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Purification and Partial Characterization of Lactacin F, a Bacteriocin Produced by *Lactobacillus acidophilus* 11088†

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Lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088 (NCK88), was purified and characterized. Lactacin F is heat stable, proteinaceous, and inhibitory to other lactobacilli as well as *Enterococcus faecalis*. The bacteriocin was isolated as a floating pellet from culture supernatants brought to 35 to 40% saturation with ammonium sulfate. Native lactacin F was sized at approximately 180 kDa by gel filtration. Column fractions having lactacin F activity were examined by electron microscopy and contained micelle-like globular particles. Purification by ammonium sulfate precipitation, gel filtration, and high-performance liquid chromatography resulted in a 474-fold increase in specific activity of lactacin F. The purified bacteriocin was identified as a 2.5-kDa peptide by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The lactacin F peptide retained activity after extraction from SDS-PAGE gel slices, confirming the identity of the 2.5-kDa peptide. Variants of NCK88 that failed to exhibit lactacin F activity did not produce the 2.5-kDa band. Sequence analysis of purified lactacin F identified 25 N-terminal amino acids containing an arginine residue at the N terminus. Composition analysis indicates that lactacin F may contain as many as 56 amino acid residues.

Lactobacilli produce a number of antimicrobial compounds including organic acids, hydrogen peroxide, and bacteriocins. Bacteriocins are proteinaceous bacterial products that have bactericidal activity (26). They are produced by various lactic acid bacteria, including lactococci, lactobacilli, and pediococci (19). Interest in *Lactobacillus acidophilus* has resulted from its popular use in acidophilus milk products as a source of dietary lactobacilli owing to its ability to colonize the human intestinal tract (18). Recent studies in our laboratory have been directed towards defining the biochemical and genetic bases of certain physiological traits of *L. acidophilus* that are projected to have an impact on the colonization and competitive abilities of lactobacilli in the intestinal tract.

Barefoot and Klaenhammer (3) found that 63% of the *L. acidophilus* strains they surveyed produced bacteriocin-like activities; two of these bacteriocins have since been characterized. *L. acidophilus* N2 was shown to produce a bacteriocin, lactacin B, inhibitory only to lactobacilli, including *L. helveticus*, *L. delbrueckii* subsp. *lactis*, and *L. delbrueckii* subsp. *bulgaricus* (4). Maximum levels of lactacin B were detected when broth cultures were maintained at pH 6.0. Lactacin B activity is unaffected by flowing steam, but is inactivated by autoclaving and protease treatment. In culture supernatants, native lactacin B was identified in association with large, 100-kDa aggregates, whereas the purified protein was estimated to be 6.2 kDa.

Lactacin F, produced by *L. acidophilus* 11088 (NCK88), is more heat resistant and exhibits a broader spectrum of activity than lactacin B, inhibiting also *L. acidophilus*, *L. fermentum*, and *Enterococcus faecalis* in addition to the other lactacin B indicators (3). Production of lactacin F is also pH dependent; maximum levels of lactacin F are obtained in MRS broth maintained at pH 7.0, whereas negligible activity is produced in fermentors held at pH 7.5 or 6.5 (22). Lactacin F is inactivated by proteinase K, subtilisin, trypsin, and ficin, stable to autoclaving (121°C, 15 lb/in^2, 15 min), and unaffected by lysozyme, lipase, and α-amylase. The availability of two *L. acidophilus* bacteriocins (lactacin B and F), one whose inhibitory spectrum is a subset of the other, presents an opportunity for direct biochemical and genetic comparisons which may identify molecular differences responsible for the variation in bactericidal activity and heat stability of these proteins. This study describes the purification of the lactacin F peptide and partial identification of its amino acid sequence.

MATERIALS AND METHODS

Bacterial cultures and media. Bacterial strains used in this study are described in Table 1. 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.). Lactobacilli were inoculated to MRS broth at the 1% level and propagated twice at 37°C before use in experiments. MRS agar was prepared by addition of 1.5% granulated agar (BBL Microbiology Systems, Cockeysville, Md.) to the broth medium; overlay agar was prepared with 0.75% granulated agar.

Bacteriocin detection and assay. Bacteriocin production by *L. acidophilus* NCK88 and N2 was detected by deferred antagonism as described previously (22). Indicator cultures for lactacin F were identified previously (3) and include *L. acidophilus* 6032, *L. delbrueckii* subsp. *bulgaricus* 1489 (formerly *L. bulgaricus* 1489), *L. fermentum* 1750, *L. helveticus* 87, *L. delbrueckii* subsp. *lactis* 970 (formerly *L. lactis* 970), *L. delbrueckii* subsp. *lactis* 4797 (formerly *L. leichmannii* 4797), and *E. faecalis* 19433 (Table 1). *L. delbrueckii* subsp. *lactis* 4797, which exhibits the most sensitivity to lactacin F, was used as the indicator strain for routine

