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Characterization of Leucocin A-UAL 187 and Cloning of the Bacteriocin Gene from *Leuconostoc gelidum*

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Leucocin A-UAL 187 is a bacteriocin produced by *Leuconostoc gelidum* UAL 187, a lactic acid bacterium isolated from vacuum-packaged meat. The bacteriocin was purified by ammonium sulfate or acid (pH 2.5) precipitation, hydrophobic interaction chromatography, gel filtration, and reversed-phase high-performance liquid chromatography with a yield of 58% of the original activity. Leucocin A is stable at low pH and heat resistant, and the activity of the pure form is enhanced by the addition of bovine serum albumin. It is inactivated by a range of proteolytic enzymes. The molecular weight was determined by mass spectrometry to be 3,930.3 ± 0.4. Leucocin A-UAL 187 contains 37 amino acids with a calculated molecular weight of 3,932.3. A mixed oligonucleotide (24-mer) homologous to the sequence of the already known N terminus of the bacteriocin hybridized to a 2.9-kb *Hpa*II fragment of a 7.6-MDa plasmid from the producer strain. The fragment was cloned into pUC118 and then subcloned into a lactococcal shuttle vector, pNZ19. DNA sequencing revealed an operon consisting of a putative upstream promoter, a downstream terminator, and two open reading frames flanked by a putative upstream promoter and a downstream terminator. The first open reading frame downstream of the promoter contains 61 amino acids and is identified as the leucocin structural gene, consisting of a 37-amino-acid bacteriocin and a 24-residue N-terminal extension. No phenotypic expression of the bacteriocin was evident in several lactic acid bacteria that were electrotransformed with pNZ19 containing the 2.9-kb cloned fragment of the leucocin A plasmid.

Bacteriocins are antimicrobial peptides or proteins formed by bacteria. The potential applications for bacteriocin-producing lactic acid bacteria in food preservation has stimulated interest in the characterization of these substances. However, maintaining activity during isolation and purification has proved difficult, and the full or partial amino acid sequences of only five such bacteriocins have been reported. The most extensively studied is nisin A (2, 3, 7, 34), a posttranslationally modified bacteriocin from *Lactococcus lactis* subsp. *lactis*. Nisin has been approved for use as a preservative in foods in over 45 countries (9). It is ribosomally synthesized (6, 10) and is one of a group of lantibiotics that possess lanthionine- or methyllanthionine-containing rings resulting from the attack of cysteine sulfhydryl groups on dehydroalanine or dehydrobutyrine residues (derived from serine or threonine). Partial characterization of lactacin 481 from *L. lactis* shows that it also contains lanthionine rings, but the full sequence has not yet been published (31). In contrast, lactocin S from *Lactobacillus sake* (27) and lactacin F produced by *Lactobacillus acidophilus* (28) are peptides containing 54 and 57 amino acids, respectively, that do not contain modified amino acids. The bacteriocins in a third group are considerably larger proteins. Helveticin J, generated by *Lactobacillus helveticus*, has a molecular weight of 37,511 based on its gene sequence (19). Similarly, caseicin 80 from *Lactobacillus casei* is estimated to be a 40,000- to 42,000-Da protein based on gel filtration experiments, but the amino acid sequence is not known (33).

Interest in the antagonistic activity of lactic acid bacteria has resulted in the cloning of several bacteriocin genes. The structural gene for nisin has been cloned and sequenced (6, 10, 20), but production of active nisin from a cloned piece of DNA has not yet been demonstrated. Analysis of the gene sequence revealed a 57-residue precursor that consists of a 23-residue leader peptide in addition to the 34-residue bacteriocin that is posttranslationally modified (6). Two bacteriocin genes have been cloned from *Lactococcus lactis* subsp. *cremoris* (38); in these genes, three open reading frames (ORFs) are present and are organized in an operon. The first two ORFs encode bacteriocin activity and contain 69 and 77 codons, respectively. The third ORF containing 154 codons is essential for immunity. The structural gene encoding helveticin J produced by *Lactobacillus helveticus* 481 has been cloned, sequenced, and expressed in *Lactobacillus acidophilus* (19). Also, the structural gene encoding lactacin F produced by *Lactobacillus acidophilus* has been cloned; DNA sequence analysis elucidated a 75-amino-acid precursor bacteriocin consisting of a 57-residue bacteriocin and an 18-residue leader peptide (28, 29).

Leuconostoc species display antimicrobial activity against other lactic acid bacteria (16, 30), but little is known about the chemical nature of the active compounds. A strain of *Leuconostoc gelidum* has been reported that inhibits a wide spectrum of lactic acid bacteria, meat spoilage bacteria, and food-related human pathogens, including *Listeria monocytogenes* (16). We previously described a bacteriocinlike substance produced by a different strain of *L. gelidum* that was isolated from meat (17). We now report the isolation, puri-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Escherichia coli		
JM 103	*thr rpsL endA sbc-15 hsdR4* Δ (*lac pro AB*) F′ *traD36 pro* AB, *lacIZ*ΔM15	35
MV 1193	Δ (*lac-proAB*) *rpsL thr endA spcB15 hsdR4* Δ (*srl-recA*) *306*:: Tn10 (Tet^r) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ M15]	35
Leuconostoc gelidum		
UAL 187-22	Lcn$^+$ Imm$^+$ containing native plasmids pLG 9.2 and pLG 7.6	17
UAL 187-13	Lcn$^-$ Imm$^-$ containing native plasmid pLG 9.2	17
Carnobacterium piscicola UAL 26	Plasmidless	This laboratory
Leuconostoc sp. strain UAL 60	Plasmidless	This laboratory
Lactococcus lactis Na8	Plasmidless	North Carolina State University
Plasmids		
pUC118	lacZ′, Amp^r, 3.2 kb	40
pNZ19	Cm^r Km^r, 5.7 kb *E. coli-Lactococcus* sp. shuttle vector	W. de Vos
pJH6.1F	Amp^r, *lcnA*, 6.1 kb	This study
pJH8.6L	Cm^r Km^r, *lcnA*, 8.6 kb	This study
pLG9.2	Cryptic, 9.2 MDa	17
pLG7.6	*lcnA*, 7.6 MDa	17

fication, and properties of this bacteriocin and cloning of the gene for bacteriocin production by *L. gelidum* UAL 187.

