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Cloning, Phenotypic Expression, and DNA Sequence of the Gene for Lactacin F, an Antimicrobial Peptide Produced by *Lactobacillus* spp.†

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Lactacin F is a heat-stable bacteriocin produced by *Lactobacillus acidophilus* 11088. A 63-mer oligonucleotide probe deduced from the N-terminal lactacin F amino acid sequence was used to clone the putative *laf* structural gene from plasmid DNA of a lactacin F-producing transconjugant, *L. acidophilus* T143. One clone, NCK360, harbored a recombinant plasmid, pTRK160, which contained a 2.2-kb *Eco*RI fragment of the size expected from hybridization experiments. An *Escherichia coli-L. acidophilus* shuttle vector was constructed, and a subclone (pTRK162) containing the 2.2-kb *Eco*RI fragment was introduced by electroporation into two lactacin F-negative strains, *L. acidophilus* 89 and 88-C. *Lactobacillus* transformants containing pTRK162 expressed lactacin F activity and immunity. Bacteriocin produced by the transformants exhibited an inhibitory spectrum and heat stability identical to those of the wild-type bacteriocin. An 873-bp region of the 2.2-kb fragment was sequenced by using a 20-mer degenerate lactacin F-specific primer to initiate sequencing from within the lactacin F structural gene. Analysis of the resulting sequence identified an open reading frame which could encode a protein of 75 amino acids. The 25 N-terminal amino acids for lactacin F were identified within the open reading frame along with an N-terminal extension, possibly a signal sequence. The lactacin F N-terminal sequence, through the remainder of the open reading frame (57 amino acids; 6.3 kDa), correlated extremely well with composition analyses of purified lactacin F which also predicted a size of 51 to 56 amino acid residues. Molecular characterization of lactacin F identified a small hydrophobic peptide that may be representive of a common bacteriocin class in lactic acid bacteria.

Bacteriocins are proteinaceous bacterial compounds that are inhibitory towards sensitive strains and are produced by both gram-positive and gram-negative bacteria (39). Among the gram-positive bacteria, bacteriocins are produced by many lactic acid bacteria (LAB) used in food fermentations and dairy products, including lactobacilli, lactococci, and pediococci (29). Lactobacilli are especially known for their bacteriocinogenic properties (4, 11, 41). Barefoot and Klaenhammer (4) reported that 63% of the *Lactobacillus acidophilus* strains they examined produced bacteriocins. Bacteriocins produced by *L. acidophilus* 11088, *L. acidophilus* N2, *Lactobacillus helveticus* 481, and *L. helveticus* LP27 have been purified and shown to constitute a heterogeneous class of antimicrobial peptides (5, 25, 33, 42). Although efforts have been made to characterize the proteinaceous nature of these bacteriocins, limited information is available about the genetic determinants of bacteriocins from lactobacilli or other LAB.

Genetic cloning of several bacteriocin genes from LAB has recently been reported. The structural gene for nisin, a lantibiotic produced by *Lactococcus lactis* subsp. *lactis*, has been cloned and sequenced by several groups (7, 13, 27). Although production of active nisin has yet to be demonstrated, analysis of the nisin gene sequence shows the presence of a signal peptide in a precursor molecule that is not present on the secreted protein. Two bacteriocins that are plasmid encoded in *L. lactis* subsp. *cremoris*, and one in *L. lactis* subsp. *lactis*, have also been cloned and expressed in lactococci (23, 43). However, DNA sequence information for these bacteriocins has not yet been reported. Recently, the structural gene encoding helveticin J produced by *L. helveticus* 481 was cloned, sequenced, and expressed in *L. acidophilus* 88-C (26). To our knowledge, the present study and the work reported by Joerger and Klaenhammer (26) provide the first such characterizations of bacteriocin genes from lactobacilli.

L. acidophilus 11088 produces a heat-stable bacteriocin designated lactacin F that is bactericidal to *Enterococcus faecalis*, *Lactobacillus fermentum*, and the closely related species of *Lactobacillus delbrueckii* and *L. helveticus*. The ability to produce lactacin F is conjugally transferred by a 110-kb plasmid that appears to be an episomal element (33; data not shown). Native lactacin F is associated with a 180-kDa bacteriocin complex, whereas the active agent has been purified and identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis as a peptide of approximately 2.5 kDa (34). Amino acid sequence analysis identified 25 N-terminal amino acids, but composition analyses indicated that lactacin F may contain as many as 56 residues. We describe the molecular cloning, phenotypic expression, and nucleic acid sequencing of the gene encoding lactacin F (*laf*). This is the first such characterization of a small (<7,000-Da), nonlantibiotic bacteriocin

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TABLE 1. Bacterial strains and plasmids

