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# Characterization of Bacteriocin Production by *Lactococcus lactis* LAB3113 Isolated from Kimchi

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Abstract

**A lactic acid bacterium, LAB3113, isolated from traditionally fermented Kimchi was found to produce bacteriocin whose activity was very specific toward lactobacilli and not effective against any other bacteria. Lactobacilli affected by the inhibitory substance included *Lactobacillus delbrueckii-lactis*, *L. johnsonii*, *L. gasseri*, and *L. curvatus*. Based upon biochemical and physiological characteristics, LAB3113 was classified as *Lactococcus lactis*, and its bacteriocin was named as lactococcin K3113. *Lactococcus lactis* LAB3113 produced bacteriocin at the early stage of growth and the concentration of the bacteriocin did not decrease even after late stationary phase. Optimal temperature of bacteriocin production was 25°C at the initial pH 7.0. Partially purified lactococcin K3113 was completely inactivated by protease, but not affected by lipase, lysozyme and RNase. The bacteriocin was very heat-stable even after autoclaving for 20min. It was also stable in pH changes, and was not affected by the presence of solvents. Lactococcin K3113 appeared to act in bactericidal mode against *L. delbrueckii-lactis* ATCC4797. Molecular weight of lactococcin K3113 was calibrated as 10,500dal by SDS-PAGE and activity staining. *Lactococcus lactis* LAB3113 had four residential plasmids of 3.7kb, 11.2kb, 15.5kb, and 48kb in molecular sizes. Plasmid profile analysis of mutant strain revealed that 15.5kb plasmid was re-sponsible for the production of lactococcin K3113 and its immunity to the bacteriocin.**

Key words: bacteriocin production, *Lactococcus*, lactococcin K3113, Kimchi, plasmid

## INTRODUCTION

Lactic acid bacteria(LAB) are a group of genetically diverse, but functionally related bacteria which have played very important roles in the fermentation of foods in human history. Fermentation has two aspects in essence; conversion of substrate into useful metabolic products and preservation of food for longer period of time. Lactic acid bacteria have served for both of the purposes. As for the preservation of food, lactic acid bacteria can contribute to the purposes by producing a variety of antimicrobial compounds including lactic and acetic acids, hydrogen peroxide(1), diacetyl, carbon dioxide(2), alcohol, aldehyde, and most importantly bacteriocins(3, 4), all of which can antagonize growth of spolage bacteria and pathogenic bacteria in food environment.

Bacteriocins are antibacterial polypeptide whose antagonistic activity is usually directed to closely related species(3,4). Even though their antibacterial efficiency and spectrum are lower and narrower than those of medicinal antibiotics, their specificity of antagonism and easy digestibility in human digestive tract made them good candidate molecules for natural food preservative or natural bioregulator of food fermentation(3). Furthermore, because their biosynthetic production was determined by their own genes, they can be easily manipulated at the genetic level(3,5,6) and this would give plenty of rooms for industrial modification and application of bacteriocins.

With the reasons stated above, research efforts have been concentrated to screening good bacteriocins with broader spectrum and improving their production efficiency. In that context, nisin would be the best model for bacteriocin biotechnology(3), and it is the only bacteriocin which has been commercially available and successful. So far, more than hundreds of new bacteriocins have been reported and studied(7).

Based upon biochemical and genetic data accumulated, bacteriocins are classified into four major classes(3). Class I bacteriocins include lanthionine-containing bacteriocins such as nisin, while class II encompasses small hydrophobic bacteriocins such as lactococcins, leucocins and lactacin F(8-10). ClassIII bacteriocins are large, heat-labile molecules, and classIV bacteriocins are com-

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plex protein molecules with non-protein moiety(11,12).