MATERIALS AND METHODS

Cultures and media. Conditions for growth of the bacteriocin producer *L. gelidum* UAL 187 and the indicator strain *Carnobacterium divergens* LV13 were as described previously (17). The semidefined medium CAA used for production of leucocin A-UAL 187 includes the following (per liter of solution): Casamino Acids (Difco Laboratories, Detroit, Mich.), 15 g; yeast extract (BBL Microbiology Products, Cockeysville, Md.), 5 g; D-glucose (BDH Chemicals Ltd., Poole, England), 20 g; dipotassium phosphate (J. T. Baker Chemical Co., Phillipsburg, N.J.), 2 g; Tween 80 (Difco), 1 ml; diammonium citrate (BDH), 2 g; magnesium sulfate (Anachemia, Champlain, N.Y.), 0.1 g; and manganous sulfate (BDH), 0.05 g. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* was grown in Luria broth or plates (1.5% agar) with 20 mM glucose at 37°C. The selective concentrations of ampicillin and chloramphenicol for growing *E. coli* were 100 and 12.5 μg/ml, respectively. Carnobacteria, lactococci, and leuconostocs were grown in APT broth (Difco) or plates (1.5% agar) at 25°C. Selective concentrations of 5.0 and 2.5 μg of chloramphenicol per ml were used for carnobacteria and lactococci and for leuconostocs, respectively.

Production and purification of leucocin A-UAL 187. An inoculum of 100 ml of an overnight culture of *L. gelidum* UAL 187 was added to 5 liters of CAA medium, maintained at pH 6.0 with 1 N sodium hydroxide at 22 to 23°C, and gently stirred under an N_2 atmosphere (40 ml/min). After 24 h, cells were harvested by centrifugation at 6,000 × *g* for 15 min at 4°C. The active substance was precipitated with 70% ammonium sulfate or by lowering the pH to 2.5 with 12 N HCl. The precipitate was dissolved in 6 M urea (Fisher)–10 mM glycine-HCl buffer (pH 2.5) and loaded onto a 6- by 25-cm Amberlite XAD-2 column (BDH). The column was washed with 0.1% trifluoroacetic acid (TFA; 1.5 liters), and with 25% ethanol (1.0 liter), 45% ethanol (1.0 liter), and 75% ethanol (2.0 liters) solutions in 0.1% TFA. Active fractions were pooled, concentrated with a rotary evaporator (≤30°C), and loaded onto a Sephadex G-25 (Pharmacia) column (2.5 by 45 cm) equilibrated with 0.1% TFA. The elution (A_{220}) was monitored, and all fractions were assayed for bacteriocin activity. Fractions containing more than 100 arbitrary activity units (AU; determined against *C. divergens* LV13) of bacteriocin per ml were pooled and concentrated. The sample was purified by high-performance liquid chromatography (HPLC) in a Bio-Rad chromatograph with a reversed-phase C-18 column (8- by 100-mm Waters μ-Bondapak; 10-μm particle size, 125 Å; pore size, 12.5 nm). Leucocin was isocratically eluted (1.65 ml/min) with 35% acetonitrile–0.15% TFA–H_2O. The elution (A_{220}) was monitored, the activity was assayed (17), and the total protein content was determined by using the method of Lowry et al. (24) as modified by Markwell et al. (26).

Stability of leucocin. The effects of pH, temperature, degradative enzymes, and selected solvents on the activity of leucocin (400 AU/ml) were determined. Pure and crude (after pH precipitation) samples of bacteriocin were suspended in 50 mM glycine-HCl (pH 2.0 and 3.0), 50 mM sodium acetate (pH 4.0 and 5.0), 50 mM sodium citrate (pH 6.0), 60 mM Tris-HCl (pH 7.0 and 8.0), and 50 mM glycine-NaOH (pH 9.0 and 10.0). Samples in buffer solutions (200 μl) were placed on ice and at 25°C and held for 2 and 24 h; one sample in each buffer was placed in boiling water for 20 min, and the residual activity was determined. Samples in buffers at pH 2.0, 6.0, and 10.0 containing 10 mg of bovine serum albumin (BSA; Sigma) per ml or 0.5% dithiothreitol (Sigma) were tested for stability as described above, except that samples containing dithiothreitol were only tested at 0°C.

Enzymes (1 mg/ml) in appropriate buffer solutions were added to purified leucocin (400 AU/ml) suspended in the same buffer solutions, including the following: protease types I, IV, VIII, X, and XIV (Sigma) in 50 mM Tris-HCl (pH 7.5) and type XIII in 50 mM acetate buffer (pH 4.0); trypsin (Sigma) in 50 mM Tris-HCl (pH 7.5); α- and β-chymotrypsin in 50 mM Tris-HCl and 10 mM $CaCl_2$ (pH 7.5); pepsin in 10 mM citrate (pH 6.0); papain and lysozyme in 50

mM Tris-HCl (pH 7.5); and lipase and phospholipase C in 50 mM Tris-HCl and 10 mM $CaCl_2$ (pH 7.0). The leucocin activity was determined immediately.

Crude leucocin (ammonium sulfate concentrate, dissolved in 6 M urea–50 mM glycine-HCl buffer [pH 2.0]) was suspended in the following organic solvents diluted in 50 mM glycine at pH 2.0 to give a final concentration of 6,400 AU of bacteriocin per ml: 50% ethanol (BDH), 70% ethanol (BDH), 50% acetonitrile, 0.15% TFA (Sigma), 50% tetrahydrofuran (Caledon Laboratories Ltd., Georgetown, Ontario, Canada), 50% isopropanol (BDH), 50% acetone (BDH), and 50% methanol (BDH). After 2 h, the solvents were evaporated with a rotary evaporator, the samples were lyophilized and resuspended in 25 mM glycine-HCl buffer at pH 2.0, and the leucocin activity was determined.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Leucocin preparations were examined on 20% polyacrylamide gels with the buffer system described by Laemmli (23) and 3 M Tris-HCl. Electrophoresis was done at 20 mA constant current. After electrophoresis, gels were either fixed in 5% (wt/vol) formaldehyde for 1 h and stained with Coomassie blue (Bio-Rad) or assayed for antimicrobial activity by the method of Barefoot and Klaenhammer (4).