Bacterium or plasmid	Relevant characteristics	Source or reference
Bacteria		
L. acidophilus		
11088 NCK88	Laf$^+$ Laf^r, pPM4, pPM27	Muriana and Klaenhammer (33, 34)
88-C		
NCK64	Laf$^-$ Laf^r, pPM4, pPM27	Muriana and Klaenhammer (33, 34)
NCK367	Laf$^-$ Laf^r, pPM4, pPM27, pTRK159	This study
NCK369	Laf$^+$ Laf^r, pPM4, pPM27, pTRK162	This study
89		
NCK89	Laf$^-$ Laf^s, pPM4, pPM27, *str-6 rif-7*	Muriana and Klaenhammer (33, 34)
NCK368	Laf$^+$ Laf^r, pTRK162, *str-6 rif-7*	This study
NCK365	Laf$^-$ Laf^s, pPM4b, *str-6 rif-7*	This study
NCK366	Laf$^-$ Laf^s, pTRK159, *str-6 rif-7*	This study
T143 NCK61	Laf$^+$ Laf^r (unstable transconjugant), *spc-8 gen-9* pPM4, pPM27, pPM68	This study
T89 NCK62	Laf$^+$ Laf^r (stable transconjugant), *str-6 rif-7* pPM4, pPM27	Muriana and Klaenhammer (33, 34)
L. delbrueckii subsp. *lactis*		
4797 NCK235	Laf^s (indicator)	Muriana and Klenhammer (33, 34)
E. coli		
DH5	F$^-$ *recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1*	BRL
NCK359	DH5 pTRK159	This study
NCK358	DH5 pSA34	This study
NCK362	DH5 pTRK162	This study
DH5α	F$^-$ φ89d*lacZ* M15 (*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1*	BRL
NCK360	DH5α pTRK160	This study
NCK361	DH5α pTRK161	This study
NCK363	DH5α pTRK163	This study
NCK364	DH5α pTRK164	This study
Plasmids		
pBluescript KS+ (pBS/KS+)	2.9 kb; *lacZ* Ap^r	Stratagene
pPM4	6.3 kb; NCK88 cryptic plasmid	This study
pPM4b	4.3 kb; pPM4 deletion derivative	This study
pSA34	6.0 kb; Em^r Cm^r Tc^r	Sanders and Schultz (35a)
pTRK159	10.3 kb; pSA34::pPM4b; Em^r, Cm^r, Tc^r	This study
pTRK160	5.2 kb; pBS/KS+::2.2-kb *Eco*RI fragment	This study
pTRK161	4.5 kb; pTRK160 deletion derivative	This study
pTRK162	12.5 kb; pTRK159::2.2-kb *Eco*RI fragment	This study
pTRK163	3.8 kb; pBS/KS+::0.9-kb *Eco*RI-*Cla*I fragment	This study
pTRK164	4.2 kb; pBS/KS+::1.3-kb *Eco*RI-*Cla*I fragment	This study

among gram-positive bacteria and may define a class for similar peptide bacteriocins that are common to LAB.

MATERIALS AND METHODS

Bacterial cultures and media. Bacterial strains and plasmids used in this study are described in Table 1. *Lactobacillus* cultures were maintained as frozen stocks held at −20°C in MRS broth (Difco Laboratories, Detroit, Mich.) plus 20% glycerin (Fisher Scientific Co., Raleigh, N.C.). *Lactobacillus* cultures were propagated and transferred once before use. MRS agar was prepared by the addition of 1.5% (wt/vol) granulated agar (BBL Microbiology Systems, Cockeysville, Md.) to the broth medium; indicator overlay agar was prepared with 0.75% granulated agar. Frozen stocks of *Escherichia coli* were maintained in Luria-Burtani broth plus 20% glycerin. *E. coli* strains were propagated at 37°C with shaking (250 rpm). Antibiotics were used at the following concentrations: ampicillin (Ap), 100 μg/ml; tetracycline (Tc), 10 μg/ml; erythromycin (Em), 225 μg/ml for *E. coli*; erythromycin and chloramphenicol (Cm), each at 7 μg/ml for *L. acidophilus*.

Bacteriocin production and detection. Bacteriocin production by *L. acidophilus* 11088 and assays by deferred and direct methods have been described previously (33). *L. delbrueckii* subsp. *lactis* 4797 was used as an indicator for lactacin F activity. The heat stability of lactacin F was tested as described previously (33).

DNA isolation, manipulation, and hybridization. Alkaline lysis of *L. acidophilus* 11088 for plasmid DNA isolation was performed by the method of Anderson and McKay (1) with the following modifications. Cell pellets were washed once with the cell suspension buffer prior to lysozyme treatment. Incubations with lysozyme were performed at 0°C for 75 min. After lysozyme treatment, the cells were pelleted and lysed during resuspension in EDTA and SDS solutions, and then 38 ml of cell suspension buffer was added. Lysate shearing was omitted. Cell lysates were maintained at 0°C for 30 min after adjustment of the pH to 12.3.

Isolation of plasmid DNA from *E. coli* was performed by alkaline lysis by the method of Maniatis et al. (31). Total genomic and covalently closed circular (CCC) plasmid DNAs used for cloning, DNA hybridizations, and double-stranded sequencing reactions were isolated from cesium chloride-ethidium bromide equilibrium gradients (31). Re-

striction endonucleases and DNA modifying enzymes were used according to directions supplied by the manufacturers.

Lactacin F-specific probes (20-mer degenerate; 63-mer nondegenerate) for DNA and colony hybridizations were deduced from the N-terminal amino acid sequence of lactacin F (34). The 63-mer was constructed by using the most preferred codons ending in A or T compiled from *Lactobacillus* gene sequences residing in the GenBank data base and others taken from the literature. Probes were end labeled with [γ-^{32}P]dATP (Amersham Corp., Arlington Heights, Ill.), using a 5′ DNA terminus labeling system (Bethesda Research Laboratories [BRL], Gaithersburg, Md.). Electrotransfer of DNA from agarose gels to Magnagraph Nylon (MSI Inc., Westborough, Mass.) and DNA hybridization were performed following the instructions of the manufacturer. Colony hybridizations were performed as described by Ausubel et al. (3).

Cloning and construction of an *E. coli-L. acidophilus* shuttle vector. Molecular cloning techniques were as described by Maniatis et al. (31). Plasmid pBluescript KS+ (Stratagene, La Jolla, Calif.) and total plasmid DNA from *L. acidophilus* T143 were digested with *Eco*RI and ligated with T4 DNA ligase (BRL). Ligation products were ethanol precipitated and resuspended in water prior to electroporation in *E. coli* DH5α (14). Potential pBluescript recombinants were identified by the blue-white color reaction, using isopropyl-β-D-thiogalactoside and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as specified by the manufacturer (BRL). Putative clones were patch plated onto nylon filters (MSI), lysed in situ, and hybridized with lactacin F-specific probes.

An *E. coli-L. acidophilus* shuttle vector was constructed by ligation of *Bcl*I-digested pSA34 to *Bam*HI-digested pPM4b, a 4.3-kb derivative of pPM4. Plasmid pSA34 is a 6.0-kb origin-screening vector for gram-positive organisms (35a) which arose by a deletion encompassing the replication-copy number region of pSA3 (9). One erythromycin-resistant transformant recovered in *L. acidophilus* 89 contained a pPM4b::pSA34 recombinant plasmid, herein designated pTRK159 (see Fig. 2).