Lactococcin A, B, and M have been the most thoroughly studied class II bacteriocins(8,10). They are produced by *Lactococcus* species of dairy origin(13,14). Their molecular properties are characterized by heat and pH stability with membrane-active bactericidal mode of action(15,16). The genetic determinants of the bacteriocins are associated with residential plasmids(5, 17) which usually form gene blocks or multiple consecutive operons for bacteriocin production.

Compared to European and American countries, Korea has been known as more resourceful country in lactic acid bacteria(18,19). However, efforts to isolate industrially useful lactic acid bacteria and their molecular products have been little made than it should be. Recently, as the importance of lactic acid bacteria as molecular resources for various end products including bacteriocin is realized between researchers, more fundamental studies have been carried out in the field.

In that contex, we have tried to isolate lactic acid bacteria which can produce bacteriocin in the food environment of Kimchi. In this study, we report the isolation of a *Lactococcus* strain from Kimchi, and its characteristics in bacteriocin production. Molecular characteristics of partially purified bacteriocin, lactococcin K3113, were indentified. Genetic determinants of lactococcin K3113 were also located in this study.

## MATERIALS AND METHODS

### Strains and media

A strain of lactic acid bacteria, LAB3113, was originally isolated from naturally fermented Kimchi by spreading supernatant of Kimchi onto MRS agar plate and screening colonies which exhibited antagonistic activity against *Lactobacillus delbrukii-lactis* ATCC4797 and *Lactobacillus curvatus* KCA170-12. For ordinary use, LAB3113 was inoculated to MRS broth at 1%(v/v) level and propagated at 25°C. LAB3113 was maintained as frozen stock at -80°C in MRS broth containing 33% glycerol. Working cultures were stored in MRS broth at -4°C and subcultured at least twice in MRS before use in experiments.

### Detection and assay bacteriocin activity

Antagonistic substances produced by LAB3113 were detected by deferred method and confirmed by the spot-on-lawn method(20). For deferred method, overnight culture of LAB3113 was spot-inoculated onto the surface of MRS agar plate, incubated at 25°C for 24hr to allow colonies to appear, and then overlayered with 7ml of soft agar containing $5\times10^7$CFU of *L. delbrueckii-lactis* ATCC4797 per ml of 0.75% agar. After another overnight's incubation at 25°C, formation of clear zone around the colonies was checked. Formation of larger than 5mm in diameter was scored positive in the production of antagonistic substances or bacteriocins. For spot-on-lawn assay, MRS agar plates were overlayered with 7ml of soft MRS agar as described above. Activity of bacteriocin was assayed by spotting serial double dilutions of supernatant onto indicator lawn as in spot-on-lawn method. 10μl of cell-free supernatant of the potential producer strain was spotted on this agar plate. The reciprocal of the greatest inhibitory dilution was used to calculate arbitary activity units(AU) per ml.

### Identification of LAB3113

LAB3113 was identified based upon procedures described by Shaw and Harding(21) and Schillinger and Lucke(22). Bacterial identification system of API 50CHL (bioMerieux Vitek, Inc., U.S.A.) was also used to complement manual information.

### Production of bacteriocin

To characterize mode of bacteriocin production during cell growth and to calibrate optimal condition for maximal production of bacteriocin, LAB3113 was grown in MRS broth, initial pH of which was adjusted to pH 4.0, 5.0, 6.0, or 7.0, respectively, using 5N HCl or 10N NaOH. Each MRS broth was incubated at 15°C, 25°C, or 37°C, and monitored for cell growth(OD at 600nm), pH change, and bacteriocin production.

### Preparation of partially purified bacteriocin

LAB3113 was grown to the stationary phase by incubating at 25°C for 18hr in MRS. Supernatant of culture broth was obtained by centrifugation(6000rpm for 15min) and passed through cellulose nitrate filter with 0.45μm pore-size. Ammonium sulfate was added upto 75% saturation cut. After stirring for 24hr at 4°C, precipitate was collected by centrifugation and then dialyzed against 10mM phosphate buffer(pH 7.0) for 24hr at 4°C, using membrane tubing with molecular weight cut-off of 12,000 dalton. Dialyzed bacteriocin solution was filter-

sterilized and lyophilized. After resuspension in the sterilized Mili-Q water, it was stored at -20°C for future use.

