ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1984, p. 328–334
0066-4804/84/090328-07$02.00/0
Copyright © 1984, American Society for Microbiology
Vol. 26, No. 3

Purification and Characterization of the *Lactobacillus acidophilus* Bacteriocin Lactacin B†

SUSAN F. BAREFOOT‡ AND TODD R. KLAENHAMMER*

Department of Food Science, North Carolina State University, Raleigh, North Carolina 27695-7624

Received 26 March 1984/Accepted 14 June 1984

Parameters for production and purification of a bacteriocin produced by *Lactobacillus acidophilus* N2 are described. Production of lactacin B was pH dependent, with maximum activity detected in broth cultures maintained at pH 6. Lactacin B was purified by ion-exchange chromatography, ultrafiltration, and successive gel filtrations in the presence of 8 M urea and then 0.1% sodium dodecyl sulfate. The molecular weight of lactacin B was ca. 6,000 to 6,500, and the purified compound showed maximum absorbance at 211 nm. The activity of purified lactacin B was bactericidal to sensitive cells and restricted to members of the family *Lactobacilliaceae*, *L. leichmannii*, *L. bulgaricus*, *L. helveticus*, and *L. lactis*. Characteristics identified for lactacin B indicated that it was a peptide and confirmed its identity as a bacteriocin.

Bacteriocins are antagonistic proteins or peptides that show bactericidal activity against closely related species (23). Although numerous bacteriocins from other bacterial genera have been isolated and characterized (10, 18, 20, 27), few have been studied extensively in the lactobacilli. DeKlerk and co-workers (3, 4, 5) and Upreti and Hinsdill (24, 25) identified and characterized bacteriocins produced by *Lactobacillus fermentum* and *L. helveticus*, respectively. In crude form both compounds were nondialyzable, heat stable, insensitive to catalase, sensitive to proteolytic enzymes, and inhibited only members of the family *Lactobacilliaceae*. Upon subsequent purification, both bacteriocins were identified as large lipopolysaccharide protein complexes of ca. 200,000 in molecular weight. Because the *L. fermenti* bacteriocin could not be dissociated without loss of activity, DeKlerk and Smit (5) concluded that the complex molecule was essential for activity. Chromatography of the *L. helveticus* bacteriocin in the presence of sodium dodecyl sulfate (SDS) showed that the active moiety was a glycoprotein with a molecular weight of 12,400 (24).

We reported previously that a bacteriocin-like antagonist was produced when *L. acidophilus* N2 was propagated anaerobically in agar cultures (1). Crude preparations of lactacin B were stable at 100°C for 60 min, insensitive to catalase, sensitive to protease, and bactericidal to sensitive species. In contrast to recent reports of bacteriocin-type compounds that are produced by lactobacilli and antagonize *Clostridium* (13), *Neisseria* (16), and various other gram-positive and gram-negative species (14), the inhibitory spectrum of lactacin B was limited to members of the family *Lactobacilliaceae*. However, sensitivity to lactacin B was observed in four species, including *L. leichmannii*, *L. bulgaricus*, *L. helveticus*, and *L. lactis*, raising the possibility that more than one bacteriocin was present in crude culture extracts of *L. acidophilus* N2 (1).

This study describes the production and purification of lactacin B. Mutation analyses and studies of the purified compound showed that lactacin B was a peptide with bactericidal activity against the four indicator species.

MATERIALS AND METHODS

Cultures and media. The bacteriocin producer *L. acidophilus* N2, the sensitive indicator *L. leichmannii* 4797, and all other cultures used in this study were described previously (1). Lactobacilli were maintained as frozen stocks at −76°C (9) and propagated twice (1% inoculum) through MRS broth (Difco Laboratories, Detroit, Mich.) for 14 to 16 h at 37°C before experimental use.

Liquid media used were either MRS broth or a semi-defined medium. The basal semi-defined medium included (per liter of solution) Casitone (Difco), 10 g; sodium acetate, 5 g; yeast extract (BBL Microbiology Systems, Cockeysville, Md.), 5 g; glucose, 10 g; K_2HPO_4, 2 g; Tween 80 (Sigma Chemical Co., St. Louis, Mo.), 1 g; $MgSO_4 \cdot 7H_2O$, 80 mg; $FeSO_4 \cdot 7H_2O$, 4 mg; $MnCl_2 \cdot 4H_2O$, 80 mg; it was sterilized at 121°C for 15 min. The remaining ingredients were prepared in stock solutions, filter sterilized, and added aseptically in the following proportions (per liter of solution): xanthine, 10 mg; guanine, 10 mg; adenine sulfate, 10 mg; uracil, 10 mg; L-asparagine, 100 mg; L-glutamine, 100 mg; riboflavin, 1 mg; *o*-panthothenic acid, 1 mg; nicotinic acid, 1 mg; pyridoxal hydrochloride, 1.2 mg; and thiamine hydrochloride, 0.1 mg. The final pH of the medium was adjusted to 6.0 (7, 17). Agar media were prepared as previously described (1) by the addition of either 1.5 or 0.75% granulated agar (BBL Microbiology Systems) to MRS broth.

