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Plasmid-associated Bacteriocin Production by *Leuconostoc* sp. LAB145-3A Isolated from Kimchi

CHOI, YEON-OK AND CHEOL AHN*

Division of Food and Biotechnology, Kangwon National University, Chuncheon 200-701, Korea

Leuconostoc sp. LAB145-3A isolated from kimchi produced a bacteriocin which was active against food pathogens, such as *Listeria monocytogenes*, *Enterococcus faecalis*, and *E. faecium*. Bacteriocin production occurred during the early exponential phase of growth and was stable upto the late stationary phase of growth. Optimum conditions for bacteriocin production were 37°C with an initial pH of 7.0. The bacteriocin of LAB145-3A was sensitive to proteases, but stable for solvents, pH change and heat treatment. It was stable even at autoclaving temperature for 15 min. The bacteriocin exhibited a bactericidal mode of action against *Lactobacillus curvatus* LAB170-12. The bacteriocin produced by *Leuconostoc* sp. LAB145-3A was purified by CM-cellulose cation exchange column chromatography and Sephadex G-50 gel filtration. The purification resulted in an approximate 10,000-fold increase in the specific activity. Approximately 4% of the initial activity was recovered. Purified bacteriocin exhibited a single band on the SDS-PAGE with an apparent molecular weight of 4,400 daltons. This bacteriocin was named leucocin K. *Leuconostoc* sp. LAB145-3A had two residential plasmids with molecular sizes of 23 kb and 48 kb. A comparison of plasmid profiles between LAB145-3A and its mutants revealed that the 23 kb plasmid (pCA23) was responsible for bacteriocin production and immunity to the bacteriocin in *Leuconostoc* sp. LAB145-3A.

Lactic acid bacteria can be characterized as gram-positive, catalase-negative non-sporing bacteria. The main fermentation product of the bacteria from carbohydrate is lactate. They include the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, *Carnobacterium*, *Propionibacterium*, and *Bifidobacterium* (23, 24) and they are the main microflora of dairy, meat, and fermented foods. They also play an important role for preservation of fermented foods, which is usually achieved by inhibition of contaminating spoilage bacteria, such as *Pseudomonas*, and pathogens, such as *Staphylococcus aureus*, *Salmonella* spp., and *Listeria monocytogenes*. Factors contributing to the antibiosis of lactic acid bacteria against other microorganisms include organic acids, such as lactic acid and acetic acid, alcohols, carbon dioxide (6), aldehyde, hydrogen peroxide (4), diacetyl, ammonia, and bacteriocins (12, 20).

Bacteriocins are defined as proteins or protein complexes with bactericidal activity directed against bacteria which are closely related to the producer strain (12, 20). A number of bacteriocins produced by lactic acid bacteria have been identified, but only a few have been purified and characterized (1, 13). The best known is nisin, a 3,400 dalton polypeptide produced by *Lactococcus lactis*. Its structure was established by Gross and Morell (8). Three bacteriocins, lactococcin A, B, and M, have been identified in *Lactococcus lactis* ssp. *cremoris* and their genes have been sequenced (11). Hastings *et al.* (10) cloned the gene for leucocin A UAL187 of *Leuconostoc gelidum*. The lactacin F produced by *Lactobacillus acidophilus* has been sequenced by Muriana and Klaenhammer (18). Two bacteriocins from *Lactobacillus sake*, lactocin S and sakacin P, have been purified and sequenced using N-terminal amino acid determination (17).

Location of the genetic determinants for bacteriocin production is important for efficient manipulation of the genes (1, 13, 19). Bacteriocinogenicity would be an additional property of the microorganism for domination of the ecosystem. Therefore, its genetic determinants are expected to be extrachromosomal. In fact, many bacteriocins have been reported to be produced by a plasmid-associated mechanism (1-3, 13, 19). The genes of the plasmid usually form operon structures or gene blocks for the functional complements (7, 19)

Search for new bacteriocins is of great significance in

*Corresponding author
Phone: 82-361-250-6483. Fax: 82-361-241-3696.
E-mail: ahncheol@cc.kangwon.ac.kr
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development of a start culture. Since bacteriocin-producing lactic acid bacteria are frequently isolated from dairy, meat, and fermented food, bacteriocins are already in use and should be acceptable per se as a food additive. Recently, bacteriocins have become important as a new kind of biopreservative or as a bioregulator of microflora in various fermented food systems (20).

Lactic acid bacteria in traditionally fermented Korean foods, such as kimchi, have not been as well defined as those used in dairy products in Euro-American countries. Only recently have they been subject to extensive research (14). The species of lactic acid bacteria which become predominant in fermentation of kimchi can influence its storage characteristics and quality of the product. In this context the industrial importance and value of bacteriocins in fermentation and preservation of kimchi have been underestimated.