Electroporation. Transformation of *E. coli* via electroporation was accomplished by the method of Dower et al. (14), using a Gene Pulser and Pulse Controller unit (Bio-Rad Laboratories, Richmond, Calif.). *Lactobacillus* cultures were electroporated by the following modifications of the procedure of Luchansky et al. (30). *L. acidophilus* strains were grown to an optical density (600 nm) of 0.75. Electroporation buffer consisted of 2.5 mM $CaCl_2$ in 1.0 M sucrose. Cells were washed twice with 100 ml of electroporation buffer and finally resuspended to 1/75 of their original culture volume. Cell suspensions were maintained on ice until needed (0 to 30 min); DNA solutions and electroporation cuvettes were refrigerated. Electroporation was conducted with 400 μl of a cell-DNA suspension in 0.2-cm cuvettes (Bio-Rad). Electroporation conditions included 25-μF capacitance, 10.5 kV, and 20-Ω resistance in series with the cuvette. The pulse controller was set to infinity.

DNA sequencing and analysis. Double-stranded DNA sequencing was performed with [^{35}S]dATP (New England Nuclear), using the dideoxy-chain termination method of Sanger et al. (36) and the Sequenase 2.0 DNA sequencing kit (U.S. Biochemicals, Cleveland, Ohio) as prescribed by the manufacturer. Primers used for initiating DNA sequencing from pBluescript included the T7, KS, and M13 (reverse) primers (Stratagene). Site-specific 20-mer primers for sequencing within the cloned fragment were synthesized on a Gene Assembler (Pharmacia, Piscataway, N.J.) or 380A DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.). DNA sequence analysis and sequence data base searching were conducted with programs contained within the Sequence Analysis Software Package (version 6.1) licensed from the Genetics Computer Group (University of Wisconsin, Madison) (12). This package is maintained online at the Computer Graphics Center (North Carolina State University, Raleigh) on a VAX 11/780 computer.

Nucleotide sequence accession number. The sequence data are deposited in GenBank under accession number M57961.

RESULTS

Identification of lactacin F-related DNA sequences using lactacin F-specific probes. A 20-mer degenerate DNA probe to the N-terminal region of the lactacin F peptide was synthesized. This probe, however, failed to generate discernible bands when used in DNA hybridization experiments with genomic DNA. A longer, nondegenerate lactacin F probe (63-mer) demonstrated strong autoradiographic banding patterns with genomic DNA from five strains used in DNA hybridization experiments (Fig. 1A and B). The 63-mer hybridized to three bands in *Bcl*I genomic digests from the lactacin F producer (Laf$^+$) strain, *L. acidophilus* 11088; the Laf-negative (Laf$^-$) Laf-resistant (Laf^r) 11088 derivative, *L. acidophilus* 88-C; and the phenotypically stable (*L. acidophilus* T89) and unstable (*L. acidophilus* T143) Laf$^+$ transconjugants (Fig. 1A). No signal was obtained in the 2.2-kb position with the Laf$^-$ Laf^s 11088 derivative, *L. acidophilus* 89, that was the recipient strain for conjugal transfer experiments. Only a single band of 2.2 kb was detected in digests of CCC plasmid DNA from T143, which contains the 110-kb lactacin F-related plasmid, pPM68. Similar patterns in Laf$^+$ and Laf$^-$ strains were also observed in hybridization reactions carried out with genomic and CCC DNA digested with *Eco*RI, *Cla*I, and *Sca*I: multiple bands were again detected in genomic DNA, while CCC DNA from T143 yielded a single band. *Eco*RI digests of CCC DNA from T143 also demonstrated a single hybridization band of 2.2 kb, whereas the 63-mer probe hybridized to *Cla*I and *Sca*I fragments of approximately 1.8 and 18 kb, respectively (data not shown).

The absence of the 2.2-kb *Bcl*I lactacin F-specific band in strain 89 (conjugal recipient) and its reappearance in two Laf$^+$ transconjugants (T89 and T143) indicated that the 63-mer probe was suitable to detect Laf-related sequences that are conjugally transferred from 11088 to strains 89 and 88-C.

Cloning of lactacin F-related DNA sequences from *L. acidophilus* T143. Strain T143 contains several resident plasmids, including pPM27 (41 kb), pPM68 (110 kb), and a high-copy-number plasmid, pPM4 (6.3 kb) (33). Lactacin F production and immunity were previously correlated to pPM68 (33). Since plasmid pPM4 does not contain a restriction site for *Eco*RI, plasmid fragments were generated with *Eco*RI for cloning in pBluescript KS+. The 63-mer lactacin F gene probe which hybridized to Laf-related DNA sequences in genomic blots was used to screen recombinant clones. A putative *E. coli* clone, NCK360, contained a 5.2-kb plasmid, pTRK160. Dot-blot DNA hybridization showed that pTRK160 gave a strong signal with the 63-mer probe, whereas no signal was obtained with the pBluescript vector (data not shown). Plasmid pTRK160 generated the expected 2.2-kb fragment upon digestion with *Eco*RI. Restriction enzyme analysis of pTRK160 demonstrated that the

