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Isolation and Characterization of a Bacteriocin from a Homofermentative *Lactobacillus*

G. C. UPRETI AND R. D. HINSDILL

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

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Nearly 100 isolates of *Lactobacillus* were obtained from human and animal sources. Screening tests with the isolates revealed seven possible bacteriocinogenic strains and 26 strains sensitive to one or more of these inhibitory strains. Three homofermentative strains were selected for additional study after it was shown that their inhibitory substances differed in activity spectrum and in susceptibility to inactivation by proteolytic enzymes. One of these, *L. helveticus* strain LP27, was shown to produce a potent bacteriocin called lactocin 27. The lactocin was isolated from the culture supernatant fluid as a protein-lipopolysaccharide complex. In the presence of sodium dodecyl sulfate the complex was dissociated, and the activity was found to reside in a small glycoprotein (molecular weight 12,400). The amino acid composition of purified lactocin 27 is similar to that of the *L. fermenti* bacteriocin; neither requires disulfide bonds for activity.

Bacteriocins are usually defined as bactericidal substances synthesized by bacteria and active against strains of the same or closely related species. The majority of them are inactivated by proteolytic enzymes and are believed to be proteinaceous in nature. Their narrow activity spectrum and proteinaceous character distinguish them from antibiotics (11).

Bacteriocinogeny among many different bacterial species is well documented (14), but full characterization of bacteriocins, especially those from gram-positive microorganisms is limited to a few. Yet diversity among bacteriocins is evident. They include the "classical" colicins of *Escherichia coli* and related species, megacin A which is a lytic enzyme identical to phospholipase A (22), pyocin R which resembles a phage tail (19), enterococcin (type I) which appears to be a hemolysin (1), staphylococcin (414) which is a lipoprotein-carbohydrate complex (12), and bactericidal substance from *Staphylococcus aureus* (strain C55) which is proteinaceous in nature (5). These differences are not surprising when one considers that the definition of a bacteriocin allows the inclusion of many substances having antibacterial activity. To avoid further confusion, Nomura (21) has suggested that the term bacteriocin be limited to those bactericidal substances which act on culture strains related to the producer, require a specific adsorption site for action, and do not resemble morphologically a phage or parts of a phage. This definition would exclude lytic enzymes and defective bacteriophages. Reeves (23) has recently reviewed the nomenclature, chemistry, genetics, and mode of action of bacteriocins.

While looking for lysogenic cultures, deKlerk and Coetzee (8) found a number of heterofermentative and homofermentative lactobacilli which produced bacterocin-like inhibitors. A somewhat unusual situation was found with the heterofermentative *Lactobacillus fermenti* (7) in that all 25 of the producing strains inhibited the same indicator cultures, and no resistant mutants could be obtained. The bacteriocin produced by *L. fermenti* was later isolated and chemically analyzed (9).

The present report deals with the isolation and preliminary characterization of a bacteriocin called lactocin 27, which was isolated from the homofermentative *Lactobacillus helveticus*. Some comparative data are presented on two other lactocins produced by other homofermentative lactobacilli.

MATERIALS AND METHODS

Media. Lactobacillus selection broth (LBS, Baltimore Biological Laboratory, Cockeysville, Md.) and APT broth (Difco Laboratories, Detroit) were prepared as directed by the manufacturers. Agar (1.5%, wt/vol) was added to the broths to make plating

media; 0.75% (wt/vol) was added to APT broth to make soft agar. Incubation for surface growth of lactobacilli was in an atmosphere containing approximately 10% CO_2. This condition was achieved by incubating the agar plates in a jar which was closed after lighting a candle.

Organisms. Lactobacilli were isolated from the saliva and vaginal tract of humans and the feces of suckling pigs, guinea pigs, calves, rabbits, and hamsters. Primary isolation was made by streaking specimens directly on LBS agar and incubating for 48 h at 37 C. Isolated colonies were picked and streaked on APT agar plates to obtain good growth and to ensure single colony isolates. The Gram stain and benzidine test (6) were used to make certain that all new isolates were typical gram-positive rods, nonsporulating, and lacking a cytochrome respiratory system.

In addition to the new isolates, a number of known cultures, including various *Lactobacillus* sp., were provided by several members of this department. Stock cultures of lactobacilli were maintained by inoculating a 0.1-ml portion of a 24 to 48-h culture into 10 ml of fresh APT broth in screw-cap tubes. The tubes were immediately frozen at −20 C and stored. Stocks of all other cultures were grown on nutrient agar slants at 37 C overnight before being stored at 4 C.