### Sensitivity of bacteriocin to various enzymes and organic solvents

Patially purified bacteriocin(409,000AU/ml) was treated with various enzymes, including trypsin, pronase E, pepsin, β-amylase, lipase, ribonuclase A, and lysozyme. Partially purified bacteriocin was also treated with 50% organic solvents such as ethanol, methanol, chloroform, toluene, ethylacetate, acetone, and acetonitrile.

Each enzyme was added to 50μl of the partially purified bacteriocin to obtain final concentration of 1mg/ml. Reaction mixture was then incubated at 37°C for 1hr. Solvent-treated sample was also incubated at 25°C for 1hr. At the end of incubation, the residual activity of bacteriocin was assayed by spot-on-lawn method.

### Stability of bacteriocin to heat and pH

Patially purified bacteriocin(409,000AU/ml) was heated at 60°C for 30min, at 100°C for 30min, and at 121°C for 20min, and tested for its residual activity. Stability of partially purified bacteriocin at different pH levels was determined by dialyzing against 1L of each buffer from pH 2.0 to 10.0. Each buffer was prepared as follows: 10mM glycine-HCl(pH 2.0), 10mM citrate buffer (pH 3.0 to 6.0), 10mM phosphate buffer(pH 7.0), 10mM tris-Cl(pH 8.0), and 10mM glycine-NaOH(pH 9.0 and 10,0).

### Mode of inhibition

Partially purified bacteriocin was added to sterile phosphate buffer(10mM pH 7.0) containing $10^7$CFU of *Lactobacillus curvatus* KCA170-12 per ml. Bacteriocin was added to final concentrations of 3, 6, and 25AU/ml and was incubated at 25°C. Viable counts of *L. curvacus* KCA170-12 were determined on MRS agar every 30min. To test the effect of protease on the mode of action by bacteriocin, protease(final concentration 1mg/ml) was added to the reaction mixture of 6AU-treated cells after 30min of bacteriocin application.

### Calibration of apparent molecular weight of bacteriocin

8μl of partially purified bacteriocins was electrophorized by sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) in a 15% discontinuous gel with Mini-Protean II kit(Bio-Rad). After 2hr of deployment at 20mA, the gel was removed and cut into two vertical parts. One half of the gel, containing one sample and the molecular weight standards, was stained with Commassie brilliant blue R250. The another half, containing the same sample, was activity-stained with indicator microorganism by the method of Bhune et al. (23) with following modifications. The gel was fixed immediately by 2hr treatment in 20% isopropanol and 10% acetic acid, and washed for 4hr in sterilized distilled water. The gel was then placed in a petri dish and overlayered with 7ml of 0.75% agar containing $5\times10^7$ cells of the indicator strain. The plate was incubated at 25°C for 24hr, and analyzed for zones of inhibition.

### Plasmid isolation and curing

Miniprep of plasmid was routinely performed by Ahn and Stiles's method(5). Agarose gel electrophoresis was conducted on 0.7% agarose gel in TAE buffer(pH 8.0) at 70V. Plasmid curing was accomplished by growing cells in MRS broth containing 20μg/ml of acridine orange at an elevated temperature(37°C) for three consecutive transfers. For selection of mutants, stationary culture was diluted and plated onto MRS agar. After 1~2 days of incubation, replica-plating was performed. One of the plates was overlayered with soft agar seeded with indicator organisms. After 24hr of incubation at 25°C, colonies that did not produce bacteriocin were screened.