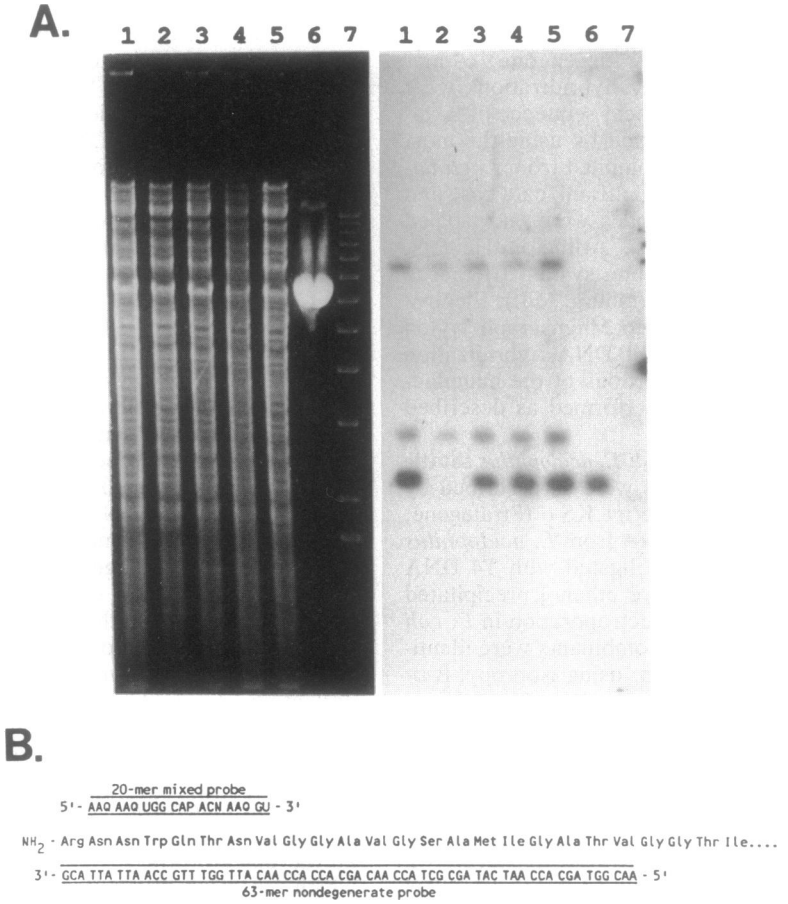

FIG. 1. Hybridization experiments with genomic DNA. (A) Left, *Bcl*I-digested DNA; right, hybridization of DNA in panel A with an end-labeled 63-mer lactacin F-gene-probe. Lanes 1 to 5 are genomic DNA; lane 1, *L. acidophilus* 11088 (Laf^+ Laf^r); lane 2, *L. acidophilus* 89 (Laf^- Laf^s); lane 3, *L. acidophilus* 88-C (Laf^- Laf^r); lane 4, *L. acidophilus* T89 (Laf^+ Laf^r); lane 5, *L. acidophilus* T143 (Laf^+ Laf^r); lane 6, CsCl-purified plasmid DNA from *L. acidophilus* T143; lane 7, 1-kb DNA ladder (BRL), from bottom to top, 1.018, 1.635, 2.036, 3.054, 4.072, 5.090, 6.108, 7.126, 8.144, 9.162, 10.180, 11.198, and 12.216 kb. (B) Sequence and relationship of *laf*-specific probes to the N-terminal amino acid sequence of lactacin F obtained through protein sequencing.

2.2-kb insert contained an internal *Cla*I site, generating two *Eco*RI-*Cla*I subfragments (0.9 and 1.3 kb).

Phenotypic expression of lactacin F in *L. acidophilus*. The 2.2-kb *Eco*RI fragment of pTRK160 was gel eluted and subcloned onto pTRK159, a 10.3-kb *E. coli-Lactobacillus* shuttle-cloning vector constructed for use in *L. acidophilus* 11088 and its derivatives (Fig. 2). The resulting plasmid, pTRK162, was isolated from *E. coli* DH5 and transformed via electroporation into two different *L. acidophilus* recipients, 89 (Laf^- Laf^s) and 88-C (Laf^- Laf^r). Plasmid pTRK162 isolated from two respective transformants, NCK368 and NCK369, was shown to contain the intact 2.2-kb insert after digestion with *Eco*RI (data not shown). NCK368 and NCK369 produced zones of inhibition against *L. delbrueckii* subsp. *lactis* 4797 (Fig. 3); however, those produced by NCK369 were significantly larger. Immunity must also be encoded by the 2.2-kb *Eco*RI fragment since lactacin F was expressed in the Laf^s transformation recipient, 89, albeit at lower levels than in 88-C. Neither 89 nor 88-C, or transformants containing the shuttle vector, pTRK159, produced any inhibitory activity against the indicator strain (data not shown).

To verify that the inhibitory activity produced by NCK368 and NCK369 was lactacin F, deferred antagonism was demonstrated against *E. faecalis* 19433, *L. delbrueckii* subsp. *lactis* 4797, *L. delbrueckii* subsp. *lactis* 970, *L. delbrueckii* subsp. *bulgaricus* 1489, *L. fermentum* 1750, *L. acidophilus* 6032, and *L. helveticus* 87. Also, the bacteriocin produced by NCK368 and NCK369 was as resistant to heat treatment (121°C, 15 min) as is lactacin F produced by the wild-type parental strain, 11088. We conclude from the hybridization, cloning, and expression experiments that lactacin F production and immunity are encoded on the 2.2-kb *Eco*RI fragment.

DNA sequence analysis of the lactacin F gene. The *Cla*I site internal to the 2.2-kb *Eco*RI fragment provided a convenient restriction site to obtain subclones of the original insert for DNA sequencing (Fig. 2). Both strands of an internal 873-bp portion of the 2.2-kb *Eco*RI fragment were sequenced, using pTRK160, pTRK163, or pTRK164 as the double-stranded template (Fig. 4A). The use of the 20-mer degenerate lactacin F probe as a sequencing primer provided sequence information from within the lactacin F structural gene and verified the position of a *Cla*I site as downstream of the putative lactacin F structural gene (Fig. 4A and B). Sequencing from pTRK163 and pTRK164 provided sequence information stemming in both directions from the *Cla*I site and confirmed the location of the lactacin F gene on the 0.9-kb