We isolated bacteriocin-producing *Leuconostoc* sp. LAB145-3A from kimchi and discovered that its bacteriocin had a broad spectrum of antimicrobial activity against gram-positive pathogens. We identified the producer strain, characterized its mode of bacteriocin production, and documented the molecular characteristics of the bacteriocin. Finally, we found that the genetic determinants of bacteriocin production were located on one of the residential plasmids.

MATERIALS AND METHODS

Isolation of a Bacteriocin-producing *Leuconostoc* Strain from Kimchi

Leuconostoc sp. LAB145-3A was isolated from naturally fermented kimchi by spreading the supernatant of kimchi onto modified MRS agar supplemented with 5% sucrose (SMRS agar) and incubating anaerobically at 25°C. Colonies showing dextran formation were tested for their production of antagonistic substances against selected indicator microorganisms. *Lactobacillus delbrueckii-lactis* ATCC 4797 and *Lactobacillus curvatus* LAB170-12 were routinely used as indicator strains. Other bacterial strains which were used as indicator organisms are listed in Table 1. *Leuconostoc* sp. LAB145-3A was inoculated into MRS broth at 1% (v/v) level and propagated at 25°C. Stock cultures were maintained at -80°C in MRS broth containing 33% glycerol. Working cultures were subcultured twice in MRS broth and incubated at 25°C for 24 h before use.

Bacteriocin Detection and Activity Assay

For detection of antagonistic activity, both the deferred method and the spot-on-lawn method were used. The deferred method was a modification of those described previously (2, 9, 24). An overnight culture was spotted onto the surface of an MRS agar plate and incubated for 24 h at 25°C to allow colonies to develop. Anaerobic conditions were used to minimize formation of hydrogen peroxide. Seven ml of soft MRS agar containing 5×10^7 CFU of the indicator strain in 0.75% agar was poured over the plate. After incubation at the optimum growth temperature of the indicator strain the plates were checked for formation of inhibition zones. Inhibition was scored as positive if a clear zone around the colonies of the producer strain appeared. For the spot-on-lawn assay, MRS agar plates were overlaid with 7 ml of soft MRS agar containing 5×10^7 CFU of the indicator strain. Ten μl of cell-free supernatant of the potential producer strain was spotted onto the overlaid surface. The plates were incubated anaerobically for 24 h at the growth temperature of the indicator strain and subsequently examined for inhibition zones.

The activity of bacteriocin was assayed by spotting two-fold serial dilutions of supernatant onto the indicator lawn. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution showing an inhibition zone.

Identification of Bacteriocin-producing Strains Isolated from Kimchi

A bacteriocin-producing strain was identified by criteria based upon carbohydrate fermentation patterns and biochemical characteristics (21-24). The results were confirmed using an API 50CHL system (BioMerieux Vitek, Inc., U.S.A.).

Production of Bacteriocin

Leuconostoc sp. LAB145-3A was grown in MRS broth at pH values of 5.0, 6.0, and 7.0 with 3 N HCl and 2 N NaOH. Individual sets of pH-adjusted broths were tempered at 15, 25, and 37°C for 1 h before adding 1% of an overnight culture. During subsequent incubation, aliquots of broths were removed at specific time intervals and examined for bacterial growth (optical density at 600 nm), pH change, and bacteriocin production.

Sensitivity of Bacteriocin to Various Enzymes, Organic Solvents, pH Changes, and Heat Treatment

A partially purified bacteriocin was prepared by ammonium sulfate precipitation and dialysis as described previously (2). The bacteriocin was treated with various enzymes, including trypsin, pronase E, pepsin, β-amylase, lipase, ribonuclease A, and lysozyme. It was also treated with 50% organic solvents, such as ethanol, methanol, chloroform, toluene, ethylacetate, acetone, and acetonitrile.

Enzymes were added to 30 μl of the partially purified bacteriocin (1,600 AU/ml) to obtain a final concentration of 1 mg/ml. The mixture was incubated at 37°C for 1 h. A solvent-treated sample was also incubated at 25°C for 1 h. At the end of the incubation the residual activity of the bacteriocin was assayed by the spot-on-lawn method as described above.

The stability of partially purified bacteriocin at dif-

ferent pH levels was determined by dialysis against 1 liter of 10 mM glycine-HCl (pH 2), 10 mM citrate buffer (pH 3, 4, 5, and 6), 10 mM phosphate buffer (pH 7), 10 mM Tris-Cl (pH 8.0), and 10 mM glycine-NaOH (pH 9 and 10). Partially purified bacteriocin (1,600 AU/ml) was heated at both 60°C and 100°C for 30 min, and 121°C for 15 min in phosphate buffer (50 mM, pH 7), and tested for its residual activity using the spot-on-lawn test.