Determination of amino acid content and sequence of leucocin A-UAL 187. Purified leucocin was derivatized with phenylisothiocyanate on an Applied Biosystems 420A derivatizer and separated by HPLC (Applied Biosystems model 130A chromatograph) with a C-18 column. Data were recorded and analyzed on an Applied Biosystems 920A data analyzer system. The amino-terminal sequence of leucocin was determined by automated Edman degradation with a gas-phase sequencer (Applied Biosystems model 470A) with on-line phenylthiohydantoin-derivative identification by HPLC (Applied Biosystems model 120A chromatograph).

Cleavage of leucocin A-UAL 187 by trypsin. A sample of 0.8 mg of leucocin in 50 mM citrate buffer at pH 6.0 (1.0 ml) was treated with 2.0 mg of trypsin (Sigma) in the same buffer (1.0 ml) for 2 h at 24°C. Trypsin was removed by chromatography on Sephadex G-25 (1.5 by 25 cm; 0.1% TFA in water), and the other fractions were combined and lyophilized. The residue was dissolved in 0.1% TFA in water (1.5 ml) and separated by HPLC on a C-18 column (8- by 100-mm Waters μ-Bondapak; 10-μm particle size, 125 Å [12.5 nm]) with a flow rate of 1.65 ml/min with 35% acetonitrile–0.1% TFA–H_2O. Four fractions were collected, and two of these were analyzed by mass spectrometry (see below).

Mass spectrometry. All mass spectra were obtained on a Finnigan MAT TSQ 700 triple quadrupole instrument fitted with an electrospray ionization source (Analytica, Bradford, Conn.). The peptide samples were dissolved in water-methanol (1/1) containing 0.1% formic acid to give final concentrations of about 25 pmol/μl. These solutions were infused at a constant rate of about 1 μl/min into the electrospray source with a syringe infusion pump (Harvard Apparatus model 22). Spectra were acquired in the profile mode by using multichannel averaging. Finnigan MAT BioMass software was used to determine the molecular weights from the envelope of multiply charged peaks in the *m/z* spectra. Tandem mass spectrometry (MS/MS) daughter spectra were generated by selecting the parent *m/z* in the first quadrupole, fragmenting the parent ions in the second quadrupole by using collision-induced dissociation, and recording the resultant daughter ions (*m/z*) by scanning the mass range of the third quadrupole. Argon (1 to 3 mTorr) was used as the collision gas with collision energies of −9 to −20 eV. Full-scan range mass spectra (3 s each) were averaged over 1 min.

Cleavage of the disulfide bond in leucocin A-UAL 187 and derivatization with iodoacetic acid. Leucocin (1.0 mg) in 50 mM citrate buffer at pH 6.0 (0.5 ml) was reduced with β-mercaptoethanol for 1 h at 24°C. The sample was divided into two aliquots, and one part was derivatized with iodoacetic acid (4.6 mg) in 500 μl of citrate buffer (final concentration, ~25 mM) and stirred for 6 h at 24°C. Both this sample and the other portion were concentrated in vacuo, diluted with 0.1% TFA in water (10 ml), and concentrated again. Before HPLC separation, each sample was purified on a small C-18 column (1.0 g) with 37% acetonitrile-0.1% TFA–H_2O.

DNA isolation, manipulation, and hybridization. Large-scale plasmid preparations were done by the Anderson and McKay method (1). Small-scale plasmid isolation from *E. coli* was done by the Geneclean Bio 101 method (Bio 101, LaJolla, Calif.). Restriction endonucleases and T4 DNA ligase from Boehringer-Mannheim (Dorval, Quebec) or Bethesda Research Laboratories (Burlington, Ontario) were used as recommended by the supplier. Procedures for DNA manipulations, cloning, and hybridizations were done as described by Sambrook et al. (35). Competent cells of *E. coli* were transformed by standard procedures (8). Lactococci, carnobacteria, and leuconostocs were transformed by electroporation (25). For Southern and colony blot hybridizations (35), DNA was bound to Hybond N (Amersham Corp.). Hybridizations and washes were done at 30°C. Radioactive DNA probes were end labeled with [γ-^{32}P]ATP (Amersham Corp.) with T4 polynucleotide kinase (Pharmacia). A site-specific 17-mer, JHA-3 (5′-CGAGAGCACTAT TATCC-3′), was used to identify the *lcnA* gene in both Southern and colony blot hybridizations. Oligonucleotides were synthesized on an Applied Biosystems 391 PCR Mate synthesizer and used without further purification.

DNA sequencing analysis. Double-stranded DNA was sequenced bidirectionally by the dideoxy-chain termination method (36). The initial sequence was obtained by priming with a 24-mer wobbled oligonucleotide [5′-AAATATTATG G(TA)AATGG(TA)GT(XA)CAT-3′, *X* = bromouracil] that corresponded to the N-terminal amino acid sequence of leucocin A-UAL 187. For further sequencing of both DNA strands, appropriate oligonucleotides were synthesized. Initially, DNA was sequenced by using the 7.6-MDa plasmid of *L. gelidum* UAL 187-22 as the template. After the genes in pUC118 were cloned, sequencing was continued with synthesized oligonucleotides from the revealed sequence and M13/pUC multiple-cloning site (MCS) universal forward and reverse primers.

Molecular cloning of the *lcn* gene. Plasmid DNA from *L. gelidum* UAL 187-22 was digested with restriction enzymes compatible with the M13/pUC MCS. Plasmid DNA from a bacteriocin-negative mutant, *L. gelidum* UAL 187-13, was run as a control. A 2.9-kb *Hpa*II fragment was eluted from a gel by using the Geneclean Bio 101 kit according to the manufacturer's instructions and ligated into the *Acc*I site of pUC118 to form the hybrid plasmid pJH6.1F. Putative clones were confirmed by Southern and colony blot hybridization by using the primer JHA-3 as well as by using restriction analysis of the insert and resequencing of regions already sequenced on the native plasmid pLG7.6.