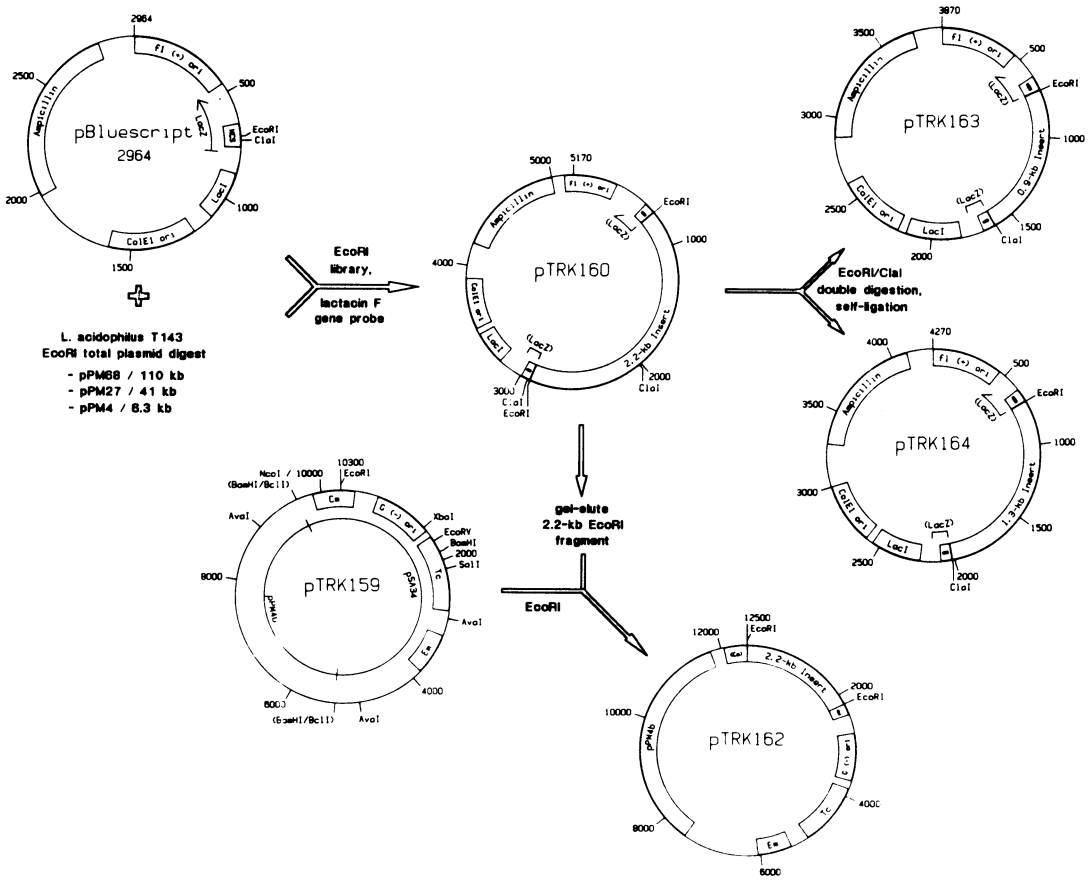

FIG. 2. Cloning strategy and plasmids derived in this study. The 63-mer lactacin F probe was used to select pTRK160 from among other putative pBluescript recombinants. Plasmids pTRK160, pTRK163, and pTRK164 were used for double-stranded sequencing. Plasmid pTRK159 is a *Lactobacillus-E. coli* shuttle vector constructed by using a cryptic replicon (pPM4b) and pSA34 (see Materials and Methods). The *laf* genes contained on the 2.2-kb *Eco*RI fragment were cloned into pTRK159. Lactacin F expression in *L. acidophilus* 89 and 88-C was evaluated after transformation with pTRK162.

*Eco*RI-*Cla*I fragment of pTRK163. Sequence-specific primers allowed sequencing of both strands, upstream and downstream, proximal to the lactacin F structural gene (Fig. 4B).

Further analysis of the sequenced region, using the computer programs Frames and TestCode (12), indicated areas which contain open reading frames (ORFs) and potential protein coding sequences, respectively (Fig. 5A and B). One ORF, identified by both the Frames and Testcode programs, is the region which correlates to the *laf* gene (Fig. 5A and B). The *laf* gene contained all 25 N-terminal amino acids of lactacin F which were identified previously through protein sequencing (34). However, the N-terminal lactacin F sequence, starting at Arg-19 (nucleotide [nt] 352), was 18 amino acids (54 bp) downstream of the translational start site of the putative lactacin F structural gene (Fig. 4A). This suggests that lactacin F results from posttranslational processing of a pre-lactacin F peptide.

Computer analyses (8, 12) of pre-lactacin F secondary structure have indicated a propensity for two extensive regions containing hydrophobic β-structures (data not shown).

DISCUSSION

This work reports the first cloning, expression, and nucleotide sequence of a gene encoding a bactericidal peptide in a *Lactobacillus* sp. Its small size (6.3 kDa, 57 amino acids) and heat stability indicate that this bacteriocin is unique relative to the much larger antimicrobial proteins (helveticin J, 37 kDa; *Clostridium* strain BCN5, 96 kDa) that have been characterized previously in gram-positive bacteria (17, 25, 29). Amino acid composition analyses of purified lactacin F did not show the presence of lanthionine or other unusual residues as found in bacteriocins now classified as lantibiotics (34). The small size of lactacin F places it among the smallest nonlantibiotic bacteriocins produced by gram-positive bacteria. Although small bacteriocins have been identified and sequenced from *E. coli* (microcins [18]; colicin V [20]), these are different from lactacin F. Microcin B17, a 3.2-kDa peptide with broad-spectrum activity among the members of the family *Enterobacteriaceae*, contains 60% Gly residues (26 of 43 amino acid residues). Lactacin F has a Gly content of 21% (12 of 57 residues) and possesses a narrow spectrum of inhibitory activity limited predominantly to other lactobacilli. Genetic studies have localized four genes essential to microcin B17 production, and three genes that confer immunity, to a continuous 5-kb region of DNA (18). Similarly, three contiguous genes required for production of colicin V (4 kDa), and one overlapping gene required for immunity, span a region of 4.4 kb (20). Of the multiple genes required for production of microcin B17 and colicin V,