Mode of Bacteriocin Action

Partially purified bacteriocin was added to a sterile phosphate buffer (10 mM, pH 7.0) containing approximately 5×10^7 CFU of LAB170-12 per ml with final bacteriocin concentrations of 5, 10, 15, and 50 AU/ml. Viable cell numbers were monitored periodically. Indicator cells without bacteriocin and those treated with pronase E (1 mg/ml of reaction mixture) were used as experimental controls. Pronase E was also added to cells treated with 10 AU of bacteriocin per ml after 30 min of reaction time.

Purification of Bacteriocin

An overnight culture of *Leuconostoc* sp. LAB145-3A was centrifuged at 8,000 rpm for 15 min at 4°C. Aliquots (700 ml) of culture supernatants held at 4°C were precipitated with ammonium sulfate to 75% saturation cut. The precipitate was centrifuged at 8,000 rpm for 15 min at 4°C. The pellet was resuspended in 15 ml of 10 mM sodium citrate buffer (pH 6.0) and dialyzed against 2 liter of the same buffer for 24 h in dialysis tubing (molecular weight cutoff, 12,000; Sigma). The dialysate was applied to a carboxymethyl-cellulose column (3×16 cm) which had been equilibrated with 10 mM sodium citrate buffer (pH 6.0). After initial washing with 10 mM sodium citrate buffer (pH 6.0), bacteriocin activity was eluted with a linear gradient of NaCl from 0 to 1.0 M (2×300 ml) in the same buffer. Fractions of 5 ml were collected at a flow rate of 20 ml/h. Active fractions were collected, then dialyzed against 2 liter of the same buffer for 24 h, then lyophilized. The lyophilized bacteriocin was dissolved in 10 mM sodium citrate buffer, then loaded onto a Sephadex G-50 column (1×90 cm) which had been equilibrated with 10 mM sodium citrate buffer (pH 6.0). The sample was eluted with the same buffer at a flow rate of 10 ml/h and 4.5 ml fractions were collected. The active fractions were combined (13.5 ml) and concentrated by lyophilization.

The protein concentration was determined by the method of Lowry *et al.* (15) with bovine serum albumin as the standard. The specific activity of the bacteriocin solution was defined as the number of bacteriocin units per mg of protein. The absorbance at 280 nm was used to monitor the elution of proteins on column chromatography.

Determination of Molecular Weight of Bacteriocin by SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 15% discontinuous gel was performed using the method of H. Daba *et al.* (5). with a Mini-Protean II electrophoresis system (Bio-Rad). Activity staining was performed with one duplicate gel which was fixed in 20% isopropanol and 10% acetic acid for 2 h, then washed in sterilized distilled water for 4 h (5). This gel was aseptically placed on an MRS agar plate, then overlaid with 7 ml of MRS soft agar containing approximately 5×10^7 cells/ml of the indicator strain. The plate was incubated at 25°C for 24 h and examined for inhibition zones.

Plasmid Isolation

Miniscale extraction of plasmids was done by modification of the method of Ahn *et al.* (2, 7). One and one half ml of overnight culture grown in MRS broth at 25°C was used. Agarose gel electrophoresis was conducted on 0.7% agarose in a horizontal slab gel in TAE buffer (pH 8.0) at 70 V for 150 min. Purified plasmids of *E. coli* V517 were used as standards for molecular weight determination, as described by Macrina *et al.* (16).

Plasmid Curing and Mutant Screening

One hundred μl of the overnight culture in MRS broth was mixed with 10 ml of fresh MRS broth containing 20 μg/ml of acridine orange. The culture was incubated at 40°C for 24 h. For selection of non-bacteriocinogenic mutants, the stationary culture was diluted and plated onto MRS agar. After 1~2 days of incubation, pairs of replica-plates were prepared, one of which was overlaid with soft agar containing the indicator organism. After incubation at 25°C colonies that did not produce bacteriocin were screened.

RESULTS AND DISCUSSION

Isolation of *Leuconostoc* from Kimchi and Screening for Production of Antibacterial Compounds

Strains forming slime on an SMRS agar surface were tested for antagonistic activity. Among 131 isolates, LAB145-3A was found to produce an antibacterial compound effective against a wide range of indicator strains. The antibacterial compound produced by LAB145-3A was a bacteriocin which was sensitive to protease treatment (Fig. 1). The antibacterial spectrum of the bacteriocin produced by LAB145-3A on MRS agar plates against indicator strains is summarized in Table 1. The antibacterial activity was further confirmed using 10 μl of partially purified bacteriocin by the spot-on-lawn method (Table 1).