Screening of cultures. Producer and indicator cultures were identified by the stab plate technique. APT agar plates were stabbed with prospective bacteriocinogenic cultures of lactobacilli and incubated for 48 h. The growth which developed at the surface was killed by exposure to chloroform vapor for 1 h. The plates were then overlaid with 3 ml of APT soft agar inoculated with 0.1 ml of a 24-h broth culture of a prospective indicator strain. After the overlays had solidified, the plates were incubated for 48 h and then were examined for a zone of inhibition in the bacterial lawn just above the stabs.

Bacteriocin assays. The technique previously described (12) was used with slight modifications. The indicator culture was grown overnight in APT broth at 37 C and diluted with APT broth just prior to use to give an optical density (615 nm) of 0.4. After further diluting this culture 100-fold with APT broth, 0.2 ml was inoculated into tubes containing 3 ml of APT soft agar. Serial dilutions were made of the solution being tested, and 0.01-ml samples were placed on an APT agar plate divided into eight sections by a felt marking pen. The plates were then overlaid with the inoculated soft agar and incubated as described above.

Prior to determining the bacteriocin level of broth cultures, the cultures were heated in flowing steam (approximately 100 C) for 1 h. This treatment eliminated interference in the assay that would result from the growth of producing cells; moreover, higher titers resulted, presumably because of the release of lactocin from the cells during the heat treatment.

Column chromatography. Sephadex G-200 (Pharmacia, Piscataway, N.J.) was prepared and packed to give a bed size of 3.4 by 90 cm by using 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.7) containing 0.01% sodium azide for the packing and final elution. For certain experiments, 0.5% sodium dodecyl sulfate (SDS) was added to the buffer. Samples were dissolved in the appropriate buffer by heating at 50 C for 4 h, to give an initial concentration of 20 mg/ml. Insoluble material, accounting for roughly 25% (dry weight) of the sample, was removed by centrifugation at 7,980 × *g* for 30 min, and 20 ml of the clarified sample was placed on the column. The chromotographic apparatus consisted of an LKB column (Stockholm, Sweden), fraction collector and an LKB Recychrome kit operated at room temperature. Flow rate was 25 ml/h, absorption at 280 nm was measured by a flow cell monitor and chart recorder, and 5-ml fractions were collected. Void volumes of the columns were determined with Blue Dextran (Pharmacia).

The following procedure was used to obtain fractionated material in dry form. The active fractions obtained by gel filtration through SDS-Sephadex G-200 were pooled and concentrated by evaporation under vacuum. The Tris and SDS in the concentrate were removed by filtration through a Sephadex G-25 column (2 by 25 cm) by using 0.05 M ammonium bicarbonate buffer (pH 8.7) for elution. Excess ammonia in the pool of the recovered fractions was removed by adding 1 to 2 drops of triethylamine; the resulting solution was freeze dried.

Acrylamide gel electrophoresis. The acrylamide gel electrophoresis procedure was that described by Waehneldt (30), except that 10% polyacrylamide (wt/vol) was used for the resolving gel. Samples were dissolved in a solution containing 4 M urea, 1% SDS, and 1% mercaptoethanol. Portions (50 μliter) were layered on gel columns measuring approximately 6 by 75 mm. Electrophoresis was carried out by using the constant-current method (1 mA per gel for 60 min, during which time the sample was concentrated in the stacking gel, followed by 3 mA per gel for 90 min). The gels were then soaked in 12.5% trichloroacetic acid for 2 h and stained for protein with Coomassie brilliant blue (10) or for lipid with oil red 0 (4). Carbohydrate staining was accomplished by the adaptation of a colorimetric method (18) in which the gels were heated in a phenol-sulfuric acid mixture for 5 min at 90 C. Carbohydrate, when present in the gel, stained dark brown. The gels were restored to their original length by soaking in distilled water overnight.

Chemical analyses. Protein was determined by the method of Lowry et al. (20) by using Bovine Serum Albumin Fraction V Powder (Sigma Chemical Co., St. Louis, Mo.) as a standard. Phosphorous determinations were made by the method of Chen et al. (2). Carbohydrate content was determined by the phenol-sulfuric acid method (18). Lipid was determined gravimetrically (24). The samples were hydrolyzed in 1 N HCl in a sealed tube for 6 h at 100 C prior to lipid and carbohydrate determinations.