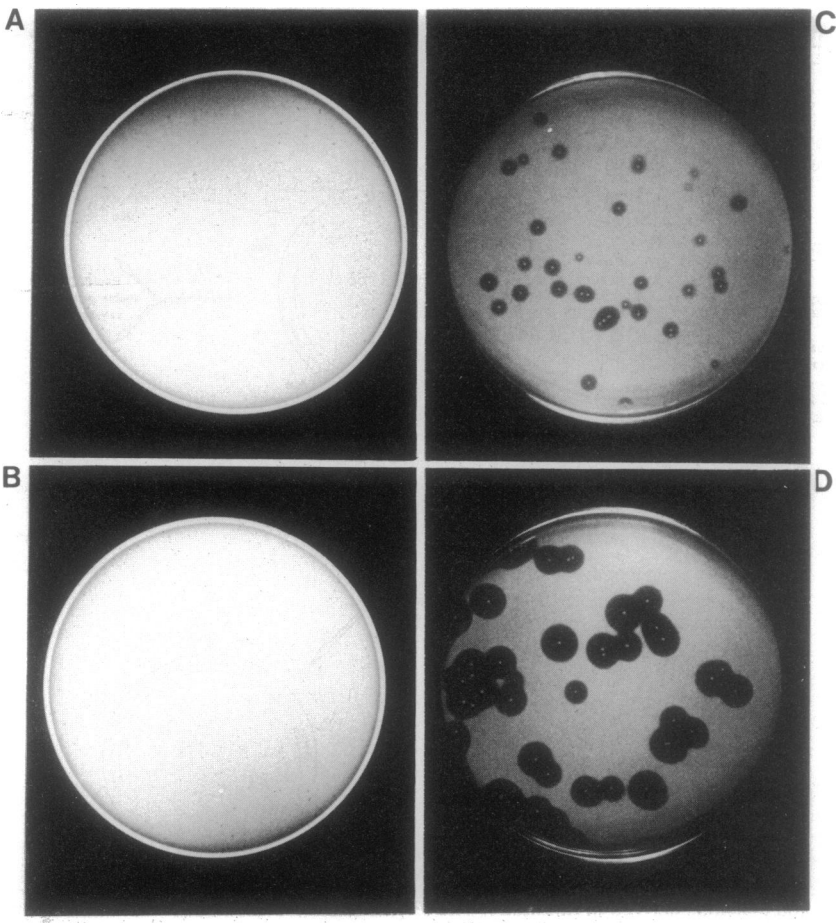

FIG. 3. *L. acidophilus* colonies overlaid with a lactacin F-sensitive indicator (*L. delbrueckii* subsp. *lactis* 4797, NCK235). (A and B) *L. acidophilus* 89 (Laf$^-$ Laf^s) and 88-C (Laf$^-$ Laf^r), respectively. (C and D) Strains 89 and 88-C transformed with pTRK162 (NCK368 and NCK369).

the smallest has been identified as the bacteriocin structural gene. Elucidation of the sequence surrounding the lactacin F structural gene shows that it is larger than either of the two adjacent downstream ORFs (Fig. 5), one of which could potentially encode for Laf immunity. Therefore, lactacin F has a different genetic organization from the antimicrobial peptides microcin B17 and colicin V.

Purification of lactacin F and identification of a partial N-terminal amino acid sequence (34) allowed us to synthesize a probe specific for the lactacin F structural gene. Although the 63-mer lactacin F gene probe hybridized to several bands in genomic digests, a single 2.2-kb *Bcl*I fragment was demonstrated in a plasmid digest from a Laf$^+$ transconjugant. Furthermore, this band was missing in a genomic digest of *L. acidophilus* 89, suggesting that this Laf$^-$ Laf^s derivative was most likely the result of a deletion in the lactacin F structural gene. In contrast, the presence of the 2.2-kb *Bcl*I band in genomic digests of *L. acidophilus* 88-C suggests that the Laf$^-$ Laf^r phenotype in 88-C arose by a point mutation or minor deletion within the *laf* region which did not affect lactacin F immunity. We believe the two additional bands exhibited in genomic digests of all of the strains, irrespective of their Laf phenotype, may represent DNA sequences having homology to part of the 63-mer gene probe, considering the occurrence of 22% incorrect bases relative to the *laf* gene sequence.

Comparison of the three translation frames with the amino acid sequence of lactacin F identified an ORF as the *laf* gene containing an N-terminal extension. The N-terminal extension of 18 amino acids appears to be proteolytically processed since it is not found in the active peptide. It is not clear, however, whether the extension is a signal sequence. Signal sequences of secreted proteins have shown considerable heterogeneity in primary sequence considering their conservation of cellular function (19). However, several features are characteristic of most signal sequences: a positively charged residue(s) at the N terminus, a central region (h-region) rich in hydrophobic or neutral amino acids forming either an α-helix or extended thread, and a C-terminal cleavage site described by the "(−3,−1)-rule" that only small, uncharged amino acids are found in positions −1 and −3 (2, 6, 45). The N-terminal portion of the lactacin F peptide loosely conforms to several of these features: a positively charged Lys residue at the N terminus, a hydrophobic core region (Leu-Ala-Val-Val-Val), and a C terminus (Val-Gly-Gly) which conforms to the (−3,−1)-rule (Fig. 4). However, computer analysis did not establish a strong correlation between this region and features of known prokaryotic signal sequences. Secondary-structure predictions of the unprocessed lactacin F sequence did indicate a propensity for α-helical structure at the N terminus followed by β-structures through the h-region, and ending in a β-turn