The bacteriocin of strain LAB145-3A was active not only against other lactic acid bacteria, including *Lactobacillus*, *Leuconostoc*, and *Carnobacterium*, but also against food pathogens, such as *Listeria monocytogenes*,

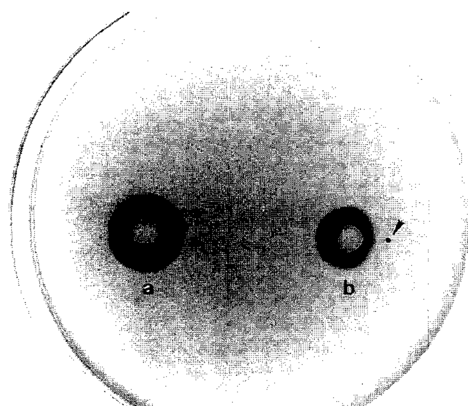

Fig. 1. The antagonistic activity of *Leuconostoc* sp. LAB145-3A against the indicator strain *Lactobacillus curvatus* LAB 170-12, and the effect of protease treatment on the antagonistic activity.
a, antagonistic activity without protease treatment; b, antagonistic activity when treated with protease at the spot indicated by the arrow.

Enterococcus faecalis, and *E. faecium*. However, it was not active against gram-negative bacteria. Effective inhibition which was identified by the deferred method against gram-negative bacteria appeared to be due to other antibiosis factors, such as acidity or hydrogen peroxide. Additional activity against *Leuconostoc* and *Canobacterium* was observed when partially purified bacteriocin was applied. It may have been due to an increase in the effective concentration of bacteriocin.

Identification of Strain LAB145-3A

Strain LAB145-3A was gram-positive, catalase-negative, formed slime from sucrose, and did not hydrolyze arginine. Based upon its biochemical characteristics and its carbohydrate fermentation patterns described by Schillinger and Lucke (21) (data not shown), it was classified as *Leuconostoc* species. The lactic acid bacteria identification system using API 50CHL gave the same results.

Production of Bacteriocin

The effect of temperature and the initial pH of the medium on the production of bacteriocin was monitored at various conditions in a time-dependent fashion. Fig. 2 shows growth, pH change, and production of bacteriocin by LAB145-3A incubated at 37°C in MRS broth adjusted to a pH range of 5.0 to 7.0. Optimum production of bacteriocin was achieved at 37°C with an initial pH of 7.0 (Fig. 3). At this optimum condition the maximum concentration of inhibitory substance (800 AU/ml) was reached after 9 h of incubation, in the late exponential or early stationary phase of growth. Bacteriocin remained active well into the stationary phase, as indicated in Fig. 2.

The maximum concentration of 800 AU/ml was also achieved by growing LAB145-3A at 25°C. However, the time required to reach the maximum production at 25°C was 12 h, compared to 9 h at 37°C. The efficient bacteriocin production at 25°C seemed to be due to original environment of the strain which was isolated by incubation at 25°C. Bacteriocin production by LAB145-3A was detected as early as the 6th hour, coinciding with cell growth.

Table 1. Antibacterial spectrum of the bacteriocin produced by *Leuconostoc* sp. LAB145-3A on a solid agar surface (deferred method), and as a partially purified bacteriocin.

Indicator strains	Deferred method	Partially purified bacteriocin
Lactobacillus curvatus LAB170-12	+[a]	+
Lactobacillus acidophilus VPI11088	−	−
Lactobacillus delbrueckii-lactis ATCC 4797	+	+
Lactobacillus gasseri ATCC 33323	−	−
Lactobacillus brevis (LB)50	−	−
Lactococcus lactis LM0230	−	−
Leuconostoc gelidum UAL187	−	+
Pediococcus pentosaceus FBB61-2	−	−
Carnobacterium piscicola LV17	−	+
Carnobacterium divergens LV13	+	+
Carnobacterium piscicola UAL26	+	+
Listeria monocytogenes ATCC 1911	+	+
Staphylococcus aureus ATCC 25823	−	−
Enterococcus faecalis ATCC 19433	+	+
Enterococcus faecium ATCC 11576	+	+
Escherichia coli ATCC 25922	+	−
Salmonella typhimurium ATCC 14028	+	−
Pseudomonas aeruginosa ATCC 27853	+	−
Pseudomonas fluorescence ATCC 21541	+	−

[a] +, indicator strain inhibited by the bacteriocin; −, indicator strain not inhibited by the bacteriocin.