Expression studies of leucocin in lactic acid bacteria. The 2.9-kb insert in pUC118 (pJH6.1F) was subcloned into the *Pst*I-*Sac*I sites of the shuttle vector, pNZ19 (de Vos, the Netherlands), to make the hybrid pJH8.6L. The presence of

TABLE 2. Purification of leucocin A-UAL 187

Purification stage	Vol (ml)	Leucocin activity (AU/ml)	Total leucocin activity (AU)	Protein[a] (mg/ml)	Leucocin sp act (AU/mg)	Activity recovered (%)	Fold purification
Culture supernatant	5,000	1.28×10^4	6.4×10^7	3.2	4×10^3	100	1
Combined ammonium sulfate and pH 2.5 concentrate	18	3.3×10^6	6.0×10^7	9.5	3.47×10^5	94	87
Pooled Amberlite XAD-2 fractions (0.1% TFA–ethanol)	900	6.4×10^4	5.76×10^7	0.12	5.3×10^5	90	133
Pooled Sephadex G-25 fractions (0.1% TFA)	420	1.02×10^5	4.3×10^7	0.06	1.71×10^6	67	428
HPLC[b] (35% acetonitrile–0.1% TFA)	1.8	2.05×10^7	3.7×10^7	1.14	1.8×10^7	58	4,500

[a] Determined by the method of Lowry et al. (24) as modified by Markwell et al. (26) with Nisin-A (Aplin and Barrett) as a standard.
[b] Reversed-phase HPLC, separation, lyophilization, and suspension in 0.1% TFA.

the insert was confirmed by Southern and colony blot hybridization and by restriction analysis. Plasmid pJH8.6L was transformed into *Lactococcus lactis* Na8, *Leuconostoc* sp. strain UAL60, and *Carnobacterium piscicola* UAL26 by electroporation. The presence of pJH8.6L was confirmed by plasmid analysis, restriction analysis, and Southern hybridization. Transformants were confirmed by sugar fermentation testing. Bacteriocin production and immunity were tested by using a deferred inhibition assay (17).

Nucleotide sequence accession number. The DNA sequence was submitted to Genbank (Los Alamos, N.M.) and was given the accession number M64371.

RESULTS

Purification of leucocin A-UAL 187. The purification stages of leucocin are shown in Table 2. The recovery of bacteriocin after 70% ammonium sulfate and pH 2.5 precipitation was similar. Each procedure recovered approximately 30×10^6 AU of bacteriocin. The precipitates were not soluble in 50 mM glycine-HCl (pH 2.5); however, the addition of 6 M urea (final concentration) solubilized the precipitate. The crude concentrate was applied to an Amberlite XAD-2 column washed with 0.1% TFA and TFA-ethanol solutions. Bacteriocin was eluted with 75% ethanol–0.1% TFA. Only 4% of the total starting activity was lost in this step. The pooled, active fractions were evaporated, lyophilized, resuspended in 0.1% TFA, and loaded onto a Sephadex G-25 column. Most of the bacteriocin was eluted in a single band (Fig. 1); however, some activity was detected in all subsequent fractions. Only fractions containing more than 100 AU/ml were pooled.

Several gradient elutions were tried on an HPLC C-18 column to obtain pure leucocin. Isocratic elution with 35% acetonitrile–0.15% TFA gave the best separation (Fig. 2). Four fractions corresponding to peaks 1 through 4 were collected; 99% of all activity was detected in peak 2. The HPLC-purified sample was initially visualized by electrophoresis on an SDS–20% polyacrylamide gel (Fig. 3). Resolution of the small peptides was difficult because they

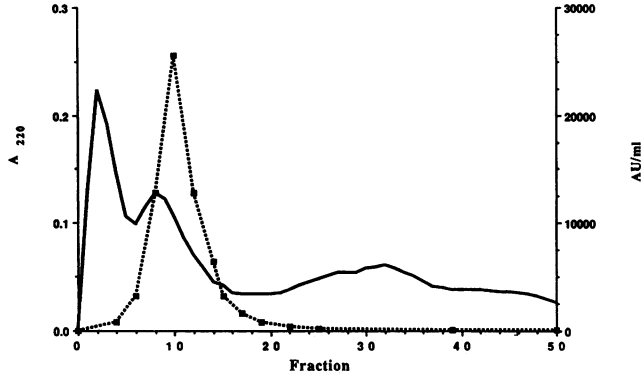

FIG. 1. Elution and activity of a 5-ml sample of leucocin A-UAL 187 on Sephadex G-25. Symbols: ——, A_{220}; – – –, leucocin activity.

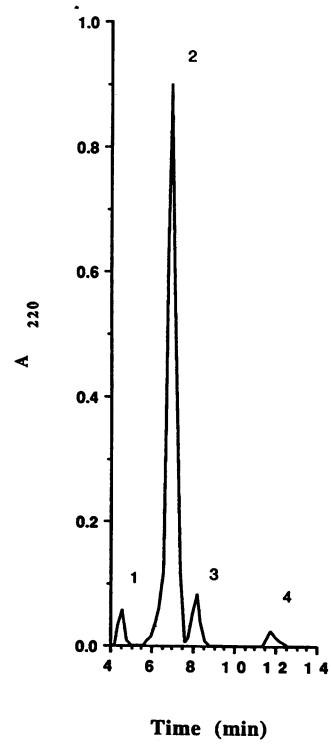

FIG. 2. Elution spectrum of a 50-μl sample of leucocin A-UAL 187 in 0.1% TFA from a reversed-phase HPLC C-18 column (Waters) with 35% acetonitrile in 0.1% TFA.

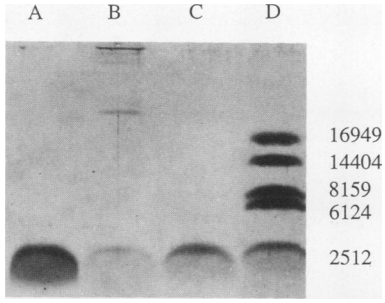

FIG. 3. Polyacrylamide (20%) gel electrophoresis of leucocin A-UAL 187. Lanes A, reversed-phase HPLC-purified leucocin; B, crude leucocin (after pH precipitation); C, nisin (3,353 Da) (Aplin and Barrett); D, low-molecular-mass protein standards of 16,949, 14,404, 8,159, 6,124, and 2,512 Da (Fluka, Biochemika).

appeared as faint, diffuse bands. Resolution was improved by increasing the amount of SDS to 0.5% and the amount of Tris to 3.0 M (final concentrations). The boldness of the band was improved by fixing the gel in 5% formaldehyde before staining (11). The estimated molecular mass of the bacteriocin was between 2,500 and 3,000 Da.