Production studies at controlled pH. A 1,500-ml working volume of broth medium was placed in a sterile Multigen bench-top fermentation system (New Brunswick Scientific Co., Inc., Edison, N.J.) connected to an automatic pH controller (New Brunswick Scientific). The temperature was maintained at 37°C and the cultures were agitated at 150 rpm. For media at pH 4.7, 5.0, 5.4, and 6.0, the initial pH was adjusted with concentrated hydrochloric acid. An ammonium hydroxide solution (12%; 346 ml of 30% ammonium hydroxide in 654 ml of sterile distilled water) was used to adjust the media at pH 6.6 and 7.0 and to control the pH. A 12-h broth culture of *L. acidophilus* N2 was inoculated (0.1%), and at 2-h intervals, 25-ml samples were removed for viable counts and activity assays. For each sample, dilutions

* Corresponding author.
† Paper no. 9248 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC 27650.
‡ Present address: Department of Food Science, Clemson University, Clemson, SC 29631.

were prepared and plated on MRS agar by the Spiral Systems technique (Spiral Systems, Inc., Bethesda, Md.) as described previously (29). Samples to be assayed for lactacin activity were filtered to remove producer cells (0.45 μm, Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.) and stored at −20°C.

Activity assay and spectrum, bactericidal action, and adsorption. Lactacin B activity was assayed by a critical dilution method described previously (1). Controls for all assays included examination of dilution buffers for inhibition of the lawn of *L. leichmannii* 4797 cells. Activity against other indicator cultures (Table 2) was determined by spotting 20 μl of purified lactacin B (8 or 512 AU/ml) in 0.1 M acetate buffer, pH 5, containing 0.1% SDS (Bio-Rad Laboratories, Rockville, Centre, N.Y.) on cell lawns prepared as described previously (1). Application of buffer without lactacin B did not affect the growth of any indicator.

Bactericidal action was determined as described previously (1) except that purified lactacin B was added to sterile 1 mM phosphate buffer, pH 7, and the initial cell population of *L. leichmannii* 4797 added to the cuvettes was 3.1×10^8 CFU/ml.

Production and purification of lactacin B. Culture supernatants containing lactacin B were prepared as follows. A 20-ml sample of a 13-h culture of *L. acidophilus* N2 was added to 20 liters of sterile semi-defined medium in a Microferm fermentation system (New Brunswick Scientific) maintained at pH 6 and 37°C. After 12 h, the culture was adjusted to pH 5 with hydrochloric acid. Cells were removed by continuous centrifugation (Szent, Gyorgi and Blum continuous flow system; Ivan Sorvall, Inc., Sorwalk, Conn.), and the supernatant portion was filtered through a 0.45-μm membrane filter and chilled to 4°C.

(i) Ion-exchange chromatography. Two ion-exchange procedures were used for initial purification. For column chromatography, culture supernatants (100 ml) containing lactacin B activity were applied to a 100-ml column (2-cm interior diameter) of carboxymethyl Sephadex C-50 (Sigma) equilibrated with 0.05 M sodium acetate buffer, pH 5, at 4°C. The column was washed with 0.073 M sodium acetate buffer, pH 5, until the absorbance at 280 nm returned to 0. Activity was eluted by application of a linear salt gradient (0 to 0.6 M NaCl, 650 ml) in the same buffer. Fractions (2 ml) were collected, and their refractive indices and lactacin B activity were determined. Gradient concentrations were verified by comparison of the refractive indices with those on an experimentally determined standard curve.

For ion-exchange chromatography of larger volumes, ca. 500 ml of carboxymethyl Sephadex, equilibrated as described above, was stirred into culture supernatants (4 liters) containing lactacin B and held at 4°C for 4 h with occasional mixing. The resin was collected on a Miracloth-lined Buchner funnel, washed with 0.073 M sodium acetate buffer, pH 5.0, containing 0.25 M NaCl (3× resin volume), and then with the same buffer containing 0.35 M NaCl (2× resin volume). Activity was eluted with 0.8 M NaCl (3× resin volume) and concentrated by ultrafiltration with a YM 10 Diaflo membrane (Amicon Corp., Lexington, Mass.) with a molecular exclusion limit of 10^4.

(ii) Ultrafiltration and gel chromatography. Dissociating conditions were used in subsequent purification procedures. Urea (Ultrapure; Schwarz/Mann, Orangeburg, N.Y.) was added to the concentrated eluent obtained as described above to a final concentration of 8 M. This solution was passed through an XM50 Diaflo membrane (Amicon) to remove large-molecular-weight contaminants, diluted with 3 volumes of 0.1 M Tris–0.1% SDS buffer, pH 8.6, and concentrated by ultrafiltration with a YM 10 membrane. The concentrate was applied to a 44-ml Sephadex G-75 column (1.5-cm interior diameter; Pharmacia Fine Chemicals, Piscataway, N.J.) packed, and equilibrated with 0.1 M Tris, pH 8.6, containing 8 M urea. Activity was eluted at 7.2 ml/h with the same buffer, and the eluent was monitored for absorbance at 280 nm. Fractions (1.2 ml) were collected in tubes containing 1 ml of 0.1 M Tris–0.1% SDS buffer, pH 8.6. Active fractions were pooled, dialyzed in no. 3 Spectrapor tubing (Spectrum Medical Industries, Los Angeles, Calif.) against 0.1 M sodium acetate–0.1% SDS, pH 5, and concentrated with polyethylene glycol (Carbowax PEG 20,000; Fisher Scientific Co., Raleigh, N.C.) Purified lactacin B was obtained by chromatographing this solution three times on a 67-ml column of Sephacryl S-200 (1.5-cm interior diameter; Pharmacia) equilibrated in 0.1 M acetate–0.1% SDS buffer. Active fractions were stored in sterile screw-capped plastic vials at room temperature.

Protein determinations. Protein concentrations were determined by the method of Lowry et al. (12) as modified by Peterson (19) or by the ratio of absorbance at 215 and 225 nm described by Waddell (26) and reappraised by Wolf (28). For these methods, bovine serum albumin (Mann Research Labs Inc., New York, N.Y.) was used as a standard. In some instances protein was estimated by the ratio of absorbance at 235 and 280 nm, as described by Whitaker and Granum (27).