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TABLE 1. Bacterial cultures

Strain	NCK no.[a]	Origin[b]	Relevant phenotype[c]	Reference(s)
L. acidophilus				
11088	NCK88	VPI 11088	Laf producer	22
N2[d]	NCK56	NCSU	Lab producer	3, 4
88-4	NCK65	NCSU	Laf^- Laf^s	22
88-C	NCK67	NCSU	Laf^- Laf^r	22
6032	NCK336	VPI 6032	Laf^s Lab^r	3
L. fermentum 1750	NCK127	NCDO 1750	Laf^s Lab^s	3
L. helveticus 87	NCK338	NCDO 87	Laf^s Lab^s	3
L. delbrueckii subsp. *bulgaricus* 1489	NCK231	NCDO 1489	Laf^s Lab^s	3
L. delbrueckii subsp. *lactis* 970	NCK234	NCDO 970	Laf^s Lab^s	3
L. delbrueckii subsp. *lactis* 4797	NCK235	NCDO 4797	Laf^s Lab^s	3
E. faecalis 19433	NCK337	ATCC 19433	Laf^s Lab^r	3

[a] NCK, Culture Collection of T. R. Klaenhammer, Department of Food Science, North Carolina State University, Raleigh.
[b] VPI, J. L. Johnson, Virginia Polytechnic Institute, Blacksburg; NCDO, National Collection of Dairy Organisms, National Institute for Dairying, Reading, England; NCSU, North Carolina State University, Raleigh.
[c] Laf, Lactacin F; Lab, lactacin B; Laf^-, deficient in Laf production; Laf^s, sensitive to Laf; Laf^r, resistant to Laf; Lab^s, sensitive to Lab; Lab^r, resistant to Lab.
[d] Single-colony isolate from *L. acidophilus* NCFM.

bacteriocin assays. Bacteriocin titers were determined by the serial dilution assay described previously (3); activity is defined as the reciprocal of the last serial dilution demonstrating inhibitory activity and presented as activity units (AU) per milliliter.

Bacteriocin production. Fermentor conditions for production of lactacin F by *L. acidophilus* NCK88 in MRS broth (500 ml) were described previously (22). For this study, a 5.0-liter Microferm fermentor (New Brunswick Scientific Inc., Edison, N.J.) containing 3.0 liters of MRS broth was used to produce lactacin F. The fermentor was inoculated with 30 ml of an overnight culture, maintained at 37°C and pH 7.0 (pH-controller; New Brunswick Scientific), and harvested at 10.5 h. Cells were removed by centrifugation, and the supernatant fraction was sequentially filtered through 0.45- and 0.22-μm filters to remove cellular debris. This material was designated crude lactacin F and was frozen at −20°C when not used immediately.

Ultrafiltration. Crude lactacin F preparations were subjected to membrane filtration by using a TCF10 thin-channel filtration device (Amicon Corp., Danvers, Mass.). Ultrafiltration was carried out with crude lactacin F by sequentially filtering 200 ml through membranes of decreasing pore sizes (300, 100, 50, 30, and 10 kDa). The concentrate obtained through filtration was brought to a common volume of 5 ml with 50 mM sodium phosphate buffer (pH 7.2), and titers for lactacin F activity were determined.

Ammonium sulfate precipitation. Five 100-ml aliquots of crude lactacin F (pH 7.0, 7°C) were made up to 20, 30, 40, 50, and 60% saturation by slow addition of solid ammonium sulfate (Sigma Chemical Co., St. Louis, Mo.) and held overnight at 7°C with stirring. The samples were centrifuged (11,950 × *g*, 15 min, 7°C) and supernatant fractions were decanted. Surface pellicles and bottom pellets were independently recovered and resuspended in 2 ml of 50 mM sodium phosphate buffer, pH 7.0. Lactacin F activity was titrated from the pellicle, pellet, and supernatant suspensions.

Purification of lactacin F. Lactacin F concentrated by ammonium sulfate precipitation was sized by Sephacryl S-300 (Pharmacia, Inc., Piscataway, N.J.) gel filtration chromatography, using a 146-ml column bed (1.6 by 58 cm). The running buffer contained 50 mM sodium phosphate (pH 7.2), 50 mM NaCl, and 0.04% NaN_3. Fractions containing lactacin F activity were sized by plotting the elution volume of lactacin F against a standard curve (K_{av} versus log molecular weight) of gel filtration protein standards (Pharmacia). Lactacin F fractions were concentrated with a Centricon-30 or Centricon-100 microconcentrator (Amicon) containing 30- or 100-kDa membranes, respectively. These samples were further purified by high-performance liquid reversed-phase chromatography (HPLC) on a 30-cm μBondapak C_{18} column (Millipore Corp., Milford, Mass.), using a gradient of 14 to 77% 2-propanol (Fisher Scientific, Raleigh, N.C.). Buffer A was made up as 5% (vol/vol) 2-propanol in 0.1% trifluoroacetic acid (Sigma); buffer B was composed of 90% (vol/vol) 2-propanol in 0.05% trifluoroacetic acid. The gradient consisted of a 70-min linear gradient from 90% A/10% B to 15% A/85% B. Fractions were evaporated in a vacuum centrifuge (Savant Instruments, Inc., Farmingdale, N.Y.) and resuspended in 100 to 200 μl of glass-distilled water. The column was washed after every injection with 100% glass-distilled water (0.1% trifluoroacetic acid) and 100% 2-propanol (0.1% trifluoroacetic acid) to remove extremely hydrophilic and hydrophobic substances.