Stability of leucocin A-UAL 187. Crude leucocin from ammonium sulfate precipitation is stable at pH 2.0 and 3.0, even after samples are boiled for 20 min. Loss of activity was detected at pH 5.0 and above. This was most severe in samples above pH 8.0. Purified leucocin is less stable. The activity of the pure sample was lost rapidly at all pH levels and temperatures tested. The addition of BSA to purified leucocin caused a ca. fourfold increase in activity. In an experiment to determine whether BSA reversed the denaturation process or enhanced activity, in the sample containing BSA the activity was unchanged at 3,200 after 2 h at 25°C, but only 400 AU/ml was detected in the sample without BSA. The sample without BSA was divided into two aliquots; BSA was added to one, and an activity of 1,600 AU/ml, which is equivalent to half of the initial activity, was detected. The loss of activity in the presence of dithiothreitol was the same or greater than that in the control. Treatment with protease I, IV, VIII, X, XIII, or XIV, trypsin, α- or β-chymotrypsin, pepsin, or papain caused complete loss of activity of purified leucocin A-UAL 187. However, lysozyme, lipase, and phospholipase did not affect the activity as compared with that of the control. Of the organic solvents tested, only 50% methanol had a detrimental effect on activity. Ethanol, TFA, and acetonitrile were used in the separation process.

Mass spectrometry of leucocin A-UAL 187. Mass spectral measurements on samples of pure leucocin by using electrospray ionization (12) and a triple quadrupole spectrometer are shown in Fig. 4. The average molecular weight is 3,930.3 ± 0.4. The nominal molecular weight of the peptide derived from the putative (nucleotide-derived) amino acid sequence is 3,932.3. Leucocin appears to have no posttranslational modifications except for a disulfide bridge between the cysteine residues at positions 9 and 14. Digestion of pure leucocin with crude trypsin followed by HPLC separation revealed a series of four fragments. None of these tryptic fragments of leucocin displayed antibacterial activity. Two of the fragments were analyzed by mass spectrometry in the same fashion to further validate the sequence. One of these peptides is Lys-Tyr-Tyr (average molecular weight of 472.5 with observed MH^+ 473.3) arising from cleavage at the N terminus. The other tryptic peptide fragment is Tyr-Tyr-Gly-Asn-Gly-Val-His (average molecular weight of 808.3 with observed MH^+ 809.0). Its mass spectrum also contained the doubly charged ion at 405.0 ($[M + 2H]^{+2}$). The daughter spectra of both tryptic peptides support the proposed sequence.

Derivatization of leucocin A-UAL 187. Reduction of the disulfide linkage of leucocin with β-mercaptoethanol followed by derivatization with iodoacetic acid generated a modified compound that was readily separated from leucocin by using HPLC. The reduced sample of leucocin had a retention time of 7.0 min, and the carboxymethylated sample had a retention time of 7.6 min. Both samples displayed antibacterial activity at levels approximately the same as those of the parent leucocin.

Amino acid sequence of leucocin A-UAL 187. The N-terminal amino acid sequence identified the following 13 amino acids: H_2N-Lys-Tyr-Tyr-Gly-Asn-Gly-Val-His-Cys-Thr-Lys-Ser-Gly-. After the ninth amino acid residue, the yield of phenylthiohydantoin-derivatized residues decreased sharply; phenylthiohydantoin-derivatized residues were not detectable after residue 13. The amino acid analysis and amino acid residues for leucocin A-UAL 187 shown in Table 3 are consistent with the proposed structure.

Isolation of the leucocin structural gene, *lcnA*. Plasmid DNA (pLG9.2 and pLG7.6) was isolated from *L. gelidum* UAL187-22 and digested with restriction enzymes compatible with the pUC118 MCS. A 24-mer oligonucleotide deduced from the N-terminal amino acid sequence of leucocin A-UAL 187 hybridized with a 2.9-kb *Hpa*II fragment as shown in Fig. 5, lanes B. No signals were present from *Hpa*II-restricted plasmid DNA from *L. gelidum* UAL 187-13 containing only pLG9.2 (Fig. 5, lanes A). Cloning of the 2.9-kb *Hpa*II fragment into the *Acc*I site of pUC118 resulted in a hybrid plasmid of 6.1 kb, pJH6.1F (Fig. 5, lanes C). Southern and colony blot hybridizations done by probing with the sequencing primer JHA-3 were used to confirm the presence of the *lcnA* gene (Fig. 5, lanes C).

Structure of the *lcnA* gene. DNA sequencing revealed two ORFs flanked by a putative promoter and terminator. According to the N-terminal amino acid sequence of leucocin A-UAL 187, leucocin begins with Lys-25, suggesting that the *lcnA* structural gene encodes a 61-amino-acid prepeptide consisting of a 24-residue N-terminal extension and a 37-amino-acid bacteriocin (Fig. 6). A putative ribosomal binding site (RBS), GAGGA, is located 9 bp upstream of the start codon (coordinate 293). A putative promoter (coordinates −35 and −10) is located 26 nucleotides upstream of the RBS (coordinate 261). A second ORF (ORF 2) containing 113 amino acids was evident from the sequence (Fig. 6). There is a putative RBS (GAAGG) 7 bp downstream of the ORF 1 stop codon and 8 bp upstream of a TTG codon that appears to function as a start codon. A region of dyad symmetry that could form a stem-loop structure was located downstream of ORF 2 (coordinate 902; Fig. 6). This has the features associated with a bidirectional rho-independent termination site (32). The stabilization energy (ΔG) was calculated as −21.4 or −24.8 kcal (ca. −89.5 to −104 kJ)/mol, depending on whether the outer 4 A · U bp are included (13). Sequencing with the reverse primer of the M13/pUC MCS displayed overlapping sequence at the 5′ end of the leucocin operon, indicating the position of the operon in the cloned insert (Fig. 7). No protein in the SWISPROT data bank showed homology to the translation products of ORFs 1 and 2.