PAGE. Polyacrylamide gel electrophoresis (PAGE) in the presence of SDS (0.1%) was conducted in a vertical slab gel (1 mm) by the method of Laemmli (11). Polyacrylamide and *N,N'*-methylenebisacrylamide (Sigma) concentrations in the stacking gel (5.6 ml) were 5.7 and 0.15%, respectively, and in the separating gel (17 ml) they were 20 and 0.5%. PAGE was conducted at a constant current of 50 mA per gel for 3.5 h. Gels were removed and sliced longitudinally, and activity determinations were done on replicate lanes. The remaining portions of the gel were stained with silver stain (Bio-Rad) by the method of Merril et al. (15). Protein standards and their molecular weights included the following: phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400 (Bio-Rad); and bovine trypsin inhibitor, 6,200 (Sigma).

For activity determinations, the gels were sliced with a gel slicer (Bio-Rad) into 2-mm sections. Each section was placed into a tube containing 0.4 ml of 0.2 M Tris–0.1% SDS, pH 8.6, and held at 20°C overnight to allow elution of activity into the buffer. Buffer (20 μl) was spotted onto cell lawns of the sensitive indicator, *L. leichmannii* 4797. After 12 to 14 h at 37°C in a CO_2 atmosphere (0.4 liters/h), these lawns were examined for inhibition. Buffer alone or buffer containing polyacrylamide gel slices in the absence of lactacin B did not inhibit growth of the lawn.

Sensitivity to proteolytic enzymes. Lactacin B obtained after ion-exchange chromatography was dialyzed against 0.2 M Tris (pH 7)–5 mM $CaCl_2$–0.1% SDS and then treated with 50 or 500 μg of proteinase K per ml (MC/B Manufacturing, Cincinnati, Ohio). Separate aliquots without enzyme and with inactivated proteinase K (100°C, 10 min) were included as controls. All samples were filter sterilized and held at 37°C for 30 min. Proteolysis was arrested by heating at 100°C for 3 min. Residual lactacin B activity for each sample was then assayed. Neither proteinase K (active or inactive) nor the buffer inhibited the growth of the indicator lawn.

Mutagenesis and mutant screening. To generate mutants lacking lactacin B activity, cells of a 6-h MRS broth culture

of *L. acidophilus* N2 were washed in sterile 0.1 M citrate buffer, pH 5.5, suspended in citrate buffer containing 500 or 1,000 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma) per ml at 1.8×10^8 CFU/ml, and maintained at 37°C for 90 min. Treated cells were washed in sterile 0.3 mM phosphate buffer, pH 7, sonicated for 7 s at 5,000 cycles per s (model S110; Branson Sonic Power Co., Danbury, Conn.), and plated on MRS agar by a spiral systems method as described previously (29). After 48 h at 37°C in an anaerobic glove box (Coy Laboratory Systems, Ann Arbor, Mich.), isolated colonies were picked onto 96-well microtiter plates containing 0.2 ml of MRS agar per well. After 48 h at 37°C under anaerobic conditions, these plates were stored at 4°C and used as masters for subsequent mutant screening.

Isolates were examined for inhibition of *L. leichmannii* 4797, *L. lactis* 970, *L. helveticus* 87, and *L. bulgaricus* 1489. Isolates were transferred to the dry surface of sterile MRS agar plates (15 by 150 mm) with a multiple inoculation device. The plates were overlaid immediately with sterile MRS agar (20 ml). After 24 h at 37°C under anaerobic conditions, the plates were overlaid with 10 ml of MRS agar (0.75%) containing 0.25 ml of a 10^{-1} dilution of an overnight MRS broth culture of the desired indicator. After development of the cell lawn, isolates were examined for surrounding zones of inhibition. Isolates lacking inhibitory activity were purified and examined for activity against all four indicators by deferred antagonism methods (1).

The carbohydrate fermentation patterns, hydrolysis of esculin, Gram stain reactions, inability to grow at 15°C, and lack of catalase exhibited by mutants isolated in this study were identical to those of the parent culture, *L. acidophilus* N2 (1).

RESULTS

Production of lactacin B. To optimize the activity of lactacin B, the relationship of the medium pH to production by *L. acidophilus* N2 was examined. When the producer was cultivated on sterile MRS agar previously adjusted to pH 5.9 or above, the culture extracts contained lactacin activity (1 AU/ml at pH 5.9). As the initial agar pH was increased to 7.0, the activity in the extracts increased (32 and 64 AU/ml at pH 6.5 and 7, respectively). If the initial agar pH was below 5.9, no activity was detected. Absence of activity was not due to lack of producer growth since confluent growth occurred at all initial pH values. Production of lactacin B was dependent on initial agar pH, suggesting that manipulation of pH in broth media might facilitate production of lactacin B.

To determine the optimal broth conditions for production of lactacin B, cultures in MRS broth were examined under controlled pH conditions (Fig. 1). Little or no activity was detected at pH 6.6, 5.4, or 5.0. However, significant activity was detected at pH 6.0 during the early stationary phase of growth. Titers of activity were highest at 10 h but decreased by 75% within 4 h. This decrease was not due to interaction with producer cells since activity in filter-sterilized portions held at pH 6 and 37°C declined similarly. Adjustment of the culture to pH 5 stabilized activity at 37°C (data not shown).