The specific activity of LAB145-3A bacteriocin was not high when compared to the specific activity of other bacteriocins against the same indicator strain (2, 10). However, stability of bacteriocin production by LAB145-3A was remarkable when considering the general instability of bacteriocin in the late stationary growth phase (12, 20). Maintenance of stability through the late stationary growth phase has been observed in *Carnobacterium piscicola* LV17 (2). Early production of other bacteriocins has also been observed (2). The early bacteriocin production of LAB145-3A should be exploited as an industrially useful asset.

Sensitivity of Bacteriocin to Various Enzymes, Organic Solvents, pH Changes, and Heat Treatment

The bacteriocin produced by *Leuconostoc* sp. LAB145-3A was partially purified using ammonium sulfate precipitation (75% saturation cut) up to 51,200 AU/ml after dialysis and lyophilization. As summarized in Table 2, the antibacterial activity of partially purified bacteriocin was not affected by treatment with lipase, lysozyme, β-

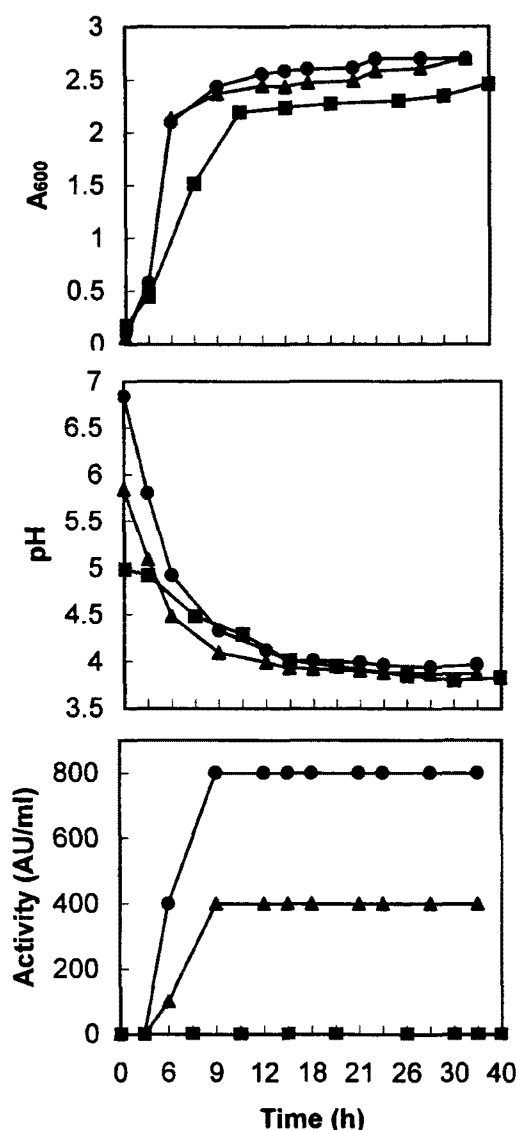

Fig. 2. The mode of bacteriocin production of *Leuconostoc* sp. LAB145-3A at 37°C with initial pH of 5.0, 6.0, and 7.0. The time-dependent change of bacteriocin activity was monitored together with the change in pH and cell density. —■—, pH 5; —▲—, pH 6; —●—, pH 7.

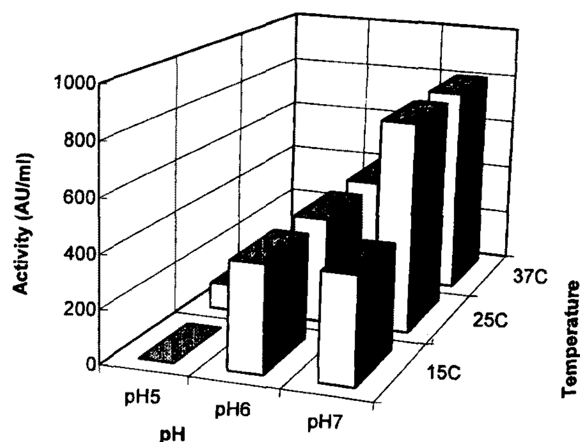

Fig. 3. Optimum conditions for bacteriocin production by *Leuconostoc* sp. LAB145-3A.

Table 2. Effect of various enzymes, organic solvents, pH, and heat treatment on the stability of bacteriocin produced by *Leuconostoc* sp. LAB145-3A.