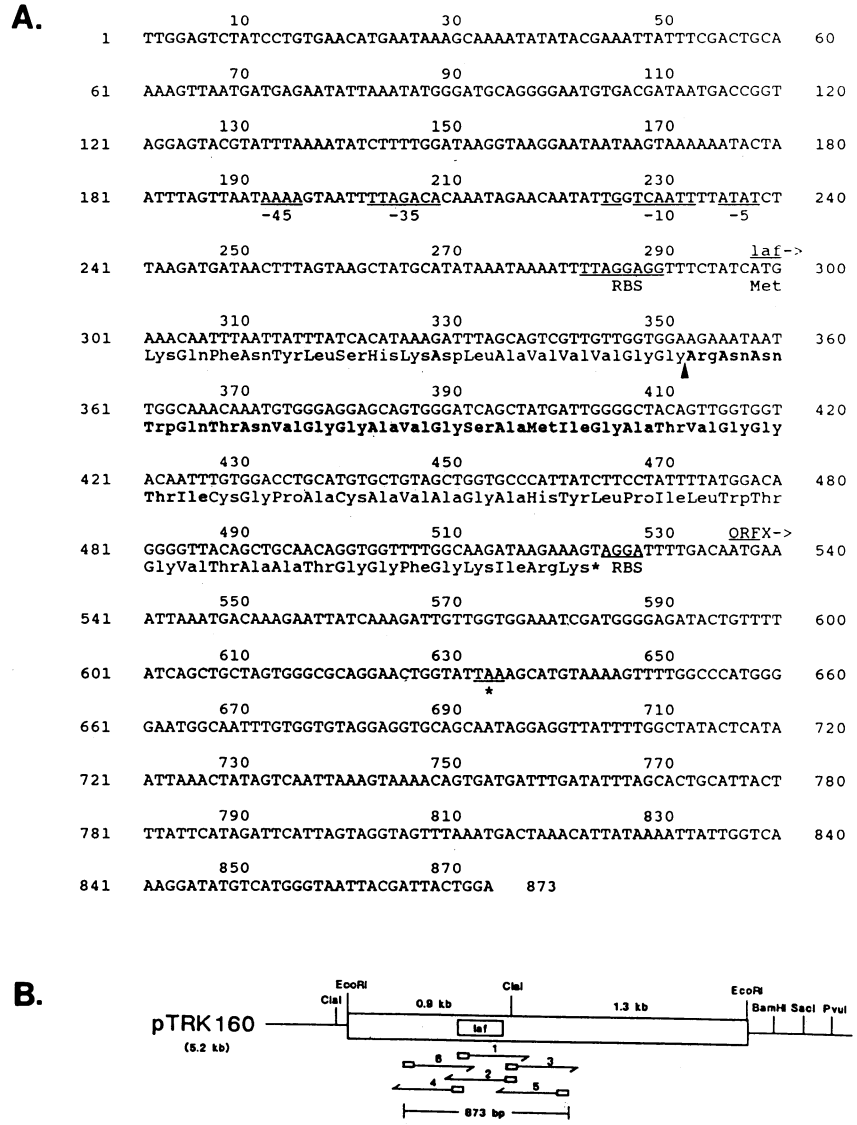

FIG. 4. DNA sequence analysis. (A) Nucleotide sequence containing the lactacin F structural gene (*laf*) and its amino acid sequence. Bold amino acids were identified by protein sequencing; the putative signal peptidase processing site is indicated (▲). The putative promoter regions (−35, −10), consensus sequences (TT, TG, A-cluster), and potential RBS are underlined. Termination codons are indicated by an asterisk. (B) Relationship of the *laf* gene and the 873-bp sequenced region relative to the 2.2-kb *Eco*RI fragment. Numbers denote progression of primers used for sequencing.

at the proposed processing site (data not shown). A β-turn at the C terminus of the prepeptide is purportedly essential in placing the cleavage site into juxtaposition with the signal peptidase (35). The extent of β-structure and small size of lactacin F may be related to its heat stability in which there is little tertiary structure to be affected by elevated temperatures. This becomes important in developing applications of bacteriocins from food grade organisms as potential preservatives in which the food product undergoes some level of heat processing.

Twenty-five consecutive N-terminal amino acids of lactacin F, identified previously by protein sequence analysis (34), are identical to those found in the *laf* gene, starting with Arg-19 (nt 352), 54 bp downstream of the proposed translational start site (Fig. 4A). The remainder of *laf*, starting with Arg-19, would thus constitute a protein of 57 amino acids. The 57-amino-acid residue portion of the *laf* gene corresponds well to composition analyses of purified lactacin F that indicated that this bacteriocin may contain as many as 56 amino acid residues (34).

Upstream regions of the *laf* gene are A+T-rich (82% A+T; nt 130 to 285) and may mask the identification of regulatory sequences (Fig. 4A). However, certain regions show similarity to consensus sequences compiled for gram-negative and gram-positive promoters. Position nt 282 contains 7 of 8 bases (TTAGGAGG) of the long Shine-Dalgarno (S-D) ribosomal binding site (RBS) sequence, TAAGGAGG (37). Although two RBS sequences have been recognized (TAAG GAGG and AAGGA), the longer S-D sequence has been found to work threefold better than the shorter one (19, 21). The putative RBS also has an optimal spacing of eight to nine nucleotides between itself and the initiation codon (40). A