Effect of surfactants and hydrolytic enzymes on lactacin F activity. Both crude and ammonium sulfate-flocculated lactacin F were treated with the detergent CHAPS, Triton X-100, Brij 35, Tween 20, Tween 80, Lubrol PX, Nonidet P-40, hexadecyltriethylammonium bromide, sodium dodecyl sulfate (SDS), glycocholic acid, deoxycholic acid, or *N*-lauroyl sarcosine at a final concentration of 1%. Controls consisted of either lactacin F or detergent, treated with 50 mM sodium phosphate buffer, pH 7.0. All samples and controls were incubated at 37°C for 6 h, and titers for lactacin F activity were determined.

After gel filtration, lactacin F was treated with various hydrolytic enzymes. Buffer suspensions for ficin, subtilisin, tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin, and proteinase K were described previously (22); α-amylase and lysozyme were suspended in 50 mM sodium phosphate buffer (pH 7.0) containing 10 mM NaCl. Lipase was suspended in 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM $CaCl_2$. Enzymes were used in the following concentrations: trypsin (tolylsulfonyl phenylalanyl chloromethyl ketone treated), 11,500 U/ml; ficin, 2 U/ml; subtilisin, 11 U/ml; proteinase K, 6 U/ml; lysozyme, 45,200 U/ml;

α-amylase, 750 U/ml; lipase, 50 U/ml. Lactacin F enzyme samples were incubated for 2 h at 37°C.

Electrophoresis. Nondenaturing polyacrylamide gel electrophoresis was carried out by the method of Davis (5) in either $5\%T/5\%C_{bis}$ (where T is total acrylamide concentration and C is cross-linker concentration), $10\%T/5\%C_{bis}$, or $15\%T/5\%C_{bis}$ slab gels. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (21) or Giulian et al. (10) in either $15\%T/2.7\%C_{bis}$ or $20\%T/0.5\%C_{bis}$ gels, respectively. Polyacrylamide gels were stained with either Coomassie blue (Sigma) or silver stain (Bio-Rad Laboratories, Richmond, Calif.). Stained gels were photo-documented by using Kodak electrophoresis duplicating paper EDP (Eastman Kodak Co., Rochester, N.Y.).

Protein sequence analysis. HPLC-purified lactacin F was analyzed by the protein sequencing facilities at North Carolina State University, Raleigh, and Duke University, Durham, N.C., on a gas-phase sequencer (Applied Biosystems, Inc., Foster City, Calif.).

Computer analysis. Computer search and sequence analysis was performed with programs contained within the Sequence Analysis Software Package (version 6.1) licensed from the Genetics Computer Group (University of Wisconsin, Madison, Wis. [8]). This package is maintained on-line at the Computer Graphics Center (North Carolina State University, Raleigh) on a VAX 11/780 computer.

RESULTS

Ultrafiltration of lactacin F. Lactacin F activity obtained from pH-controlled fermentors harvested at 10.5 h ranged from 25,600 to 51,200 activity units/ml. Nearly 100% of the lactacin F activity was retained by the 300- and 100-kDa membranes (20 and 80%, respectively) during ultrafiltration of culture supernatants. However, in comparison with lactacin F activity in the original supernatant, the total activity in the retentate increased 14-fold after ultrafiltration. These data suggest that lactacin F occurs in high-molecular-weight aggregates which can be physically disrupted to yield additional units of activity.

Effect of detergents and hydrolytic enzymes on lactacin F activity. Crude lactacin F was treated with a variety of surfactants, including nonionic, cationic, anionic, and dipolar ionic detergents. Treatment of crude lactacin F with Nonidet P-40 or SDS resulted in increases in activity of nearly 400% (Table 2). All detergent controls had negligible activity on the indicator strain, *L. delbrueckii* subsp. *lactis* 4797, except for the cationic detergent, hexadecyltriethylammonium bromide, which was highly inhibitory. Therefore, its effect on lactacin F could not be established. When first treated with ammonium sulfate, lactacin F samples did not exhibit increases in activity upon subsequent treatment with these detergents (data not shown). Therefore, treatment of crude lactacin F with either surfactants or ammonium sulfate can disperse lactacin F complexes, releasing more units of activity.

The inhibitory activity of lactacin F (gel filtration purified; see Materials and Methods and below) was eliminated with ficin, trypsin, proteinase K, or subtilisin. No loss of activity was observed when lactacin F was treated with lipase, lysozyme, α-amylase, or mutanolysin. This confirms the proteinaceous nature of lactacin F and suggests that neither lipid, carbohydrate, nor peptidoglycan moieties are critical to lactacin F activity.