Purification of lactacin B. Initial attempts to purify lactacin B from MRS broth culture supernatants were unsatisfactory. In relation to the high content of proteins and peptides in the medium, lactacin B was produced in very low concentrations (ca. 5 mg/liter). Application of ion-exchange procedures resulted in only 70-fold purification, and further resolution was complicated by coelution of lactacin B with MRS constituents during gel filtration (data not shown). Therefore, purification of lactacin B was conducted by using supernatant portions of broth cultures prepared in the semidefined medium. Use of this medium resulted in growth and lactacin B production at pH 6 comparable to MRS cultures, but eliminated excessive levels of medium contaminants.

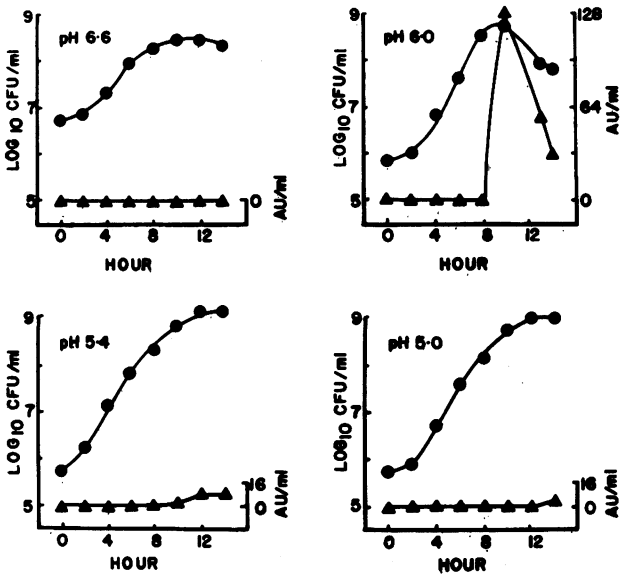

FIG. 1. Producer growth and production of lactacin B at constant pH. *L. acidophilus* N2 was propagated in sterile MRS broth in a fermenter system held at 37°C with continuous agitation. Symbols: ●, CFU per milliliter; ▲, lactacin B activity.

In preliminary experiments, lactacin B adsorbed to carboxymethyl Sephadex at pH 6. Therefore, supernatants containing lactacin B activity were subjected to column ion-exchange chromatography at pH 5 (Fig. 2). Under these conditions, contaminating protein eluted as a peak of absorbance between 0 and 0.2 M NaCl. Lactacin activity eluted as a single peak in the absence of detectable protein absorbance at 0.4 to 0.5 M NaCl. Batch ion-exchange separation (Table 1) resulted in considerable purification but also in a 97% loss in activity due, in part, to incomplete adsorption of activity from culture supernatants and incomplete desorption from the resin during the batch ion-exchange process (data not shown). Subsequent concentration by ultrafiltration resulted in a solution containing minimal activity (32 to 128 AU/ml) and a white precipitate. This suggested that lactacin B had either been denatured during the ion-exchange procedures or was not soluble in the acetate buffer system at high concentrations. Attempts were made to solubilize the precipitate. Addition of 8 M urea resulted in complete dissolution of the precipitate, with formation of a clear solution and a 200-fold increase in measurable activity. Neither 0.1% SDS nor 8 M urea had a detectable effect on the assay lawns.

Since lactacin B retained activity in the presence of protein-denaturing agents, we attempted to purify it by using dissociating conditions for ultrafiltration and gel chromatography. In a previous study, lactacin B was retained on a membrane with molecular exclusion limits of 100,000 (1). In this study solubilization of lactacin B in 8 M urea before ultrafiltration resulted in its passage through a membrane with molecular exclusion limits of 50,000 and a 10-fold increase in purification with full retention of activity (Table 1). Inclusion of 8 M urea during chromatography of lactacin B on Sephadex G-75 resulted in elution of all activity in a

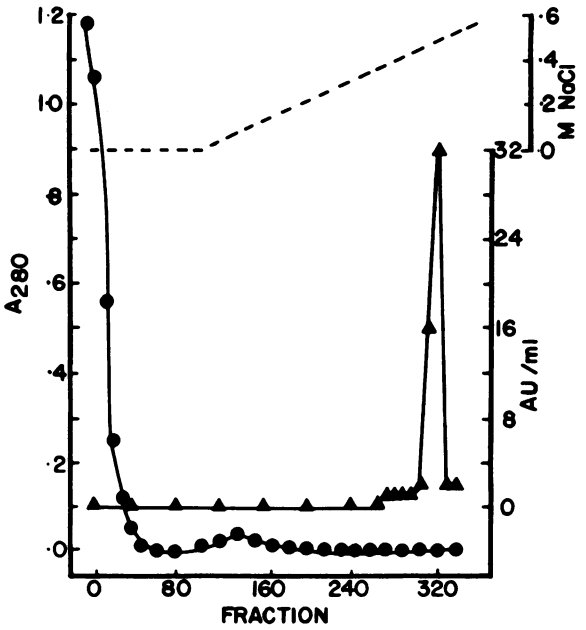

FIG. 2. Elution of lactacin B from carboxymethyl Sephadex. Culture supernatants (100 ml) containing lactacin B were applied to carboxymethyl Sephadex C-50 equilibrated with 0.05 M acetate buffer, pH 5. The column was washed until absorbance at 280 nm returned to 0. Lactacin was eluted with a linear gradient of 0 to 0.6 M NaCl in 0.073 M sodium acetate, pH 5. Fractions (2 ml) were collected, and lactacin B activity was determined for each. Symbols: ●, absorbance at 280 nm; ▲, lactacin B activity; ----, linear NaCl gradient.