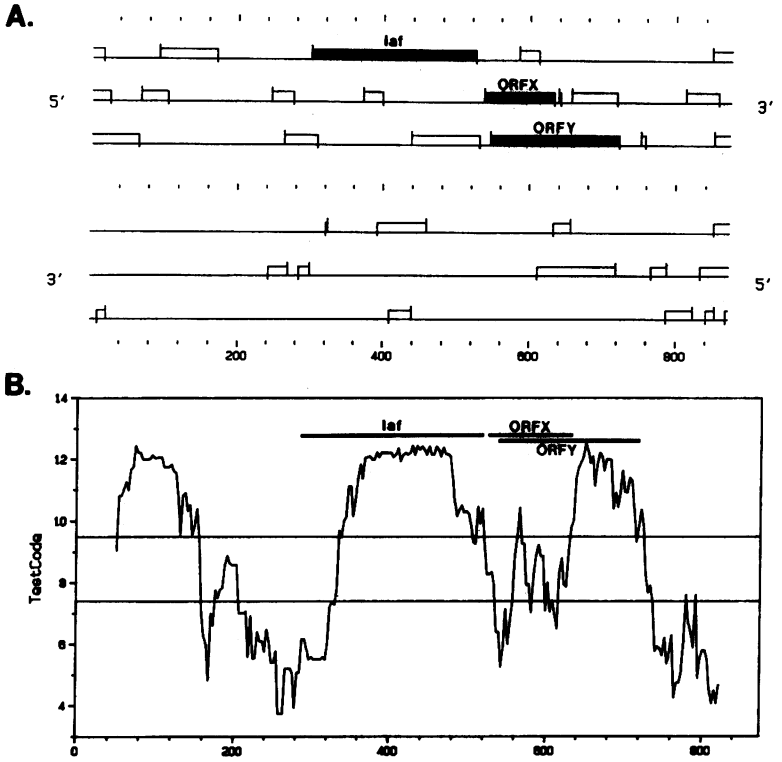

FIG. 5. Further analysis of sequenced region. (A) ORFs identified in the 873 bp of sequenced DNA containing the *laf* gene, using the Frames program (12). ORFs corresponding to the *laf* gene, ORFX, and ORFY are indicated. (B) TestCode program (12) analysis of sequence from panel A: upper area predicts protein coding regions to 95% level of confidence, and lower level predicts noncoding regions to the same level of confidence; no significant prediction is made for the middle region. The positions of the *laf* gene, ORFX, and ORFY are indicated.

putative promoter region for the −10 consensus region of *E. coli* promoters (TATAAT) (24) has been identified at position nt 227 (TCAATT; Fig. 4A). A TG sequence has also been found upstream of the −10 region and separated from it by one nucleotide. Similarly, a TG sequence separated by one nucleotide from the −10 region is strongly conserved among *Bacillus subtilis* (32), *Lactococcus cremoris* (44), and other gram-positive promoters (22). A sequence representing the canonical *E. coli* −35 region (TTGACA) (24) preceded by a TT sequence may be located at nt 203 (TTAGACA) and a cluster of A's at nt 192 (−45 region). Graves and Rabinowitz (22) have shown that a pair of T's is conserved at the −36 and −35 positions in >75% of gram-positive −35 regions and an A cluster in >50% of gram-positive −45 regions examined. In an analysis of >29 gram-positive promoter sequences, they arrived at a statistically significant consensus for gram-positive promoters (5'-TA-AAAAA--GT TGACA---A--A---A-T-TG-TATAATAATAT-3') which is inclusive of the *E. coli* consensus and is in good agreement with sequences nt 190 to 240 (Fig. 4A).

All well-characterized bacteriocin genes have demonstrated the presence of a corresponding immunity determinant that protects the producer organism against the effects of its own bacteriocin. Immunity genes described for the colicin family of bacteriocins, as well as others, have been located downstream and in close proximity to the respective bacteriocin structural genes (10). The isolation of Laf$^-$ Laf^s double mutants as well as the concomitant conjugal transfer and loss of the Laf$^+$ and Laf^r phenotypes from transconjugants (33) have indicated a close genetic association between lactacin F production and immunity determinants. In this study, hybridization analysis of Laf$^-$ Laf^s and Laf$^-$ Laf^r derivatives and cloning of the 2.2-kb *Eco*RI fragment, which expresses both lactacin F production and immunity in a Laf^s background (*L. acidophilus* 89), localized the immunity determinants within the 2.2-kb region. Improved expression of Laf by 88-C is possibly due to the inherent Laf immunity of this strain. Although the *laf* gene has been identified, an ORF corresponding to the lactacin F immunity gene remains to be defined. Computer analysis of the 873-bp sequenced region from the 2.2-kb *Eco*RI fragment for ORFs has indicated the presence of two ORFs (ORFX and ORFY) in close proximity and downstream of *laf* (Fig. 5A). ORFX appears 14 nucleotides downstream of the *laf* gene and has a putative RBS (AGGA; nt 524) with an optimal spacing of 8 nt before the start of the initiation codon (nt 536); ORFX could potentially encode for a protein of 32 amino acids (3.6 kDa). ORFY also appears downstream and adjacent to *laf*; however, an RBS close to the initiation codon is not readily apparent (Fig. 4A).

Many cytolytic and antimicrobial peptides have demonstrated a propensity to accommodate a number of structural variations that are well suited for membrane-related interactions. Protein motifs typically involved in protein-membrane interactions include amphiphilic β-sheets, amphiphilic α-helices, and uniformly hydrophobic α-helices (45). Magainin (15) and cecropin (16) have shown a predisposition for an amphiphilic helical organization, whereas gramicidin A and gramicidin S contain predominantly β-structure, forming dimeric helical coils and amphipathic β-sheets, respectively (28, 38). Computer analysis of lactacin F for secondary structure also indicated stretches of β-sheet structure. The

enhanced cidal effect exhibited by lactacin F against bacterial protoplasts and its hydrophobic partitioning during purification have implicated the cell membrane as a possible target for activity (34).

LAB have become well known for their bacteriocinogenic properties including the production of nisin, diplococcin, lactacin F, lactacin B, helveticin J, plantaricin A, pediocin A, pediocin AcH, pediocin PA-1, lactocin 27, lactocidin, and various unnamed bacteriocins (29). However, little is known of the genetic structure, regulation, or similarity of bacteriocin-related gene products among these organisms. The genetic cloning and sequencing of the nisin structural gene have verified the purported mechanism for the incorporation of lanthionine and other unusual amino acids among the lantibiotics (7, 27). The cloning, expression, and sequencing of *Lactobacillus* bacteriocin genes should further help to elucidate mechanisms of genetic regulation, antimicrobial activity, and relatedness among bacteriocins of LAB. The small size of lactacin F should be particularly amenable to site-directed alterations that could help determine the role of primary or secondary structure in posttranslational processing and inhibitory spectrum. Equally important, expression of lactacin F also localizes the position of the *laf* immunity determinants to the 2.2-kb *Eco*RI fragment, facilitating its future identification. Identification of *Lactobacillus* bacteriocin determinants should provide a greater understanding of their potential role in affording a competitive advantage to important food-related organisms, facilitate studies relating to the intestinal benefits of lactobacilli, allow the construction of positive selection vectors for LAB, and provide insights into the structure-function relationships of antimicrobial peptides.

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