Purification of lactacin F. Culture supernatants which were treated with ammonium sulfate provided three distinct phases after centrifugation: a surface pellicle, a bottom pellet, and the liquid supernatant fraction. Greater than 99% of lactacin F activity was removed from broth at a level of 40% ammonium sulfate saturation. The majority (97%) of lactacin F activity was contained in the surface pellicle, while only 3% was located in the bottom pellet. Lactacin F activity was isolated several times in succession in 40% ammonium sulfate as a means of removing extraneous proteins from the floating pellicle. Although some portion of the floating pellicle was lost during each recovery step due to its semisolid consistency, 64% of the lactacin F activity detected in the initial culture supernatant fraction was recovered after three successive washings. Ammonium sulfate flocculation provided an easy method to recover and concentrate lactacin F activity.

TABLE 2. Effect of surfactants on lactacin F activity

Surfactant	Activity (AU/ml)[a]	
	Lactacin F + treatment	Surfactant control
None	3,200	
Nonionic		
Triton X-100	3,200	0
Brij 35	6,400	0
Tween 20	6,400	0
Tween 80	6,400	0
Lubrol PX	6,400	0
Nonidet P-40	12,800	0
Anionic		
Deoxycholic acid	6,400	0
Glycocholic acid	6,400	0
N-Lauryl sarcosine	6,400	100
SDS	12,800	100
Cationic		
Hexadecyltriethyl-ammonium bromide	51,200	51,200
Dipolar ionic		
CHAPS	3,200	0

[a] Indicator strain was *L. delbrueckii* subsp. *lactis* 4797.

After repeated treatment with 40% ammonium sulfate, floating pellicles containing lactacin F activity were relatively free of most extraneous proteins as determined by SDS-PAGE analysis. Electrophoresis of lactacin F preparations on nondenaturing polyacrylamide gels (5) resulted in extensive streaking of the stainable material throughout most of the sample lanes (data not shown). Streaking was reduced significantly when identical samples were electrophoresed on denaturing gels (10). Samples of lactacin F on SDS-PAGE gels could not be stained with Coomassie dye but could be stained with silver. Silver-stained SDS-PAGE gels showed several moderately sized protein bands as well as a prominent band of 2.5 kDa (Fig. 1A, lane 1). Lactacin F activity was extracted from polyacrylamide gel slices of the 2.5-kDa band. No activity was detected from slices pertaining to other areas of the gel, providing direct evidence that the 2.5-kDa band contained lactacin F.

Lactacin F obtained by ammonium sulfate flocculation was also fractionated by Sephacryl S-300 gel filtration chromatography. The elution profile, monitored at 280 nm, demonstrated a distinct absorbance peak which coeluted with a single peak of lactacin F activity (Fig. 2). Lactacin F in this sample was estimated at approximately 180 kDa against gel filtration protein standards. Fractions representing this peak were concentrated with Centricon microcon-

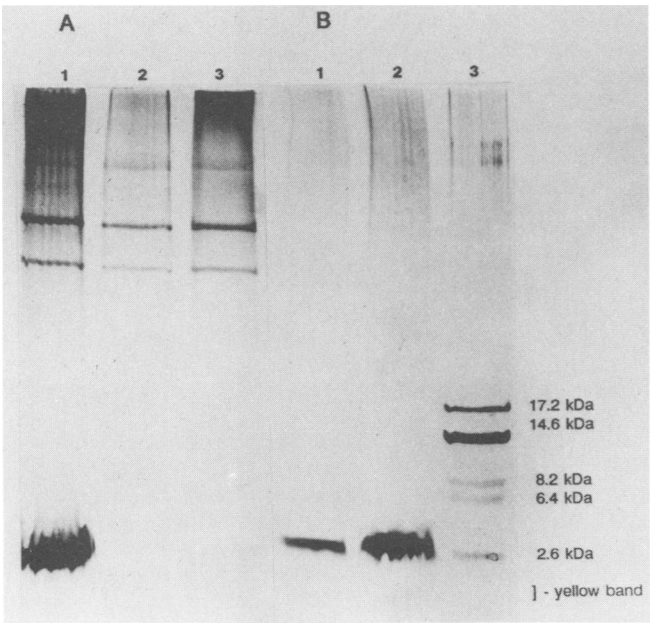

FIG. 1. Silver-stained SDS-PAGE gel of lactacin F preparations. (A) Ammonium sulfate-flocculated preparations (5 μl each): lane 1, from *L. acidophilus* NCK88 (Laf$^+$ Laf^r); lane 2, from *L. acidophilus* 88-4 (Laf$^-$ Laf^s derivative of NCK88); lane 3, from *L. acidophilus* 88-C (Laf$^-$ Laf^r derivative of NCK88). (B) Lanes 1 and 2, Gel filtration-purified lactacin F (0.27 and 0.54 μg of lactacin F); lane 3, low-molecular-weight peptide standards (17,200, 14,600, 8,240, 6,380, and 2,560).

centrators and run on SDS-PAGE gels. These samples were free of all extraneous proteins except for the 2.5-kDa lactacin F protein band and a band which stained yellow with silver stain (Fig. 1B, lanes 1 and 2). Examination of these concentrated samples by transmission electron microscopy demonstrated the presence of globular structures resembling micelles which averaged approximately 25 to 50 nm in diameter (Fig. 3).