## RESULTS AND DISCUSSION

### Isolation of LAB3113 from Kimchi and detection of bacteriocin production

A lactic acid bacterium of strain number 3113(LAB 3113) was isolated from naturally fermented Kimchi by using MRS as primary selective media. The primary authenticity of the strain as one of lactic acid bacteria was further confirmed by Gram-staining(positive), catalase test(negative), and spore-formation(negative). LAB 3113 was found to produce antibacterial compound which was active against indicator strain used for primary screening, including *L. delbrueckii-lactis* ATCC4797, *Leuconostoc gelidum* UAL187, *Carnobacterium piscicola* LV17, and *L. curvatus* KCA170-12(Fig. 1). To check the effect of protease on the antagonistic activity, clear zone

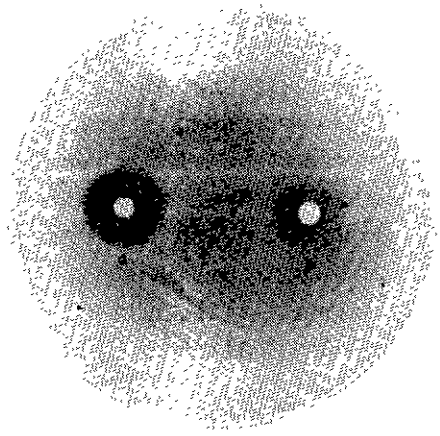

**Fig. 1. Antagonistic activity of *Lactococcus lactis* LAB 3113 against indicator strain *Lactobacillus curvatus* KCA LAB170-12 on the solid surface of MRS agar.**
Effect of protease treatment on the antagonistic activity was illustrated by application of the protease near the colony as indicated by an arrow in the figure.
a: Antagonistic activity without protease treatment
b: Antagonistic activity with protease treatment at the arrow end

around the colony was treated with 1mg/ml of protease. At the treatment, inhibition zone was disappeared as shown in Fig. 1, suggesting it is bacteriocin-like substance. Authenticity of the substance as bacteriocin was further confirmed by additional experiments(see below).

The antimicrobial spectrum of the bacteriocin produced by LAB3113 on MRS agar plates against indicator strains is summarized in Table 1. This was further confirmed by using 10μl of partially purified bacteriocin with the spot-on-lawn methods. The antimicrobial spectrum of strain LAB3113 was remarkably narrow, showing effective inhibition only against genera of *Lactobacillus* including *L. johnsonii, L. delbrueckii-lactis, L. gasseri*, and *L. curvatus*. It did not show activity against any other genera of lactic acid bacteria, and against any other Gram-positive bacteria. This remarkable specificity of inhibition could be a barrier in application of the bacteriocin as a broad food preservative, but can be a most useful asset in case that a lactobacilli-specific bacteriocin is required. In that context, bacteriocin produced by LAB3113 was one of a few bacteriocins which specifically inhibit lactobacilli.

## Identification of LAB3113

Strain LAB3113 did not produce gas from glucose, and did not show any growth at 45°C. It showed growth

**Table 1. Activity spectrum of strain LAB3113 and its bacteriocin**

| Indicator strains | Deferred test[1)] | Partially purified bacteriocin[2)] |
|---|---|---|
| *Lactobacillus* | | |
| *L. johnsonii* VPI11088 | + | + |
| *L. delbrueckii lactis* ATCC4797 | + | + |
| *L. gasseri* ATCC33323 | + | + |
| *L. brevis*(LB)50 | + | − |
| *L. curvatus* KCA170-12 | + | + |
| *Lactococcus lactis* LM0230 | + | − |
| *Leuconostoc gelidum* UAL187 | + | − |
| *Pediococcus pentosaceus* FBB61-2 | − | − |
| *Carnobacterium* | | |
| *C. piscicola* LV17 | + | − |
| *C.divergens* LV13 | + | − |
| *Listeria monocytogenes* ATCC1911 | + | − |
| *Staphylococcus aureus* ATCC25923 | + | − |
| *Enterococcus* | | |
| *E. faecalis* ATCC19433 | + | − |
| *E. faecium* ATCC11576 | + | − |
| *Escherichia coli* ATCC25922 | + | − |
| *Salmonella typhimurium* ATCC14028 | + | − |
| *Pseudomonas. aeruginosa* ATCC27853 | + | − |

