* biovar diacetylactis WM4. Appl. Environ. Microbiol. **58:** 1952–1961.
35. **Stoffels, G., I. F. Nes, and Á. Guòmundsdóttir.** 1992. Isolation and properties of a bacteriocin-producing *Carnobacterium piscicola* isolated from fish. J. Appl. Bacteriol. **73:**309–316.
36. **Tanskanen, E. I., D. L. Tulloch, A. J. Hillier, and B. E. Davidson.** 1990. Pulsed-field gel electrophoresis of *Sma*I digests of lactococcal genomic DNA, a novel method of strain identification. Appl. Environ. Microbiol. **56:**3105–3111.
37. **Tichaczek, P. S., R. F. Vogel, and W. P. Hammes.** 1993. Cloning and sequencing of *curA* encoding curvacin A, the bacteriocin produced by *Lactobacillus curvatus* LTH1174. Arch. Microbiol. **160:**279–283.
38. **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.
39. **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.
40. **van Belkum, M. J., J. Kok, and G. Venema.** 1992. Cloning, sequencing and expression in *Escherichia coli* of *lcnB*, a third bacteriocin determinant from the lactococcal bacteriocin plasmid p9B4-6. Appl. Environ. Microbiol. **58:** 572–577.
41. **Woese, C. R., R. Gutell, R. Gupta, and H. F. Noller.** 1983. Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. Microbiol. Rev. **47:**621–669.
42. **Worobo, R. W., T. Henkel, M. Sailer, K. L. Roy, J. C. Vederas, and M. E. Stiles.** 1994. Characteristics and genetic determinant of a hydrophobic peptide bacteriocin, carnobacteriocin A, produced by *Carnobacterium piscicola* LV17A. Microbiology **140:**517–526.