Amino acid analysis. The amino acid composition of purified lactocin 27 was determined by comparison with a mixture of known amino acids by using an amino acid analyzer (Technicon Instruments Corp., New York, N.Y.). An acid hydrolysate of the lactocin was prepared by adding 3 mg to 1 ml of 6 N HCl and placing the mixture in a desiccator. After deaeration

by repeated evacuation and flushing with N_2 gas, the evacuated desiccator was placed in an oven at 110 C for 24 h. Cysteine and methionine were estimated after oxidation of the hydrolysate with performic acid (17). Tryptophan was determined spectrophotometrically by *N*-bromosuccinimide (26).

Inactivation by enzymes. Three enzymes were used at a concentration of 1 mg/ml in different buffers; ficin (EC 3.4.4.12) was suspended in a buffer consisting of 0.02 M cysteine-hydrochloride, 0.01 M ethylenediaminetetraacetic acid, and 0.15 M NaCl, pH 7.0; Pronase (B grade, Calbiochem, Los Angeles, Calif.) was dissolved in a 0.02 M sodium barbital-hydrochloride buffer, pH 7.8, containing 0.01 M $CaCl_2$ and 0.001 M $CoCl_2$; trypsin (EC 3.4.4.4) was dissolved in 0.5 M Tris-hydrochloride buffer, pH 8.7, containing 0.01 M $CaCl_2$. Lactocin 27 (1 mg/ml) in sterile saline was mixed with an equal volume of enzyme solution and incubated at 37 C for 60 min. For controls, buffer solutions without enzymes were substituted. All solutions were tested for inhibitory activity by using the bacteriocin assay procedures previously described.

Except for lactocin 27, bacteriocins from the lactobacilli were either not available in purified form or were available only in small amounts. However, an attempt was made to assess the susceptibility of several of these bacteriocins to proteolytic enzymes by the following procedure: APT plates were stabbed with the bacteriocinogenic cultures, incubated overnight, and exposed to chloroform vapor. A drop of enzyme solution was placed on the growth which developed from the inoculum, and the plate was incubated at 37 C for 60 min. The plate was then overlaid with an indicator culture and incubated. Failure of a zone of inhibition to appear around the stab, where one was present on the duplicate plate treated with buffer only, gave presumptive evidence that the bacteriocin was inactivated by the proteolytic enzyme.

RESULTS

Screening tests. Among the nearly 100 new isolates of lactobacilli, seven were found to be producers of inhibitory substances and were designated LP cultures. One-fourth of the isolates were inhibited by one or more of the producers and were designated LS cultures. Table 1 shows the different activity spectra obtained with producers LP27, LP30, and LP67. Strain LP27 appears to have a narrow spectrum of activity. When tested by the stab plate method, none of the following organisms were inhibited: *Enterobacter* (*Aerobacter*) *aerogenes*, *Enterobacter* (*Aerobacter*) *cloacae*, *Agrobacterium radiobacter*, *Agrobacterium tumefaciens*, *Bacillus cereus*, *B. cereus* var. *mycoides*, *Bacillus subtilis*, *Brevibacterium*, *linens*, *E. coli* K235, *E. coli B*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Pediococcus cerevisiae*, *Proteus vulgaris*, *Salmonella enteritidis* ser *typhimurium*, *S. enteritidis* bioser *gallinarium*, *S. aureus* (Wood 16), *S. aureus* (698), *S. aureus* (140), *S. aureus* (19), *Streptococcus faecalis*, *Streptococcus lactis*, *Shigella sonnei* (phase II), *S. sonnei* E90. Similar testing with strains LP30 and LP67 has not been done as yet.

TABLE 1. *Activity spectrum of three lactocin-producing strains by stab plate technique*

Sensitive (indicator) strain	Bacteriocin producer[a]		
	L. helveticus LP 27	*L. casei* LP 30	*L. casei* LP 67
Lactobacillus acidophilus	10	0	7
L. casei NRRL 1445	0	9	15
L. helveticus LS 18	28	10	17
Lactobacillus sp. (LS 124)	0	0	13
Lactobacillus sp. (LS 127)	11	0	0

[a] Average diameter of zone of inhibition appearing in the overlay growth as measured in mm; O denotes no inhibition. Producing strains were not inhibited by each other or themselves.