Expression of the leucocin gene. The 2.9-kb insert containing the *lcnA* gene in pJH6.1F was excised by digestion with

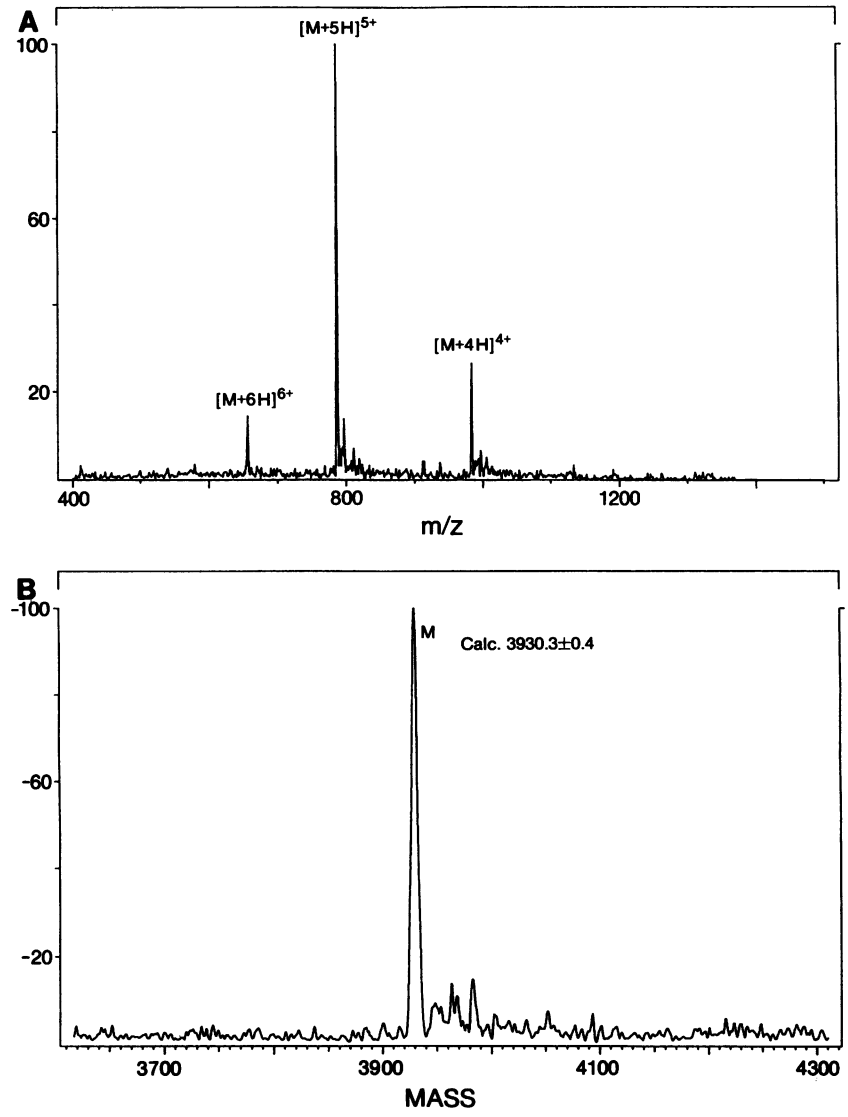

FIG. 4. Mass spectrum of leucocin A-UAL 187 acquired on a Finnigan MAT TSQ 700 triple-quadrupole spectrometer with electrospray ionization. (A) relative intensities as a function of mass to charge ratio (*m/z*) of the ions resulting from multiple protonation ($4H^+$, $5H^+$, $6H^+$) of the molecular ion (M). (B) Spectrum calculated from the data in panel A for the parent molecular ion (M).

*Sac*I-*Pst*I and ligated into pNZ19 (5.7 kb). Chloramphenicol-resistant *E. coli* JM103 colonies were screened by probe hybridization with the primer JHA-3. Colonies that gave a positive signal showed the presence of an 8.6-kb plasmid that resolved into 5.7- and 2.9-kb fragments upon digestion with *Pst*I and *Sac*I. This new hybrid pJH8.6L was successfully transferred into *Leuconostoc* sp. strain UAL 60 and *Lactococcus lactis* Na8 without apparent deletion of any part of the plasmid. The site-specific primer JHA-3 hybridized strongly to the plasmid pJH8.6L in both of these transformants. However, *C. piscicola* UAL 26 showed the presence of a smaller plasmid that may be a deletion derivative of pJH8.6L. No signal was evident upon hybridization with the site-specific primer JHA-3. None of the *E. coli* strains containing pJH6.1F or pJH8.6L or the transformants showing the presence of the intact pJH8.6L or spontaneous deletion derivatives produced detectable levels of leucocin. We have been unable to transform a bacteriocin-negative mutant of the original producer strain, *L. gelidum* UAL 187-13, with the hybrid plasmid (pJH8.6L) or with any other foreign plasmid.

DISCUSSION

Leucocin A, with a molecular mass of 3,930.3 ± 0.4 Da (37 amino acids), may be one of a group of small antibacterial peptides produced by lactic acid bacteria. This group may include lactacin F (6.3 kDa, 57 amino acids) (28, 29), pediocin A (2.7 kDa) (5), and recently cloned bacteriocins from *Lactococcus lactis* that are encoded by genes containing 69, 75, and 77 codons, part of which may be a leader peptide that is processed before secretion (38). Leucocin was purified with a yield of 2.06 mg from 5 liters of CAA growth medium. This represents a significantly higher yield than that previously obtained during purification of nonlantibiotic bacteriocins (4, 18, 28). Ion-exchange chromatogra-

TABLE 3. Amino acid analysis of leucocin A-UAL 187 determined by derivatization with phenylisothiocyanate and separation by HPLC

Amino acid	pmol	No. of residues in sequence[a]
Asparagine	416	4
Glutamic acid	188	1
Serine	247	3
Glycine	913	8
Histidine	243	2
Arginine	120	1
Threonine	115	1
Alanine	353	3
Tyrosine	192	2
Valine	337	3
Leucine	137	1
Phenylalanine	231	2
Lysine	195	2
Tryptophan	ND[b]	2
Cysteine	ND	2

[a] Determined from DNA sequencing.
[b] ND, not determined.

phy, dialysis, and high pH conditions were avoided because they resulted in large losses in activity. Maintaining a low pH and using only precipitation, gel filtration, and hydrophobic interactions resulted in a good yield. Similar yields were obtained when these procedures were used for the isolation of lantibiotics.