Treatment		Residual activity[c] (AU/ml)
Enzymes[a]	Pronase	0
	Pepsin	0
	Trypsin	0
	Lysozyme	1600
	β-Amylase	1600
	Lipase	1600
	RNase A	1600
Solvents[b]	Ethanol	1600
	Methanol	1600
	Acetone	1600
	Acetonitrile	1600
	Chloroform	1600
	Toluene	1600
	Ethyl acetate	1600
pH	pH 2 to 10	1600
Heat	60°C for 30 min	1600
	100°C for 30 min	800
	121°C for 15 min	400

[a]The final enzyme concentration was 1 mg/ml. [b]50% (vol/vol) concentration was used. [c]The residual activity of untreated bacteriocin was 1600 AU/ml.

amylase, ribonuclease A, or any of the organic solvents tested. However, activity was completely lost after treatment with pronase, pepsin, or trypsin, suggesting a proteinaceous nature for the bacteriocin.

The bacteriocin was pH-stable, showing activity unchanged over a wide pH range from 2.0 to 10.0. It was also stable to heat treatment at 65°C for 30 min. After it was boiled at 100°C for 30 min, 50% of its activity remained, while 25% of the initial activity remained even after autoclaving at 121°C for 15 min (Table 2). This molecular stability of LAB145-3A bacteriocin agrees with the characteristics of class II bacteriocins as defined by Klaenhammer (12).

Mode of Bacteriocin Action

To determine whether the bacteriocin had bactericidal or bacteriostatic effect, various concentrations of the bacteriocin were added to the indicator strain *L. curvatus* LAB170-12 suspended in 10 mM phosphate buffer (pH 7.0). The number of viable indicator cells was determined by plate counting at specific times after addition

of bacteriocin. As shown in Fig. 4, addition of bacteriocin upto 50 AU/ml reduced the viable cell count to less than one within 30 min, indicating that the bacteriocin of LAB145-3A acted in a bactericidal fashion against LAB170-12. However, the lethal bacteriocin effect was immediately reversed when pronase was added to a reaction mixture containing 10 AU bacteriocin per ml after 30 min of bacteriocin application (Fig. 4).

The intrinsic nature of the bactericidal mode of inhibition exhibited by LAB145-3A has not yet been identified. On scanning electron-microscopy no indication of cell lysis was observed. Rescuing effect of protease to counter the bactericidal effect of the bacteriocin may be owing to either inactivation of bacteriocin at the cell surface, or reduction of the effective bacteriocin concentration in the medium (20), both of which require further investigation.

Purification of Bacteriocin

A supernatant of the culture broth, which had been incubated at the optimum conditions for bacteriocin production, was concentrated by ammonium sulfate precipitation (75% saturation cut). Partially purified bacteriocin was applied to a carboxy-methyl cellulose cation exchange column and eluted with a linear salt gradient (0 to 1.0 M NaCl), as shown in Fig. 5. After passage through the column, 43.0% of the bacteriocin activity was recovered and the specific activity was increased 557 fold (Table 3). Active fractions from the column were concentrated and subjected to gel filtration on a Sephadex G-50 column (Fig. 6). The active fractions were collected and lyophilized, resulting in a 909 fold increase in the specific activity, as summarized in Table 3. The activity recovery was 4.0%.

Molecular Weight Determination of the Bacteriocin

The relative molecular weight and purity of the purified bacteriocin were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). When the purified bacteriocin was analyzed by SDS-PAGE, a single band was detected, migrating with a molecular weight of approximately 4,400 daltons (Fig. 7).

The authenticity of the purified protein as a bacteriocin was confirmed by activity-staining of one of the duplicate

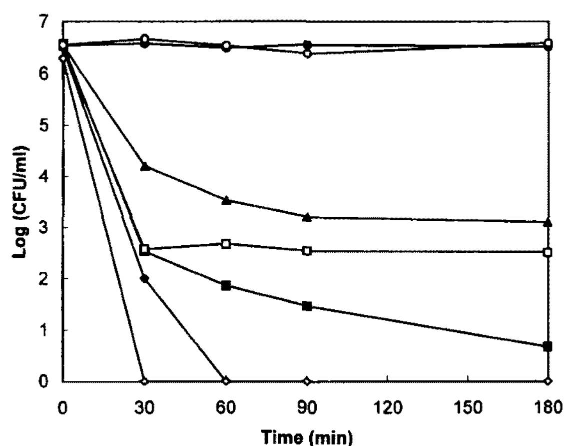

Fig. 4. Mode of inhibitory action of the bacteriocin produced by *Leuconostoc* sp. LAB145-3A.

The effect of bacteriocin on the survival rate of *L. curvatus* LAB170-12 was analyzed by adding 5 AU (▲), 10 AU (■), 15 AU (◆), and 50 AU (◇) of partially purified bacteriocin per ml of cell suspension in 10 mM phosphate buffer (pH 7.0). The CFU of the cells was monitored every 30 min. Experimental control without bacteriocin treatment (●), another experimental control with protease-treated bacteriocin (○), and cells treated with protease after 30 min of bacteriocin application (□) were also tried.