None of the three LP cultures appears active when tested against itself by the stab procedure; thus the producers are immune to at least nominal levels of their own inhibitors. The producers are also immune to the inhibitors of other producing strains. The diameters of the inhibition zones vary only about 1 mm in repetitive experiments, provided the soft agar is inoculated with approximately the same number of indicator cells in the logarithmic-growth phase. Tentative species designations for the six cultures isolated for this study and listed in Table 1 were determined by using the taxonomic scheme of Sharpe (25).

Growth and lactocin production. Screw-cap tubes containing 10 ml of APT broth were seeded with a 1% (vol/vol) inoculum of a 24-h APT broth culture of LP27. The tubes were incubated statically at 37 C. Growth was followed by measuring absorbance at 615 nm, and the bacteriocin titer was measured as previously described. Lactocin production paralleled growth for the most part (Fig. 1). Neither growth nor lactocin production could be improved by changing the incubation temperature to 45 or 55 C or by substituting media such as brain heart infusion, Trypticase soy broth (Baltimore Biological Laboratories) or Thioglycollate broth (Difco).

An effort was also made to identify the primary location of the lactocin in a broth culture. Typically, the supernatant fluid obtained from centrifugation of a 40-h broth culture of LP27 (unheated) possessed an activ-

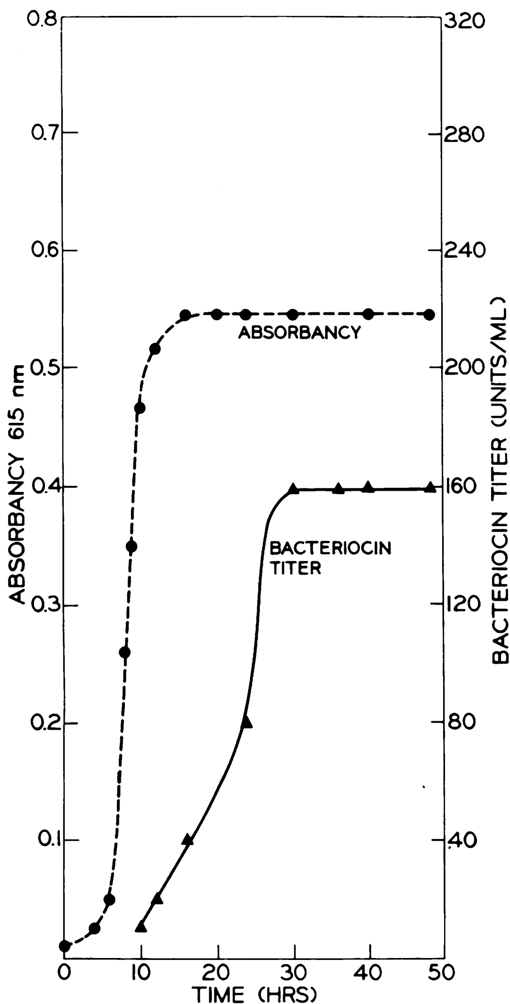

FIG. 1. *Measurement of growth and bacteriocin production in APT broth with culture LP 27.*

ity of 80 U/ml. If the cells in the pellet were resuspended in saline to original volume and assayed, an activity of 20 U/ml or less was found. For this procedure, dilutions of cell suspension were spotted on a plate and allowed to dry for 15 min. The plates were subsequently exposed to chloroform vapor, overlaid with indicator strain, and incubated.

The question remained whether the bacteriocin activity in the supernatant fluid was the result of autolysis during growth of the culture. Cells washed and suspended in saline were disrupted in a Ribi Cell Fractionator (Ivan Sorval Inc.) at 30,000 lb/in^2. Although electron microscopy (12) showed almost every cell to have one end sheared off, the inhibitory activity of the cell suspension did not increase over the starting material. These results indicated that the cytoplasm was not the major source of the lactocin and that the bacteriocin most likely was excreted during growth.

Several lines of evidence have shown that the inhibitory activity of strain LP27 is not due to a bacteriophage. No phage particles were observed in the culture supernatant fluid by electron microscopy by using methods described previously (12). Diluting out the culture supernatant fluid leads to diminished inhibitory activity when tested against the sensitive strain, but not to the formation of individual plaques. In addition, the inhibitory activity cannot be propagated in the sensitive strain as would be expected of phage.