[1,2)]Please refer to text for the detailed procedures of deferred test and preparation of partially purified bacteriocin
+: Indicator strain inhibited by application of producer cells or by partially purified bacteriocin
−: Indicator strain not inhibited by application of producer cells or by partially purified bacteriocin

at pH 3.9 and at 7.0% of NaCl concentration. Based upon carbohydrate fermentation patterns and other physiological parameters imposed by Shaw and Harding(21) and Schillinger and Lucke(22), strain LAB3113 was classified as *Lactococcus lactis*. The result was further confirmed by API 50CHL kit.

## Characteristics of bacteriocin production by *Lactococcus lactis* LAB3113

Effects of pH and temperature on the bacteriocin production by *L. lactis* LAB3113 are illustrated in time-dependent graphs in Fig. 2. Production of bacteriocin was also confimed by activity dial, as shown in Fig. 3, which was prepared by checking activity appeared in the culture supernatant every 3hr. Maximal production of bacteriocin was diagrammatically summarized in Fig. 3.

As shown in Fig. 2, bacteriocin production by LAB 3113 was observed when the population growth reached mid to late exponential phase, and maintained stable through stationary phase(Fig. 2 and 3). Optimal condition for maximal production of bacteriocin was calibrated as 25°C and pH 7.0, as shown in Fig. 2 and Fig. 4. Although