In the early stages of the purification process, the bacteriocins aggregate with larger proteins in the supernatant fluids (4, 5). This may confuse molecular weight determinations (5). Ammonium sulfate and pH precipitations yielded bacteriocin-containing protein aggregates that were not soluble in buffer alone. The addition of urea allowed dissociation of the bacteriocin from the other proteins; upon elution from the Amberlite column, dissociating conditions were no longer necessary. The Sephadex G-25 step resulted in the largest loss of activity. This was unexpected, because gel filtration caused only marginal loss of activity in other studies (4, 5). This may be explained in part by the fact that only highly active fractions were pooled. All subsequent fractions, however, showed some activity indicating interaction between leucocin A-UAL 187 and the column matrix. The use of TFA as the equilibrating solution may increase the hydrophobicity of the protein and therefore could result increased interaction with the column (37). However, TFA was used because it eliminates the need for dialysis, which was shown to cause large losses of activity in this study.

The stability of leucocin at low pH and its instability at pH 7.0 were similar to those found with other bacteriocins of low molecular weight, such as nisin-A and pediocin AcH (5). The addition of BSA to leucocin stabilized the pure form at low pH and caused a ca. fourfold increase in AU compared with that of the control. BSA may prevent denaturation of leucocin during the time between spotting of the bacteriocin and growth of the indicator. Purified leucocin is inactivated by many types of proteases, but it is not inactivated by treatment with chloroform (17), lipase, or phospholipase, suggesting the absence of lipid or phospholipid moieties.

Leucocin contains 37 amino acids. The first 13 residues deduced by amino-terminal sequencing were confirmed by the DNA sequence. The molecular mass calculated from the putative amino acid sequence deduced from the DNA code is 3,932.3 Da. This is 2 Da greater than the molecular mass determined by fast atom bombardment (FAB)-mass spectrometry. This indicates that there could be a loss of two protons in the maturation of the prepeptide. The formation of a disulfide bridge is likely between the two cysteine residues and could explain this. Derivatization of leucocin to prevent the formation of the disulfide bridge did not cause loss of activity in the derivatized leucocin.

Knowing the first 13 amino acids of the N terminus allowed us to probe and immediately identify the ORF containing the *lcnA* structural gene and a probable 24-residue N-terminal extension. Initiation at the indicated

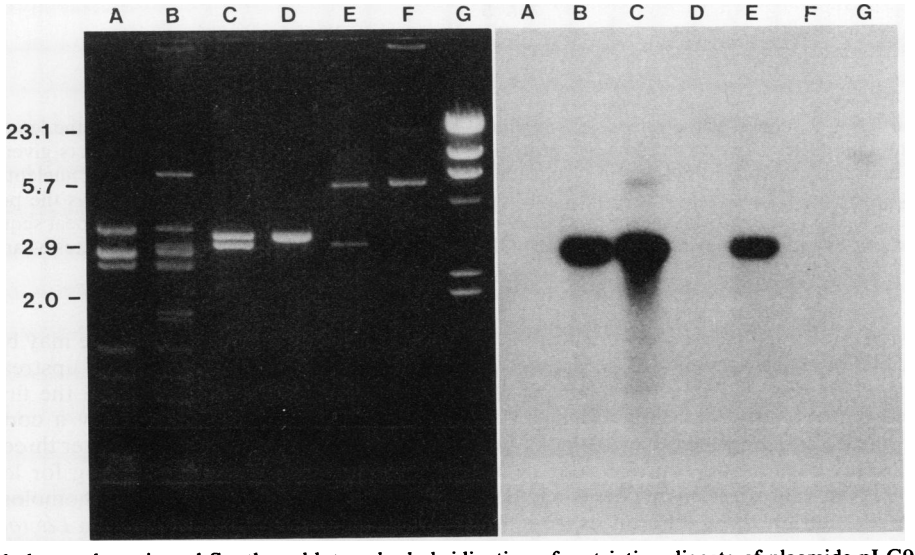

FIG. 5. Agarose gel electrophoresis and Southern blot probe hybridization of restriction digests of plasmids pLG9.2, pLG7.6, pJH6.1F, and pJH8.6L digested with restriction enzymes as follows (lanes): A, pLG9.6, *Hpa*II; B, pLG9.6 and pLG7.4, *Hpa*II; C, pJH6.1F, *Sac*I-*Pst*I; D, pUC118, *Pst*I; E, pJH8.6F, *Sac*I-*Pst*I; F, pNZ19, *Pst*I; and G, *Hin*dIII digest of λ phage size standards (23.1, 9.4, 6.5, 4.4, 2.3, 2.0, and 0.56 kb). A site-specific probe (JHA-3) homologous to a region within the leucocin structural gene was used in the hybridization.