The effect of inducing agents, e.g., mitomycin C (0.5 to 2.0 μg/ml) and ultraviolet light, on strain LP27 was studied by using the methods described by Dajani et al. (5). Neither treatment had any effect on lactocin production.

Isolation and purification. The procedure finally adopted is summarized in Fig. 2, and the yields obtained at various steps are listed in Table 2.

APT broth (2 liters) was inoculated with 20

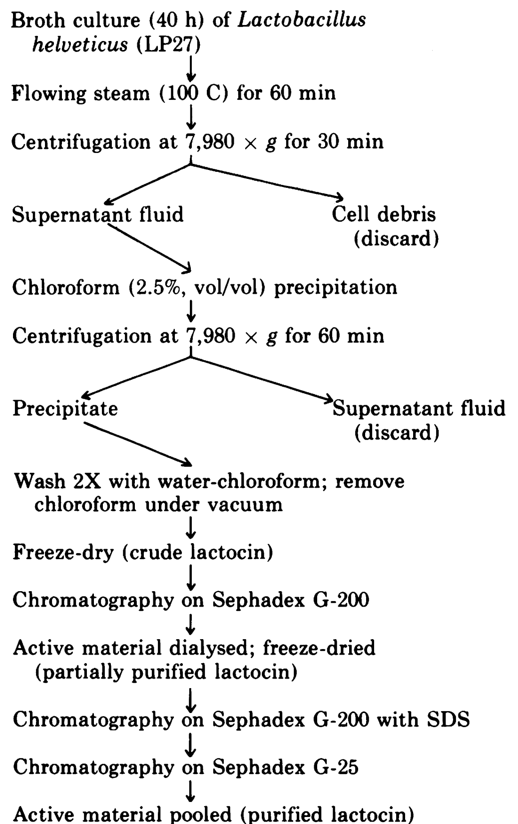

FIG. 2. *Isolation and purification of lactocin 27.*

TABLE 2. *Yield and specific activity of lactocin 27 at various stages in the purification procedure*[a]

Prepn	Dry weight (mg)	Activity (total units)	Specific activity (units/mg)	Yield (%)	Fold purification
Culture supernatant fluid	48,000	320,000	7	100	1
Crude lactocin	127	86,741	683	27	98
Partially purified lactocin	18	52,740	2,930	16	418
Purified lactocin	N.D.	N.D.	4 × PPL[b]	N.D.	1,672[b]

[a] See Fig. 2. N.D., not determined, because sample contained an unknown amount of bound SDS.
[b] Protein concentration (20) of sample adjusted to equal that of partially purified lactocin used for comparison.

ml of an overnight APT broth culture of LP27. After 40 h of incubation at 37 C, the culture was heated in flowing steam (100 C) for 1 h. The cells were removed by centrifugation at 7,980 × *g* for 20 min. Chloroform (25 ml/liter) was then added to the supernatant fluid, and the mixture was shaken vigorously for 15 min. The resulting precipitate was allowed to settle out at 4 C overnight and then was collected by centrifugation at 7,980 × *g* for 1 h. The precipitate was washed twice with water saturated with chloroform, and the residual chloroform in the precipitate was removed under vacuum in a Rotavapor (Büchi, Switzerland). The precipitate was then freeze-dried and designated crude lactocin. The crude lactocin was dissolved in 0.05 M Tris-hydrochloride buffer (pH 8.7) and subjected to column chromatography on Sephadex G-200 (Fig. 3). The fractions constituting the first absorbance peak eluted in the void volume and had inhibitory activity proportional to their absorption at 280 nm. These fractions were pooled, dialyzed against distilled water, and freeze-dried. This material was called partially purified lactocin.

Further purification was achieved by subjecting the partially purified lactocin to column chromatography by using a buffer which contained SDS (Fig. 4). The fractions containing the inhibitory activity were eluted after the void volume and had activity proportional to their absorbance at 280 nm. These fractions were pooled, concentrated to approximately one-tenth volume by rotary evaporation, and subjected to gel filtration through Sephadex G-25 to remove the Tris and free SDS. The active fractions, which eluted in the void volume, were pooled and the absorbance at 280 nm was adjusted to 1.0. This material, called purified lactocin, contained an undetermined amount of bound SDS.