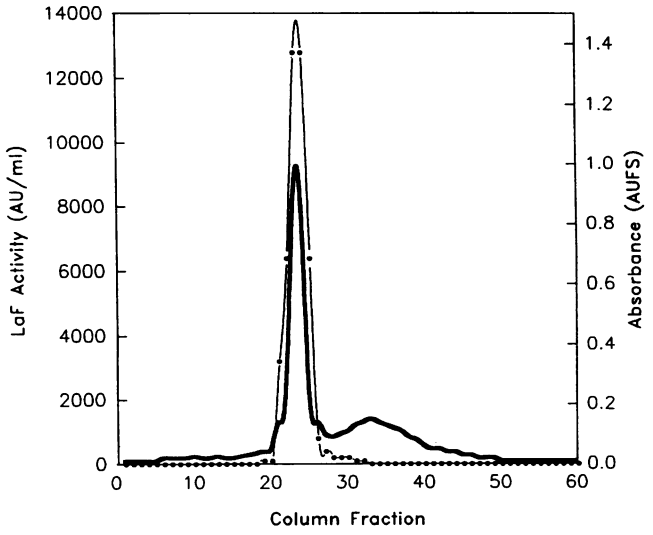

FIG. 2. Elution of lactacin F activity (●) for a Sephacryl S-300 gel filtration column monitored at 280 nm (——).

Lactacin F obtained by gel filtration was further purified by HPLC (C_{18} reversed phase, Fig. 4). Lactacin F eluted in peak 2, which was also found to contain a significant amount of protein, activity, and stainable material. UV-absorbing peaks (peaks 1, 3, 4, 5, and 6) eluting before and after lactacin F did not stain for protein, but showed the presence of a yellow band after staining with silver. SDS-PAGE gels of HPLC-isolated fractions demonstrated a 2.5-kDa band from peak 2, identified previously as lactacin F (Fig. 4B). Reconstitution of all six column fractions had no effect on lactacin F activity. Although only 1% of the original activity was recovered during the HPLC gradient, the resulting preparation yielded an additional 1.3-fold increase in specific activity over that obtained by gel filtration (Table 3).

The bulk of the sample eluted during column regeneration with 100% 2-propanol as determined by the elution of large chromatographic peaks. This fraction possessed significant lactacin F activity and yielded both the 2.5-kDa peptide and material giving a yellow reaction with silver stain on SDS-PAGE gels (data not shown). The tenacity of lactacin F samples to remain bound to the C_{18} column packing during high concentrations of 2-propanol is indicative of its hydrophobicity. Lactacin F isolated from the elution gradient was considered of greater purity because of its resolution from the yellow-staining material.

Genetic confirmation of lactacin F. To substantiate further that the 2.5-kDa band was lactacin F, we subjected culture supernatants from two lactacin F-deficient (Laf$^-$) strains, *L. acidophilus* 88-4 (Laf$^-$ Laf^s) and 88-C (Laf$^-$ Laf^r) to the purification protocol. Spent broth from these cultures produced a floating layer when treated with ammonium sulfate, but failed to show the 2.5-kDa band on SDS-PAGE gels (Fig. 1A). As expected, extracts of SDS-PAGE gel slices from lactacin F-deficient samples did not contain inhibitory activity. These data confirmed that the 2.5-kDa band purified from *L. acidophilus* NCK88 was lactacin F.

Gel filtration analysis of ammonium sulfate preparations from the Laf$^-$ strains also demonstrated the same distinct UV absorbance peak which coeluted with lactacin F activity (data not shown). Examination of column fractions from Laf$^-$ preparations representing this peak on SDS-PAGE gels only showed the presence of a yellow band on silver-stained SDS-PAGE gels (data not shown). The molecular species giving the yellow reaction with silver stain appears to be the major contributor to the UV absorbance of the peak coeluting with lactacin F activity in Fig. 2.

Protein sequence analysis. Phenylthiohydantoin-amino acid sequence analysis of HPLC-purified lactacin F identified 25 N-terminal amino acid residues (NH_2-Arg-Asn-Asn-Trp-Gln-Thr-Asn-Val-Gly-Gly-Ala-Val-Gly-Ser/Cys-Ala-Met-Ile-Gly-Ala-Thr-Val-Gly-Gly-Thr-Ile . . .). Sequence information showed that lactacin F contains an N-terminal arginine, atypical in nonprocessed gene-encoded proteins, suggesting that the purified lactacin F peptide may be the product of posttranslational processing. Amino acid composition analyses indicate that lactacin F may contain as many as 50 to 56 residues (Table 4). A computer search of the NBRF (release 21.0) protein sequence data base, using the program FASTA (23), has not identified sequences which share significant homology with the N-terminal peptide sequence of lactacin F.