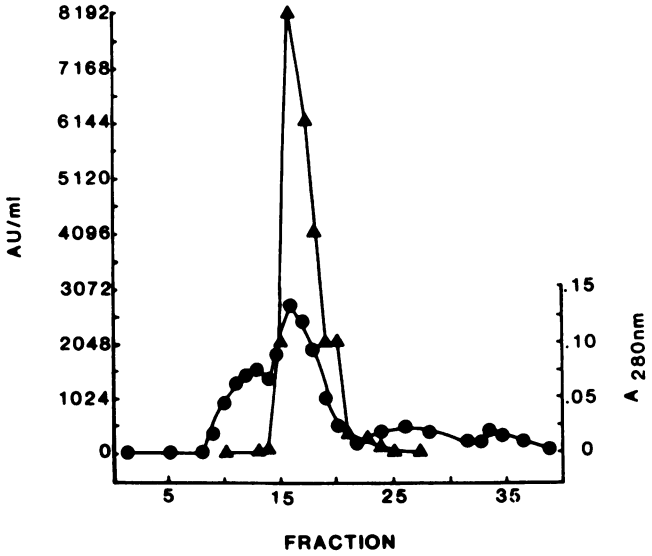

FIG. 3. Chromatography of lactacin B on Sephadex G-75 in the presence of 8 M urea. A 1-ml sample of lactacin B (33,000 AU) was applied to a Sephadex G-75 column equilibrated with 0.1 M Tris, pH 8.6, containing 8 M urea, and the absorbance at 280 nm was monitored. Fractions (1.2 ml) were collected in tubes containing 0.1 M Tris, pH 8.6, with 0.1% SDS (1 ml) and assayed for lactacin B activity. Symbols: ●, absorbance at 280 nm; ▲, lactacin B activity.

single peak after the void volume and achieved considerable separation from contaminants (Fig. 3). Subsequent application of active fractions from urea chromatography to Sephacryl S-200 in the presence of 0.1% SDS resulted in the elution of a single peak of activity corresponding to a single peak of absorbance at 280 nm (Fig. 4). For fractions containing purified lactacin B (no. 26 to 32), specific activities were relatively constant. Subsequent chromatography of purified lactacin B on Sephacryl S-200 (0.1 M Tris, 0.1% SDS, pH 8.6) resulted in the elution of a single symmetrical peak of activity corresponding to a molecular weight of ca. 6,500 (Fig. 5).

When purified lactacin B (50 μg) was electrophoresed on 20% polyacrylamide gels in the presence of 0.1% SDS, activity migrated in a single diffuse band slightly ahead of bovine trypsin inhibitor (molecular weight, 6,200). Although SDS standards were readily detected, lanes containing lactacin B treated with silver stain showed no protein bands. These data suggested that lactacin B either was present at less than 0.01 ng/mm^2 (15) or could not be detected with silver stain. The absence of protein bands suggested that this preparation was free from detectable contaminants.

Properties. Purified lactacin B was stable under a variety of conditions (data not shown). Full activity was retained after treatment at 100°C for 3 min at pH 5 or 8.6 in the presence of 1.0% SDS. Addition of 1% 2-mercaptoethanol during the heating step did not decrease activity. Activity was stable to storage at room temperature or at −20°C for

TABLE 1. Purification of lactacin B

Purification stage	Vol (ml)	Lactacin activity (AU/ml)[a]	Total lactacin activity (AU)	Amt of protein (mg/ml)[b]	Sp act (AU/mg)	Activity recovery (%)	Fold purification
Culture supernatant	10,000	128	1,280,000	15.0[c]	9	100	1
Carboxymethyl Sephadex eluent, pooled, concentrated	5	8,192	40,960	5.9	1,390[d]	3	154
Filtrate from XM50 membrane (8 M urea) concentrated	5	8,192	40,960	0.6	13,700	3	1,522
Urea chromatography on Sephadex G-75	8.4	3,580	30,100	ND[e]	ND	2.4	ND
SDS chromatography on Sephacryl S-200[f]	10.6	520	5,568	0.02[g]	29,000	0.4	3,222

[a] AU, Activity units.
[b] Determined by the method of Lowry et al. (12) as modified by Peterson (19).
[c] Determined by weight.
[d] Determined after solubilization with urea.
[e] ND, Not determined.
[f] Represents the third of three chromatographic elutions.
[g] Estimated by the ratio of absorbance at 235 and 280 nm as described by Whitaker and Granum (27).

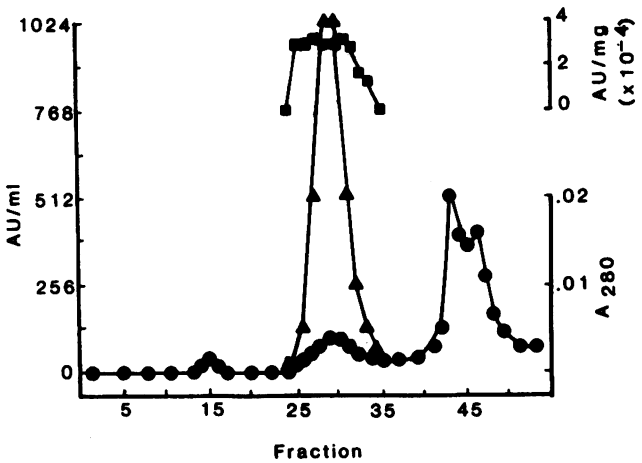

FIG. 4. Chromatography of lactacin B on Sephacryl S-200 in the presence of 0.1% SDS. Active fractions from urea chromatography were pooled, dialyzed against elution buffer (0.1 M acetate, pH 5, with 0.1% SDS), concentrated to 1 ml, and applied to Sephacryl S-200. Absorbance at 280 nm was monitored, and fractions were assayed for lactacin B activity and protein. Specific activity (AU/mg) was calculated for each fraction. Symbols: ●, absorbance at 280 nm; ▲, lactacin B activity; ■, specific activity.

several months. Activity of SDS-solubilized preparations treated with proteinase K was reduced by 97%, whereas preparations containing inactive enzyme or no enzyme retained full activity. These data indicated that lactacin B was a protein.