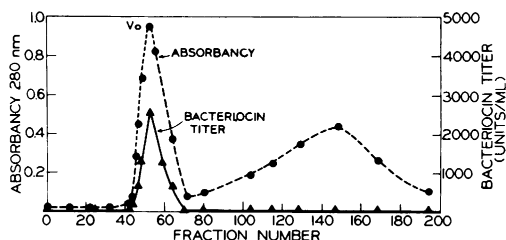

FIG. 3. *Column chromatography of crude lactocin 27 through Sephadex G-200 gel. V_0 indicates void volume of column. Fractions 45 through 65 were pooled for further purification.*

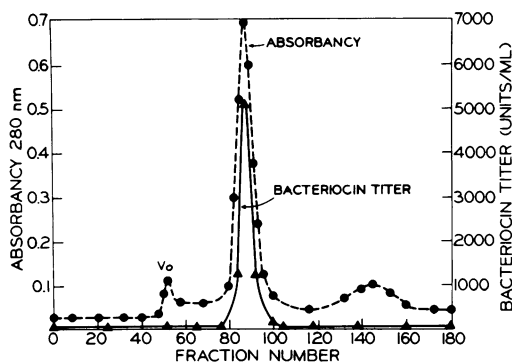

FIG. 4. *Column chromatography of partially purified lactocin 27 on Sephadex G-200 gel containing 0.5% SDS. V_0 indicates void volume of column. Fractions 82 through 95 were pooled for further characterization.*

Properties of lactocin. The partially purified lactocin derived from LP27 was inactivated by trypsin and Pronase, but not by ficin. When the enzymes were applied directly to the growth of LP27 on a stab plate, the inhibitory activity was again destroyed only by trypsin and Pronase. In similar tests using stab cultures of LP30 and LP67, only trypsin destroyed the inhibitory activity.

Crude lactocin 27 at a concentration of 1 mg/ml in 0.15 M NaCl, pH 7.0, is remarkably heat stable; no measurable drop in activity is seen after heating for 1 h in flowing steam (approximately 100 C).

Lactocin 27 in its partially purified form contains protein (47%), lipid (13%), carbohydrate (8%), and phosphorus (1%). Ash content was not determined. Its estimated molecular weight is more than 200,000 as it is eluted in the void volume of a sephadex G-200 column (Fig. 3). The partially purified lactocin complex is dissociated by SDS; the active component (purified lactocin) elutes after the void volume when partially purified lactocin is chromatographed on Sephadex G-200 with buffer containing SDS (Fig. 4).

Purified lactocin behaved as a homogeneous preparation in polyacrylamide gel electrophoresis; only one band appeared when the gels were stained for protein or for carbohydrate. Because these bands were at the same position (Fig. 5), purified lactocin is apparently a complex of protein and carbohydrate. No band was observed when the gels were stained for lipid. Because of the bound SDS and residual salts in the sample the absolute percentage of protein and carbohydrate in purified lactocin could not be determined; however, the ratio of protein to carbohydrate was 1.75:1. Its molecular weight was estimated to be 12,400 by gel electrophoresis.

The amino acid composition of purified lactocin is given in Table 3. A very small peak was observed for methionine in the unoxidized sample which disappeared when the oxidized sample was examined. The peak corresponding to cysteine was not observed with the unoxidized sample, but a small rise, corresponding to cysteic acid-sulphone, was observed with the oxidized sample. These results were interpreted to mean that methionine was present and that cysteine was either absent or present in a very small amount. To test whether disulfide linkages were required for activity, purified lactocin was tested after reductive *S*-carboxymethylation (3); activity was not diminished.

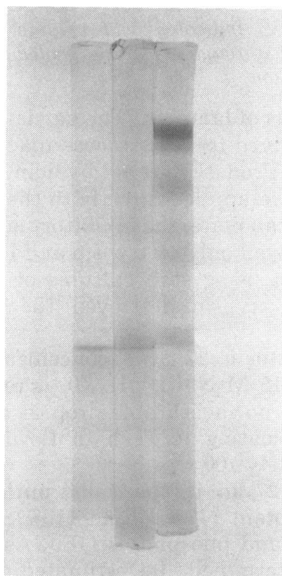

FIG. 5. *Electrophoretic migration of purified lactocin 27 in 10% polyacrylamide gels. The collapsed stacking gels have been removed from the top. The migration was towards the anode at the bottom. From left to right: lactocin stained for carbohydrate; lactocin stained for protein; and marker proteins (albumin, molecular weight 67,000; cytochrome C, molecular weight 12,400) stained for protein.*