DISCUSSION

During the course of this study, lactacin F was purified by ammonium sulfate precipitation, gel filtration, and HPLC

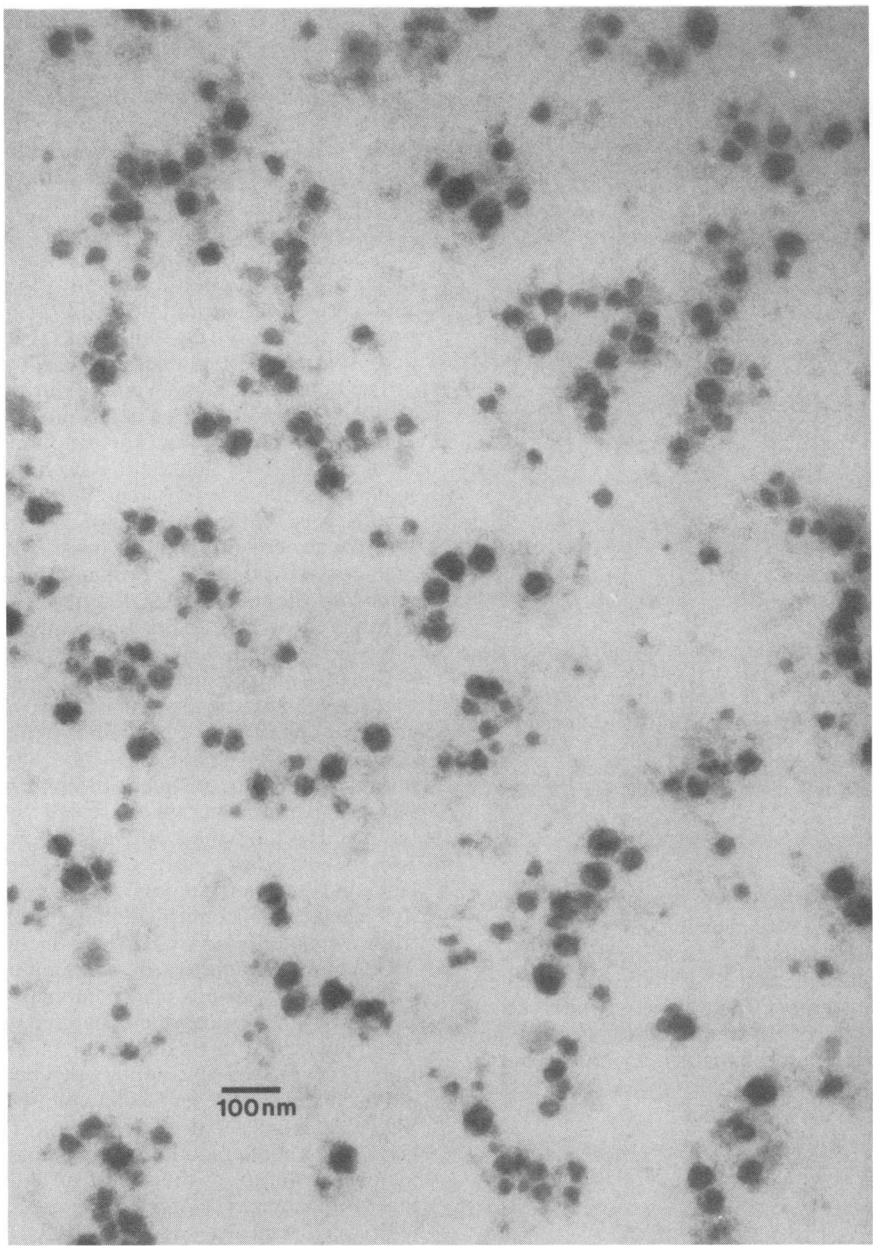

FIG. 3. Transmission electron micrograph of globular structures observed in gel filtration column fractions exhibiting lactacin F activity.

(reversed phase). Observations made during ultrafiltration, surfactant and ammonium sulfate treatment, and gel filtration indicated that native lactacin F is associated with a large bacteriocin complex. Purified lactacin F was identified as a 2.5-kDa peptide by SDS-PAGE, in sharp contrast to the size indicated by ultrafiltration and gel filtration and confirming its involvement with macromolecular complexes. However, amino acid composition analysis indicated that lactacin F may be as large as 6.2 kDa (56 amino acids). Sequence analysis further identified 25 N-terminal amino acid residues which should facilitate genetic cloning of the lactacin F structural gene.

Treatment of culture supernatants of *L. acidophilus* N2, containing lactacin B, with ammonium sulfate also provided full recovery of bacteriocin activity from broth as a floating pellicle. This compared favorably with the 3% recovery of lactacin B activity from culture supernatant fractions by batch ion-exchange separation (4). The methods developed herein may, therefore, prove advantageous in the purification of other bacteriocins produced by lactic acid bacteria.