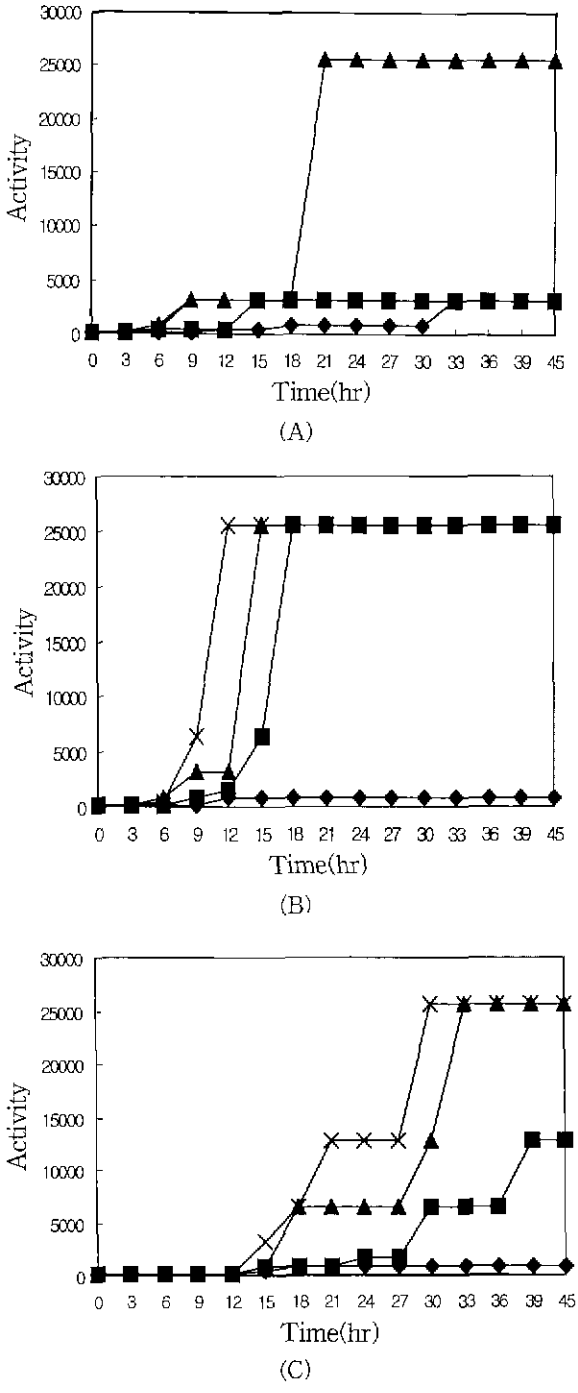


**Fig. 2. Mode of bacteriocin production by *Lactococcus lactis* LAB3113.**
Effect of pH and temperature on the production of bacteriocin was illustrated in timedependent way.
A: Bacteriocin production at 37°C with different pHs
B: Bacteriocin production at 25°C with different pHs
C: Bacteriocin production at 15°C with different pHs
(×) pH 7.0, (▲) pH 6.0, (■) pH 5.0, (◆) pH 4.0

maximal production was also achieved at other conditions, the time required to reach the highest concentration of bacteriocin in the media was much shorter at

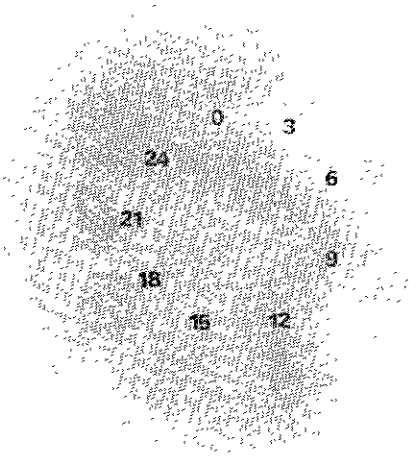


**Fig. 3. Activity dial of bacteriocin production by *Lactococcus lactis* K3110.**
Bacteriocin activity appeared in the culture was checked every 3hr for 24hr with spot-on-lawn method. 10μl of cell-free culture supernant was applied onto each spot.

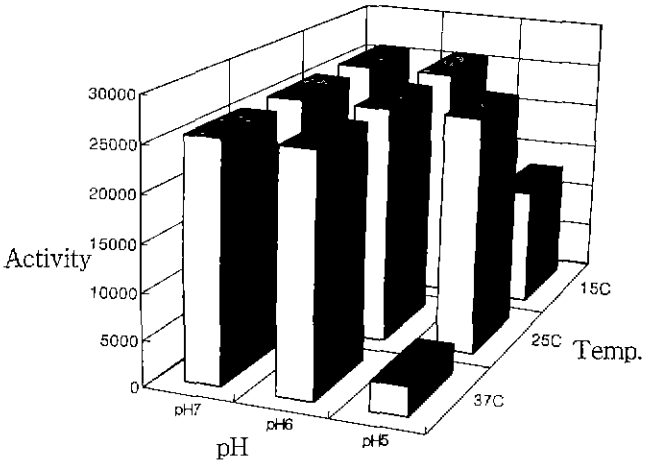


**Fig. 4. Effect of different culture conditions on the maximal production of bacteriocin in *Lactococcus lactis* LAB3113.**

25°C, pH 7.0 as 9hr than at 15°C, pH 6.0 as 33hr. Pattern of bacteriocin production in LAB3113 was characteristic in that, at pH 6.0 and pH 7.0, maximal production of bacteriocin was not affected by temperature. However, at 25°C, maximal production was not affected by the pH of media, as summarized in Fig. 4. The impact of higher temperature seemed more detrimental to bacteriocin production than that of lower temperature(Fig. 2).