The UV absorption spectrum of lactacin B was examined. Purified lactacin B showed maximum absorbance at 211 nm,

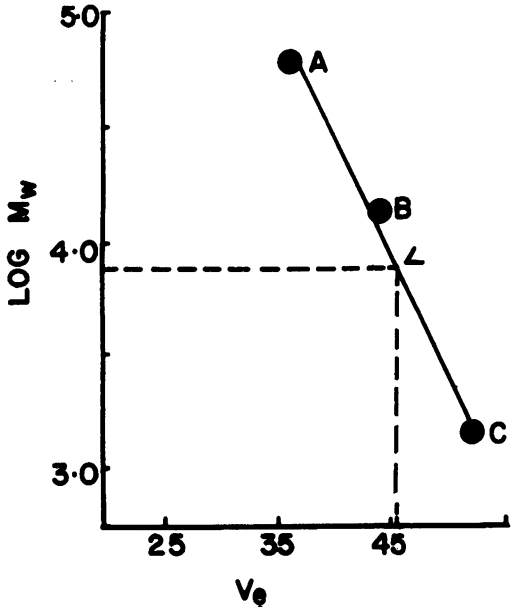

FIG. 5. Estimation of molecular weight of purified lactacin B. The molecular weight of lactacin B was estimated by gel filtration in 0.1 M Tris, pH 8.6, containing 0.1% SDS on a 67-ml Sephacryl S-200 column (1.5-cm interior diameter). Standard proteins were dissolved in buffer, held at 100°C for 3 min in the presence of 1% SDS and 1% 2-mercaptoethanol before application to the column, and included (A) catalase (monomer molecular weight, 60,000; Pharmacia), (B) RNase A (molecular weight, 13,700; Sigma), and (C) bacitracin (molecular weight, 1,400; Sigma). The arrow indicates elution of lactacin B.

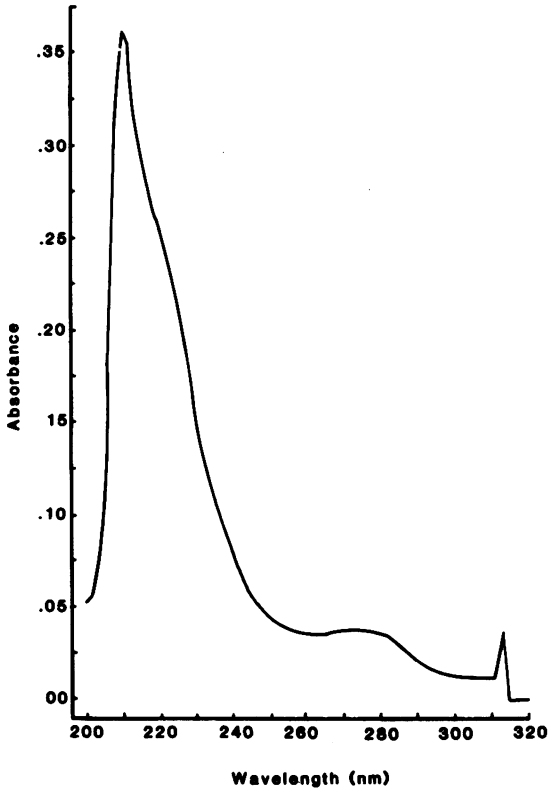

FIG. 6. UV absorption spectrum of purified lactacin B. Purified lactacin B (0.035 mg/ml) in 0.1 M acetate–0.1% SDS buffer, pH 5, was scanned on a double-beam spectrophotometer against a reference cell containing the same buffer. Values shown were corrected for absorbance of the buffer.

characteristic of peptide bonds (Fig. 6). Minimal absorbance was observed at 280 nm, suggesting a low content of aromatic amino acids.

Spectrum of activity and bactericidal action. The inhibitory spectrum of purified lactacin B was examined against the indicator species listed in Table 2. Purified lactacin B inhibited *L. leichmannii* 4797, *L. bulgaricus* 1489, *L. lactis* 970, and *L. helveticus* 87 at both 8 and 512 AU/ml, indicating that activity against these four indicators is mediated by one protein. At the higher concentration only, lactacin B inhibited both producer strains, suggesting that their immunity mechanisms may have been overloaded. No other indicator species were affected at either concentration. Examination of the producer culture after treatment with *N*-methyl-N′-nitro-*N*-nitrosoguanidine yielded three mutants (1.6% of 200 isolates examined) lacking lactacin B activity (LaB$^-$). For each, activity against *L. leichmannii* 4797, *L. bulgaricus* 1489, *L. helveticus* 87, and *L. lactis* 970 was lost simultaneously, suggesting that one gene product mediated activity against all indicators.

The effect of purified lactacin B on the viability and lysis of cells of *L. leichmannii* 4797 was examined (Fig. 7). A 40-min incubation of sensitive cells with lactacin B (40 AU/ml) resulted in the death of 99.97% of the population. Continued incubation with lactacin B beyond 40 min did not reduce the population further. The optical density remained constant throughout the experiment, indicating that purified lactacin B did not act in a bacteriolytic fashion. These data showed that lactacin B induced cell death without detectable lysis and confirmed a bactericidal mode of action for the bacteriocin.