The association of lactacin F with a "bacteriocin complex" is indicated by various characteristics displayed by lactacin F. Lactacin F activity increased when culture supernatants were subjected to rigorous treatment by thin-channel ultrafiltration or when treated with certain detergents. Nondenatured lactacin F (180 kDa) is significantly larger than the purified bacteriocin observed under denaturing conditions (2.5 kDa). Efforts to characterize and purify bacteriocins produced by lactobacilli have shown that they typically occur as large macromolecular complexes. Purifi-

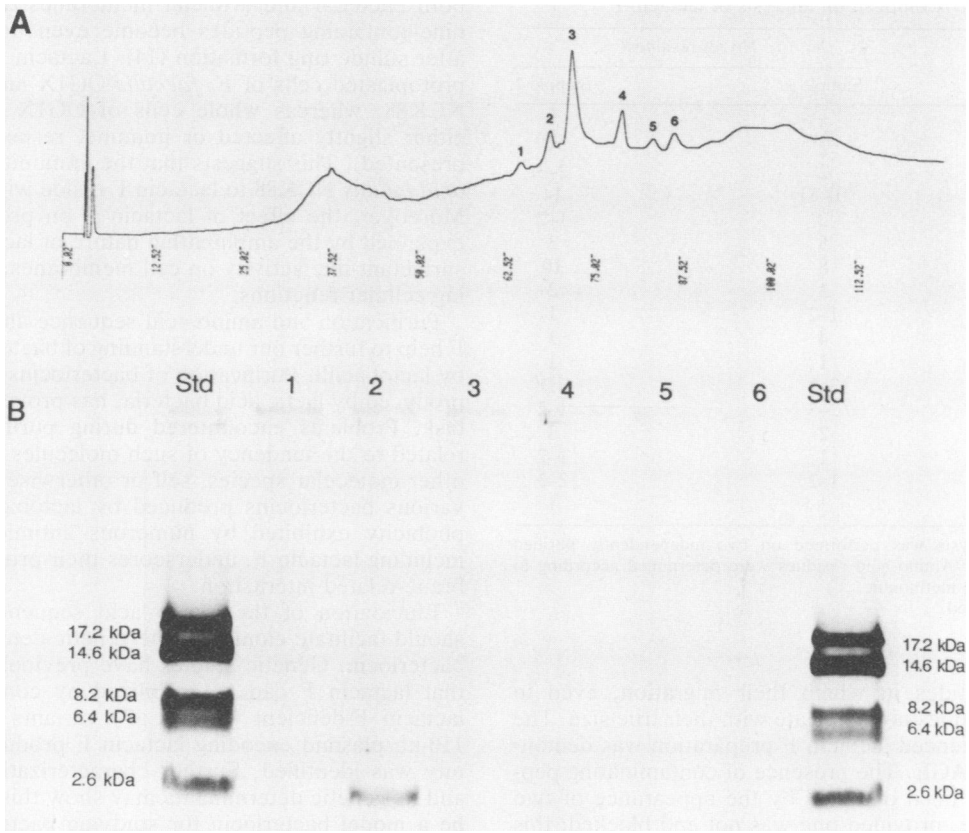

FIG. 4. Purification of lactacin F. (A) HPLC chromatogram (C_{18} reversed phase) of gel filtration-purified lactacin F. (B) Silver-stained SDS-PAGE gel of selected peak fractions from panel A. Lane numbers reflect numbered peaks in panel A. End lanes are peptide molecular weight standards as described in the legend to Fig. 1.

cation of lactacin B and helveticin J from such complexes produced by *L. acidophilus* N2 (4) and *L. helveticus* 481 (13) have identified bactericidal agents as 6- and 37-kDa proteins, respectively. Although the nature of the lactacin B and helveticin J bacteriocin complexes have not been identified, those produced by *L. fermenti* (7) and *L. helveticus* LP27 (27) have been shown to result from associations with lipid and carbohydrate. Lactacin F may similarly associate with other molecular species to form high-molecular-weight complexes. Yellow silver stain reactions, as observed for some lactacin F samples, have been shown to occur for certain lipids and sialoglycoproteins (6, 9).

Electron microscopic analysis of lactacin F samples obtained by gel filtration showed the appearance of globular, micelle-like particles. This is the first report of such bacteriocin-related structures among lactobacilli and is consistent with the observation of large macromolecular bacteriocin complexes. Similar structures have also been noticed in association with other antimicrobial peptides. Katsu et al. (15) have observed that accumulation of gramicidin S by *Bacillus brevis* causes membrane deformation, leading to the liberation of gramicidin-containing membrane fragments and increased membrane permeability. They indicated that such liberated structures could form mixed micelles, vesicles, or nonspecific clusters. In a similar fashion, a bacteriocin produced by *Bacteroides uniformis* TI-1 has been shown to be associated with membrane vesicles released by bleb formation from producer cells (2).