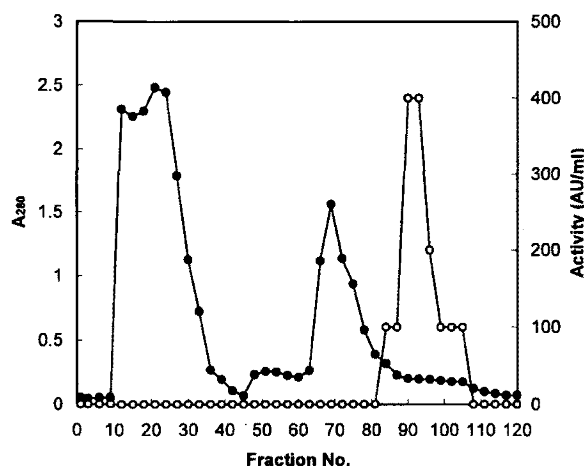

Fig. 5. Elution profile of partially purified bacteriocin from a CM-cellulose column equilibrated with 10 mM citrate buffer (pH 6.0).

Bacteriocin was eluted with linear gradient of NaCl from 0 to 1.0 M in the same buffer. The bacteriocin activity of each fraction (5 ml) was assayed by the spot-on-lawn method. (●) A_{280}, (○) bacteriocin activity (AU/ml).

Table 3. Purification of bacteriocin produced by *Leuconostoc* sp. LAB145-3A.

Purification step	Total activity (AU)	Total protein (mg)	Specific activity (AU/mg)	Fold purification	Activity recovered (%)
Culture supernatant	140,000.0	12,775.0	11.0	1.0	100.0
$(NH_4)_2SO_4$ precipitation	76,800.0	153.6	500.0	45.5	55.0
Ion exchange CM-cellulose	60,000.0	9.8	6,122.4	556.6	43.0
Gel filtration Sephadex G-50	5,400.0	0.54	10,000.0	909.1	3.9

gels using *L. curvatus* LAB170-12 as an indicator, as illustrated in panel B, Fig. 7. Therefore, the antibacterial protein produced by *Leuconostoc* sp. LAB145-3A was named leucocin K.

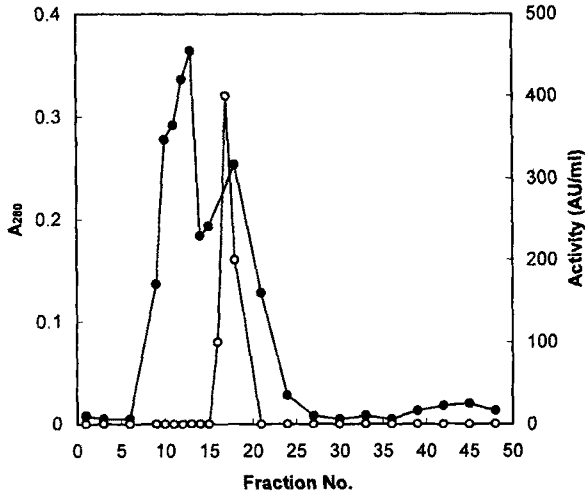

Fig. 6. Elution profile of bacteriocin from a Sephadex G-50 column equilibrated with 10 mM citrate buffer (pH 6.0).
Bacteriocin was eluted with the same buffer. The bacteriocin activity of each fraction (5 ml) was determined by the spot-on-lawn method. (●) A_{280}, (○) bacteriocin activity (AU/ml).

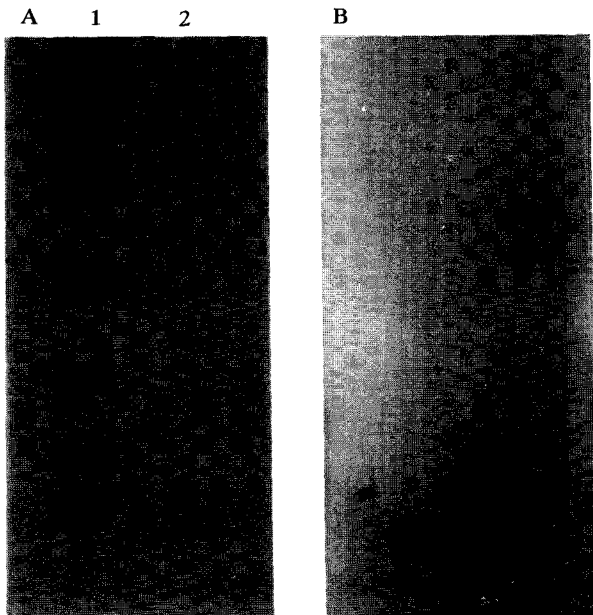

Fig. 7. Polyacrylamide gel (15%) electrophoresis of purified bacteriocin.
Panel A: Gel was fixed with 5% formaldehyde and stained with Coomassie brilliant blue R-250. Lane 1, broad molecular weight standards (Bio-Rad); lane 2, purified bacteriocin (8 µl). The arrow indicates location of the bacteriocin. Panel B: Activity-stained gel. The twin gels of panel A were overlaid with indicator strain LAB170-12 to confirm the bacteriocin band.