TABLE 2. Activity of purified of lactacin B against selected indicator bacteria

Indicator sensitivity (AU/ml)	Indicator
8 and 512	*L. bulgaricus* 1489
	L. helveticus 87
	L. lactis 970
	L. leichmannii 4797
512 only	*L. acidophilus* N2
	L. acidophilus 6032
Insensitive	*L. casei* 7469
	L. fermentum 1750
	L. plantarum 1752
	L. viridescens 12706
	Citrobacter freundii 8090
	Enterobacter aerogenes 13048
	E. cloacae
	Escherichia coli 25922
	E. coli 0;24 B;17 enteropathogenic
	Klebsiella pneumoniae 13883
	Pseudomonas aeruginosa 27853
	Salmonella typhimurium 14028
	Serratia marcescens 8100
	Shigella flexneri 14028
	S. sonnei 25931
	Staphylococcus epidermidis 12228
	Streptococcus faecalis 19433

DISCUSSION

Characteristics identified for lactacin B in this study confirm that it is a bacteriocin. Similar to previously characterized bacteriocins (23), lactacin B was proteinaceous. Biological activity was destroyed by proteinase K. Preparations showed maximum absorbance at 211 nm, characteristic of peptide bonds, and the bacteriocin gave a positive Folin reaction. Lactacin B was also insensitive to treatment with chloroform (unpublished data), suggesting the absence of lipid; however, the possibility that small amounts of carbohydrate might be present was not eliminated.

Like diplococcin (2), pep 5 (22), and the *Bacteroides fragilis* bacteriocin (8), purified lactacin B had a low molecular weight. In contrast to a previous study of the ultrafiltration characteristics of crude preparations of lactacin B, which indicated a molecular weight of 100,000 (1), gel filtration data in this study indicated a molecular weight near 6,500 for purified lactacin B. Although these data are approximate, since polypeptides with molecular weights of less than 15,000 behave abnormally during gel filtration in SDS solutions (6), the estimate was compatible with the migration of lactacin B in SDS-PAGE which was characteristic of a peptide with a molecular weight of ca. 6,000. Inconsistencies in the former and present estimates of molecular weight may be reconciled by the observation that lactacin B readily associated and coeluted with nonbacteriocin components when nondissociating conditions were used for gel filtration.

Evidence for the homogeneity of lactacin B was provided by uniform specific activities for eluted fractions, further chromatography resulting in elution of a single peak of absorbance corresponding to activity, and absence of contaminating proteins on SDS-PAGE electropherograms. Because the quantities of lactacin B available approached lower detection levels for protein assays, stain detection, and absorbance measurements, these assessments did not unequivocally preclude contamination with undetectable levels of a peptide contaminant.

Lactacin B showed many similarities to the *L. helveticus* bacteriocin lactocin 27 studied by Upreti and Hinsdill (24). Both bacteriocins were produced during the early stationary phase of growth of the producer cultures. Both were initially isolated as large-molecular-weight complexes. The initial molecular weight estimates were 100,000 (1) and >200,000 (24) for lactacin B and lactocin 27, respectively. Both large-molecular-weight bacteriocins dissociated from nonbacteriocin components in SDS to low-molecular-weight proteins with biological activity. Both purified lactacin B (molecular weight, 6,000 to 6,500) and purified lactocin 27 (molecular weight, 12,400) (24) retained their activity in the presence of SDS.

Purified lactacin B showed bactericidal activity against sensitive cells. Similar to lactocin 27 (24, 25) and the *L. fermenti* bacteriocin (3–5), activity was restricted to members of the family *Lactobacilliaceae*. Purified lactacin B showed activity against *L. leichmannii*, *L. helveticus*, *L. lactis*, and *L. bulgaricus*. Moreover, mutants isolated in this study showed simultaneous loss of activity against these four indicator species. Both biochemical and genetic evidence supported the conclusion that activity against the four indicator species was mediated by a single bactericidal agent, the peptide bacteriocin lactacin B.

Purification and characterization of lactacin B provides the first confirmation of bacteriocin-mediated antagonism in *L. acidophilus*. The targets for lactacin B activity and the means by which its specificity is determined remain unknown. Clearly, further studies of lactacin B and other bacteriocins produced by *L. acidophilus* are required to elucidate mechanisms for antagonism in these important fermentative organisms.

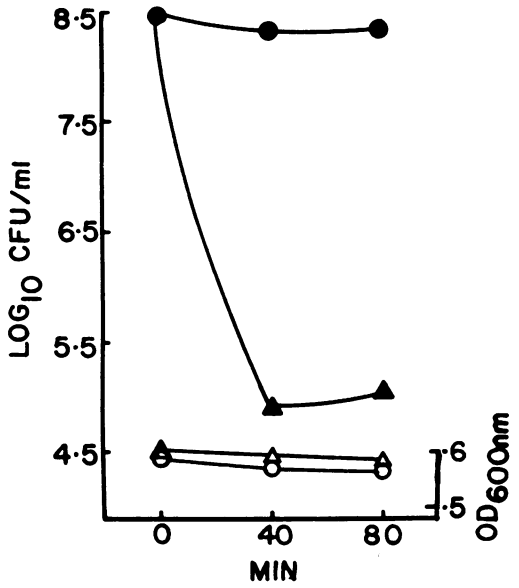

FIG. 7. Bactericidal activity of lactacin B against *L. leichmannii* 4797. Purified lactacin B (pH 7) was added at 0 (●) and 40 (▲) AU/ml to sterile 1 mM phosphate buffer, pH 7, containing 0.002% SDS. Cells from 5-h MRS broth cultures were washed in buffer and added to a final concentration of 3.1×10^8 CFU/ml. The optical density at 600 nm (open symbols) was determined spectrophotometrically, and survivors (in CFU per milliliter) (closed symbols) were determined by plating on MRS agar.