TABLE 3. *Amino acid composition of purified lactocin 27*

Amino acid	Approximate no. of residues per molecule[a]
Aspartic acid	11.0
Threonine	7.20
Serine	6.6
Glutamic acid	8.4
Proline	4.1
Glycine	22.4
Alanine	18.7
Valine	10.3
Isoleucine	6.4
Leucine	6.2
Tyrosine	3.6
Phenylalanine	4.1
Histidine	2.9
Lysine	7.2
Arginine	4.1
Tryptophan[b]	1.0
Methionine[c]	+
Half-cystine[d]	N.C.

[a] Calculated by assuming that the sum of the weights of the amino acids, aspartate through arginine, is equal to the estimated molecular weight of the lactocin.

[b] Tryptophan was estimated by using *N*-bromosuccinimide oxidation.

[c] A small peak was observed in acid hydrolysate of purified lactocin, which disappeared on performic acid oxidation.

[d] N.C., Not confirmed, details given in text.

Showing that lactocin 27 acts through specific receptors has been complicated by the fact that it adsorbs to many things, including nonsensitive cells. However, in some tests with partially purified lactocin there is a difference in the rate of adsorption; the sensitive cells adsorb the bacteriocin out of solution faster than the nonsensitive cells. Details of these experiments will be reported later with the other data on the mode of action. So far, spontaneous, resistant mutants have not been observed in sensitive strains.

DISCUSSION

Except for the difficulty in demonstrating attachment to specific receptor sites on sensitive cells, lactocin 27 meets all the criteria for a bacteriocin as described in the introduction. These criteria are of special importance with regard to lactocins, because the lactobacilli produce a number of antimicrobial substances,

including hydrogen peroxide (31) and lactic acid (27). Vincent et al., (29) reported the production of an inhibitory substance by *Lactobacillus acidophilus*, called lactocidin, which was active on *Pseudomonas aeruginosa*, *E. coli*, *S. faecalis*, and many other bacteria. Because of the wide spectrum of activity, the substance was not called a bacteriocin.

L. helveticus strain LP27, a homofermentative lactobacillus obtained from human saliva, produces a bacteriocin with properties quite similar to the bacteriocin from the heterofermentative *L. fermenti* (9). Both bacteriocins are heat stable, inactivated by certain proteolytic enzymes, diffusible in agar, nondialyzable, and have a narrow spectrum of activity.

The amino acid composition of purified lactocin 27 is quite similar to that of the bacteriocin produced by *L. fermenti* (9). Both have traces of methionine and a comparatively high content of glycine, alanine, and asparate. Cysteine (and cystine) appears to be absent in both bacteriocins.

There is one apparent difference between the bacteriocins of *L. fermenti* and strain LP27. DeKlerk and Smit (9) were unable to dissociate an active protein from the large molecular weight lipocarbohydrate-protein complex by phenol treatment or hydrolytic techniques and concluded that the integrity of the complex was essential for activity (9). SDS was not employed, however, in these studies. Lactocin 27, on the other hand, appears to be a small molecular weight glycoprotein.

Whether the bacteriocin from LP27, as well as the bacterocins from other gram-positive bacteria, can be isolated as pure proteins is difficult to predict, but it is interesting to note what has happened with the colicins. The colicins were initially thought to be large molecular weight lipopolysaccharide-protein complexes (13, 16). Herschman and Helinski (15), however, isolated colicins E_2 and E_3 as proteins from mitomycin C-induced cultures. Later, Tsao and Goebel (29) showed that mitomycin C induces *E. coli* K235 to produce an unconjugated colicin K protein, whereas the noninduced cells produced the bacteriocin as a large complex resembling the somatic antigen of the cells. The lipopolysaccharide-protein complex obtained from noninduced cultures does not appear to be an artifact of the isolation procedure and has been considered the "natural" form of the bacteriocin. Thus, the chemical composition of a bacteriocin may depend in part on whether the culture is inducible. Preliminary attempts to induce strain LP27 with mitomycin C or ultraviolet light have failed. However, if methods could be found to induce bacteriocinogenic strains of lactobacilli, it is possible that the lactocins might also be released and recovered as unconjugated proteins.

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