Plasmid Curing and Mutant Screening

Leuconostoc sp. LAB145-3A showed two residential plasmids of 48 kb and 23 kb, as shown in Fig. 8. These two residential plasmids were tentatively named pCA48 (48 kb) and pCA23 (23 kb). In order to relate the presence of plasmids to the bacteriocinogenicity of LAB145-3A, curing of the residential plasmids was performed. After three consecutive transfers (every 24 h) in the presence of 20 µg of acridine orange per ml of MRS broth, Bac⁻ variants among the leucocin K producing colonies of strain LAB145-3A were screened.

The plasmid profile of the mutant strain showed loss of the 23 kb plasmid (pCA23) (Fig. 8), but not the 48 kb plasmid (pCA48). This loss of pCA23 was correlated with the loss of bacteriocinogenicity, as shown in Fig. 9. Loss of bacteriocinogenicity was also correlated with loss of immunity to bacteriocin. All the other physiological characteristics of the Bac⁻ mutant, including carbohydrate fermentation patterns and resistance to antibiotics, did not differ from the bacteriocin-producing

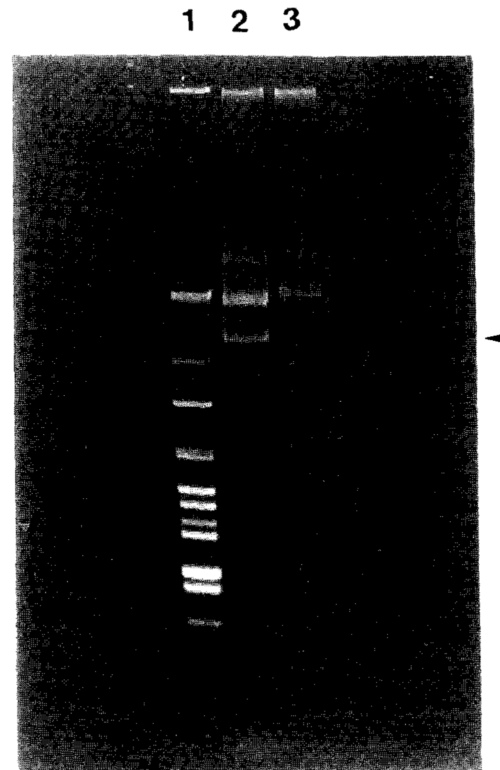

Fig. 8. The plasmid profile of *Leuconostoc* sp. LAB145-3A and its mutant.
Lane 1, Molecular size markers prepared from *E. coli* V517; lane 2, wild type strain of *Leuconostoc* sp. LAB145-3A; lane 3, Bac⁻ mutant strain of *Leuconostoc* sp. LAB145-3A. chr: chromosomal band. The 23-kb bacteriocin plasmid is indicated by an arrow.

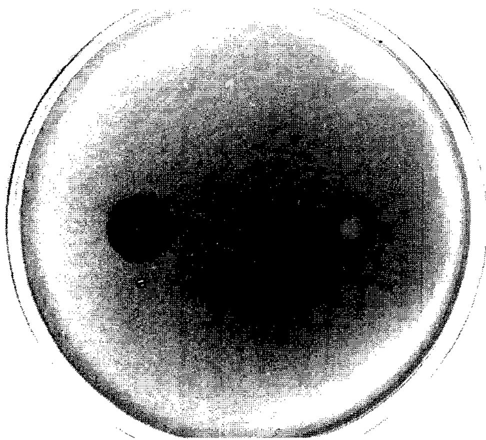

Fig. 9. Loss of bacteriocin activity in the mutant strain which lost the 23 kb plasmid.
a, Bac⁺ *Leuconostoc* sp. LAB145-3A; b, Bac⁻ *Leuconostoc* sp. LAB145-3A.

parent strain. Therefore, it appears that bacteriocin production and immunity to bacteriocin in *Leuconostoc* sp. LAB145-3A is mediated by the 23-kb residential plasmid, pCA23.

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