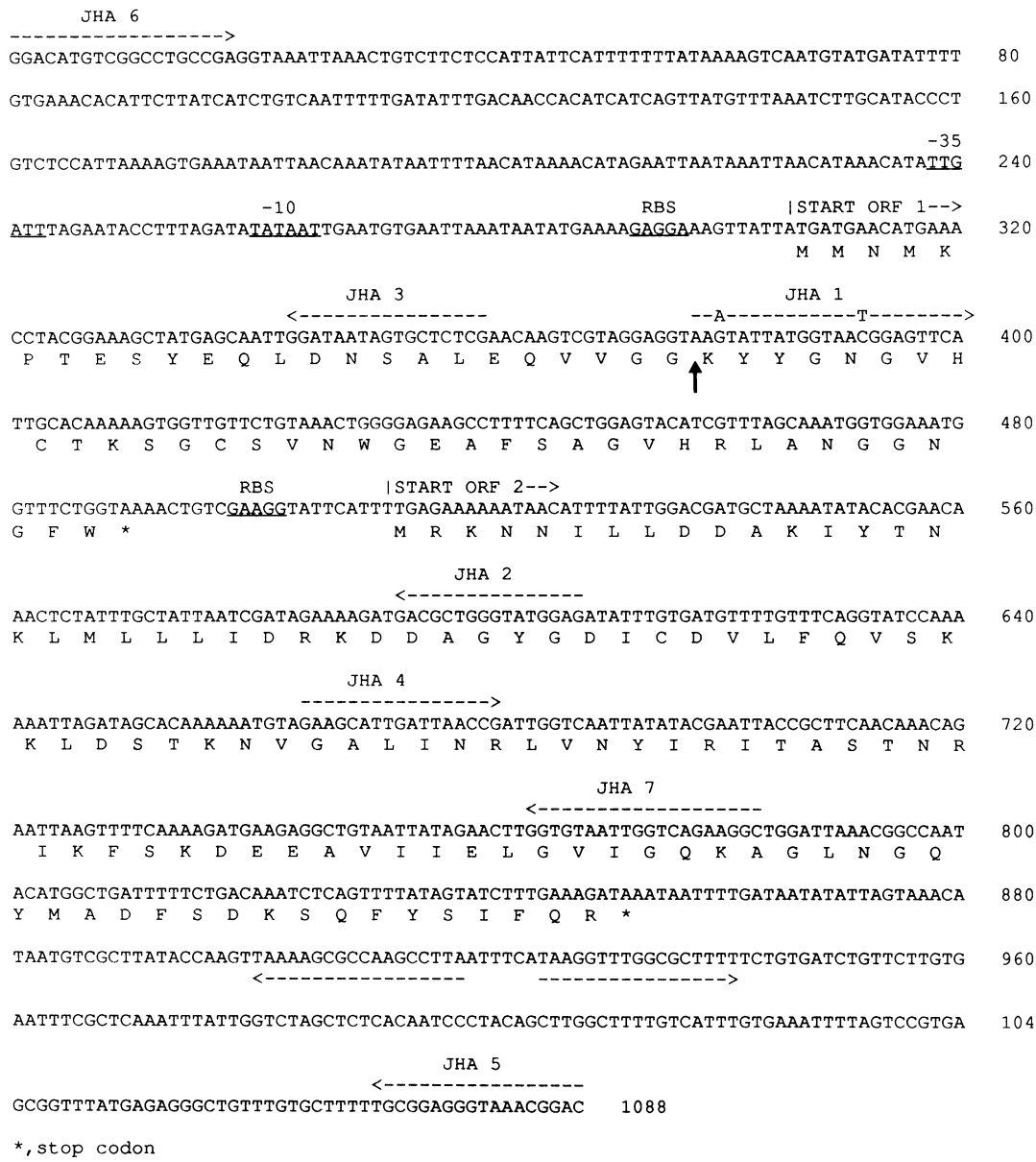

FIG. 6. Single-strand DNA sequence of the region of *L. gelidum* UAL 187 plasmid (pLG7.6) containing the leucocin A operon. ORF 1 (precursor leucocin gene) and ORF 2 (putative immunity protein) are indicated, with the translation products given below the nucleotide sequence. The probes and primers used in cloning and sequencing are shown by a dotted line above the nucleotides, with mismatches indicated for the mixed oligomer JHA-1. The arrow between the glycine and lysine at coordinate 378 shows the point of cleavage of the N-terminal leader sequence. The horizontal dashed arrows below the nucleotide sequence indicate inverted repeat sequences that are capable of forming stem-loop structures and may signal the termination of the transcript. Nucleotides between coordinates 64 and 1009 were confirmed by sequencing both strands.

methionine is suggested by the consensus distance of 9 bp from the RBS (15). This extension appears to be proteolytically cleaved before secretion, because the biologically active substance does not contain this region. This leader region may act as a signal sequence; however, it lacks the typically hydrophobic core region and contains four negatively charged residues. A novel excretion mechanism in this group of bacteria may account for these differences from the expected structure of signal peptides (14). The four amino acids adjacent to the clevage site in the C region (Val-Val-Gly-Gly) are identical to those of the leader peptide of the *lacF* gene of *Lactobacillus acidophilus* (29).

The bacteriocin structural gene may be transcribed from a putative promoter that is located upstream of the *lcnA* gene. The −10 region (TATAAT) and the first three nucleotides (TTG) of the −35 region follow a conserved pattern, but there is little consensus in the latter three nucleotides (21, 38, 39). The part of the gene coding for leucocin A-UAL 187 shows no DNA or amino acid homology to the lactacin F gene or to the genes cloned from *Lactococcus lactis* subsp. *cremoris* (38). The apparent operon consisting of a bacteriocin structural gene followed closely by a larger ORF, usually encoding immunity, seems to be a conserved pattern for this type of bacteriocin (29, 38). Further studies involving

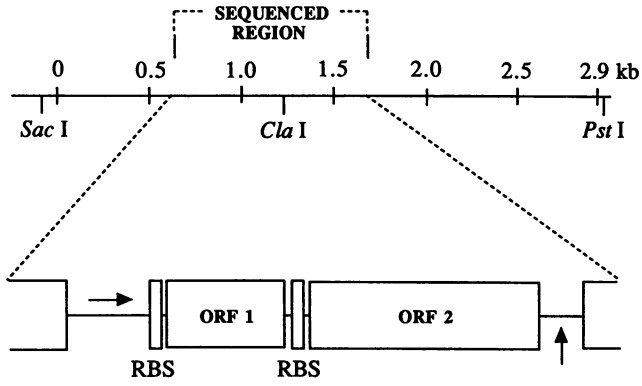

FIG. 7. Location of ORF 1 (Leucocin gene) and ORF 2 (putative immunity gene) in a 2.9-kb fragment from *L. gelidum* plasmid pLG7.6 cloned into the MCS of pUC118. *Pst*I and *Sac*I sites are part of the pUC118 MCS. The horizontal arrow indicates the putative promoter, and the vertical arrow indicates the putative terminator.

subcloning of ORF 2 of this putative operon are required to establish the function of this gene. The start codon of this gene is unusual. It is a TTG rather than an ATG. In *E. coli*, GTG or TTG replaces ATG as a start codon in about 9% of genes (15, 22). The frequency of this phenomenon in leuconostocs is not known. The putative terminator has a stable stem-loop structure that is preceded by four A's and followed by four U's. This arrangement is characteristic of bidirectional rho-independent terminators (32, 41).

The position of the *lcnA* gene within the 2.9-kb insert and the genetic evidence of a putative promoter, immunity gene, and termination signal all indicate that there should be sufficient information to obtain phenotypic expression in a suitable host. However, we were unable to obtain expression of leucocin production or immunity in lactic acid bacteria transformed with pJH8.6L. The expression of the *lacF* (29) and bacteriocin genes from *Lactococcus lactis* subsp. *cremoris* (38) was successful from smaller cloned fragments of 2.2 and 1.8 kb, respectively. In both cases, production and immunity are encoded by closely linked genes. We have not been successful in transforming a bacteriocin-negative mutant of the parent strain. Until we are able to transform the native strain with the clone, we cannot determine whether we have sufficient genetic information for normal phenotypic expression of this bacteriocin.

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