ACKNOWLEDGMENTS

This work was supported in part by Miles Laboratories, Elkhart, Ind., and the North Carolina Dairy Foundation. S.F.B. was supported in part by a Ralston Purina Graduate Fellowship.

LITERATURE CITED

1. **Barefoot, S. F., and T. R. Klaenhammer.** 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. **45**:1808–1815.
2. **Davey, G. P., and B. C. Richardson.** 1981. Purification and some properties of diplococcin from *Streptococcus cremoris* 346. Appl. Environ. Microbiol. **41**:84–89.
3. **DeKlerk, H. C.** 1967. Bacteriocinogeny in *Lactobacillus fermenti*. Nature (London) **214**:609.
4. **DeKlerk, H. C., and J. N. Coetzee.** 1961. Antiobiosis among lactobacilli. Nature (London) **192**:340–341.
5. **DeKlerk, H. C., and J. A. Smit.** 1967. Properties of a *Lactobacillus fermenti* bacteriocin. J. Gen. Microbiol. **48**:309–316.
6. **Fish, W. W., J. A. Reynolds, and C. Tanford.** 1970. Gel chromatography of proteins in denaturing solvents. J. Biol. Chem. **245**:5166–5168.
7. **Harvey, R. J., and E. B. Collins.** 1963. Roles of citrate and acetoin in the metabolism of *Streptococcus diacetilactis*. J. Bacteriol. **86**:1301–1307.
8. **Hayes, T. J., K. R. Cundy, P. B. Fernandes, and J. K. Hoober.** 1983. Purification and characterization of a bacteriocin from *Bacteroides fragilis*. J. Bacteriol. **155**:1171–1177.
9. **Klaenhammer, T. R., and E. G. Kleeman.** 1981. Growth characteristics, bile sensitivity, and freeze damage in colonial variants of *Lactobacillus acidophilus*. Appl. Environ. Microbiol. **41**:1461–1467.
10. **Konisky, J.** 1978. The bacteriocins, p.71–136. *In* I. C. Gunsalus (ed.), The bacteria, vol. 6: Bacterial diversity. Academic Press, Inc., New York.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
12. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
13. **McCormick, E. L., and D. C. Savage.** 1983. Characterization of *Lactobacillus* sp. strain 100-37 from the murine gastrointestinal tract: ecology, plasmid content, and antagonistic activity toward *Clostridium ramosum* H1. Appl. Environ. Microbiol. **46**:1103–1112.
14. **Mehta, A. M., K. A. Patel, and P. J. Dave.** 1983. Purification and properties of the inhibitory protein isolated from *Lactobacillus acidophilus* AC_1. Microbios **38**:73–81.
15. **Merril, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert.** 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science **211**:1437–1438.
16. **Morin, A., S. A. Saheb, J. G. Bisaillon, R. Beaudet, and M. Sylvestre.** 1980. Effect of culture medium composition on inhibition of growth of *Neisseriae gonorrhoeae* by lactobacilli. Curr. Microbiol. **4**:283–286.
17. **Moustafa, H. H., and E. B. Collins.** 1968. Role of galactose or glucose-1-phosphate in preventing the lysis of *Streptococcus diacetilactis*. J. Bacteriol. **95**:592–602.
18. **Nomura, M.** 1967. Colicins and related bacteriocins. Annu. Rev. Microbiol. **21**:257–284.
19. **Peterson, G. L.** 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. **83**:346–356.
20. **Reeves, P.** 1965. The bacteriocins. Bacteriol. Rev. **29**:24–45.
21. **Sahl, H. G., and H. Brandis.** 1981. Production, purification, and chemical properties of an antistaphylococcal agent produced by *Staphylococcus epidermidis*. J. Gen. Microbiol. **127**:377–384.
22. **Sahl, H. G., and H. Brandis.** 1982. Mode of action of the staphylococcin-like peptide pep 5 and culture conditions effecting its activity. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **252**:166–175.
23. **Tagg, J. R., A. S. Dajani, and L. W. Wannamaker.** 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. **40**:722–756.
24. **Upreti, G. C., and R. D. Hinsdill.** 1973. Isolation and characterization of a bacteriocin from a homofermentative *Lactobacillus*. Antimicrob. Agents Chemother. **4**:487–494.
25. **Upreti, G. C., and R. D. Hinsdill.** 1975. Production and mode of action of lactocin 27: bacteriocin from a homofermentative *Lactobacillus*. Antimicrob. Agents Chemother. **7**:139–145.
26. **Waddell, W. J.** 1956. A simple ultraviolet spectrophotometric method for the determination of protein. J. Lab Clin. Med. **48**:311–314.
27. **Whitaker, J. R., and P. E. Granum.** 1980. An absolute method for protein determination based on difference in absorbance at 235 and 280nm. Anal. Biochem. **109**:156–159.
28. **Wolf, P.** 1983. A critical reappraisal of Waddell's technique for ultraviolet spectrophotometric protein estimation. Anal. Biochem. **129**:145–155.
29. **Wright, C. T., and T. R. Klaenhammer.** 1983. Survival of *Lactobacillus bulgaricus* during freezing and freeze-drying after growth in the presence of calcium. J. Food Sci. **48**:773–777.