Amino acid sequence analysis of purified lactacin F has identified 25 N-terminal amino acid residues. Although purified lactacin F demonstrated an SDS-PAGE-determined molecular weight of 2,500 (Fig. 4), composition analyses indicate that lactacin F may be as large as 56 residues (Table 4). This size discrepancy is frequently observed with small,

TABLE 3. Purification of lactacin F

Sample	Vol (ml)	Lactacin F activity (AU/ml)	Total activity (AU)	Protein concn (mg/ml)	Total protein (mg)	Sp act (AU/mg)	Activity recovered (%)	Fold purification
Culture supernatant	3,000.0	19,200	57,600,000	33.75	101,250.0	569	100	1
3× ammonium sulfate precipitate	30.0	1,228,800	36,864,000	6.75	202.5	182,044	64	320
Gel filtration (S-300)	7.2	3,276,800	23,592,960	15.63	112.5	209,715	41	369
HPLC (reversed phase-C_{18})	0.72	32,000	23,040	0.12	0.09	269,474	0.04	474

TABLE 4. Composition analysis of lactacin F

Amino acid	No. of residues[a]	
	Sample 1	Sample 2
Asp	3	3
Glu	2	3
Ser	2	2–3
Gly	10–11	14
Arg	2	1–2
Thr	5	3
Ala	8	10
Pro	3	2
Tyr	1–2	1
Val	4	3
Met	1	1
Cys	2–3	ND[b]
Ile	2–3	1–2
Leu	2	3
Phe	1	1–2
Lys	1–2	2–3
His	1	1

[a] Composition analysis was performed on two independently purified samples of lactacin F. Amino acid residues were determined according to molar ratios relative to methionine.
[b] ND, Not determined.

hydrophobic peptides in which their migration, even in SDS-PAGE gels, does not correlate with their true size. The purity of the sequenced lactacin F preparation was demonstrated by SDS-PAGE. The presence of contaminating peptides would have been detected by the appearance of two residues per cycle, provided one was not end blocked; this was not observed.

Unusual amino acids were not observed during the partial sequencing of lactacin F. Bacteriocins such as nisin (12), subtilin (11), epidermin (1), PEP5 (17), and gallidermin (16) have been shown to contain unusual amino acids such as lanthionine, for which the common name lantibiotic has been proposed (24). Incorporation of a lanthionine residue introduces a monosulfur bridge which results in unique peptide ring structures among the various lantibiotics. Such ring-forming amino acids would result in blank cycles with the Edman degradation reaction used during amino acid sequencing (16, 17). No problems were encountered during amino acid sequencing of the first 25 residues of lactacin F, indicating that lanthionine-type amino acids are not present in this portion of the sequence. However, the possibility of lanthionine-type amino acids occurring among subsequent residues, imposing a structural constraint on lactacin F, would help explain the size discrepancy observed between SDS-PAGE gels and amino acid composition analysis.

Comparison of the N-terminal sequence of lactacin F with those contained in the NBRF Protein Sequence Database did not identify sequences having significant homology with lactacin F. Peptide structure analysis indicates a significant stretch of hydrophobic moment extending from residues 9 through 25 of the Laf peptide, whereas the extreme N-terminal portion of the Laf peptide demonstrates hydrophilic tendencies. These hydrophobic predictions may explain the flocculation observed upon treatment with ammonium sulfate as well as the tendency to "stick" to the hydrophobic C_{18} HPLC stationary phase.

Many antimicrobial peptides exhibit a fair degree of hydrophobicity which can promote interaction with cell membranes. Nisin, PEP5, and subtilin have been shown to impose their bactericidal effect by the formation of pores in both bacterial and artificial membranes (20, 25). Lanthionine-containing peptides become even more hydrophobic after sulfide ring formation (14). Lactacin F is inhibitory to protoplasted cells of *E. faecalis* OG1X and *L. acidophilus* NCK88, whereas whole cells of OG1X and NCK88 are either slightly affected or immune, respectively (data not presented). This suggests that the immunity functions of *L. acidophilus* NCK88 to lactacin F reside within the cell wall. Moreover, the effect of lactacin F on protoplasts may be explained by the amphipathic nature of lactacin F, allowing surfactant-like activity on cell membranes, thereby disrupting cellular functions.

Purification and amino acid sequence analysis of lactacin F help to further our understanding of bacteriocins produced by lactobacilli. Purification of bacteriocins, especially those produced by lactic acid bacteria, has proven to be a difficult task. Problems encountered during purification could be related to the tendency of such molecules to associate with other molecular species, self or otherwise, as indicated for various bacteriocins produced by lactobacilli. The hydrophobicity exhibited by numerous antimicrobial peptides, including lactacin F, underscores their propensity for membrane-related interaction.

Elucidation of the amino acid sequence of lactacin F should facilitate cloning of the genetic determinants for this bacteriocin. Genetic studies have previously demonstrated that lactacin F can be mobilized by conjugal transfer to lactacin F-deficient (Laf^- Laf^s) variants (22) in which a 110-kb plasmid encoding lactacin F production and immunity was identified. Further characterization of lactacin F and its genetic determinants may show this small peptide to be a model bacteriocin for studying bacteriocin structure-function relationships, host-range interactions, and the physiology of bacteriocin production and immunity among the *Lactobacillaceae*.

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