### Sensitivity of the bacteriocin to enzymes, solvents, pH changes, and heat-treatment

As summerized in Table 2, the effect of protease on

Table 2. Sensitivity of the bacteriocin produced by *L. lactis* LAB3113 to various degraditive enzymes, solvents, pH, and heat-treatment

| | Treatment | Residual activity(AU/ml)[3] |
|---|---|---|
| Enzymes[1] | Pronase | 0 |
| | Pepsin | 0 |
| | Trypsin | 0 |
| | Lysozyme | 409,000 |
| | β-amylase | 409,000 |
| | Lipase | 409,000 |
| | RNaseA | 409,000 |
| Solvent[2] | Ethanol | 409,000 |
| | Methanol | 409,000 |
| | Aceton | 204,000 |
| | Acetonitrile | 409,000 |
| | Chloroform | 102,000 |
| | Toluene | 409,000 |
| | Ethyl acetate | 409,000 |
| pH | 2 to 10 | 409,000 |
| Heat | 60°C/30min | 204,000 |
| | 100°C/30min | 102,000 |
| | 121°C/20min | 12,800 |

[1]The final concentration of enzyme was 1mg/ml
[2]50%(v/v) concentration was used
[3]Risidual activity of untreated bacteriocin was 409,000AU/ml

the activity of bacteriocin produced by *L. lactis* LAB 3113 was immediate, inactivating antagonistic activity of the molecule completely. Other hydrolytic enzymes did not exert any effect on the bacteriocin. Solvent-treatment did not affect bacteriocin. The bacteriocin was quite stable over the wide range of pH tested in the experiment. It also showed remarkable stability over heat treatment, retaining part of its activity even after autoclaving for 20min. All of these molecular characteristics suggested that the bacteriocin of LAB3113 would belong to class II bacteriocin described by Klaenhammer(3,4).

## Mode of inhibition by the bacteriocin of LAB3113

To characterize the mode of inhibition of bacteriocin, viability of *L. curvatus* KCA170-12 was monitored in the presence of bacteriocin. As exhibited in Fig. 5, addition of 25AU of the bacteriocin to a cell suspension of *L. curvacus* KCA170-12 at $5\times10^7$CFU/ml of phosphate buffer(10mM, pH 7.0) reduced the viable cell count to less than one cell per ml within 30min, indicating that the bacteriocin molecule worked in bactericidal mode. Addition of protease to the reaction mixture containing

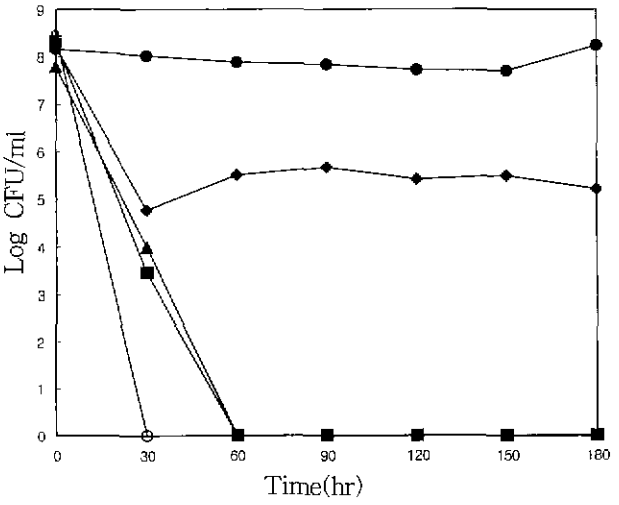


**Fig. 5. Mode of action of bacteriocin of *Lactococcus lactis* LAB311-3 against *Lactobacillus curvatus* KCA LAB170-12 in phosphate buffer(10mM, pH 7.0) at various concentrations of bacteriocin.**
(■) 3AU/ml, (▲) 6AU/ml, (○) 25AU/ml, (◆) 6AU/ml with protease treatment at 30min-point, (●) experimental control without bacteriocin treatment.

6AU of bacteriocin per ml immediately halted the lethal effect of the bacteriocin.

Molecular mechanism of bactericidal action of bacteriocin produced by LAB3113 was unknown yet. Any indication of cell lysis at the treatment of bacteriocin above the lethal concentration has not been observed on microscopic investigation. Rescuing effect of protease treatment might be due to destruction of bacteriocin at the cell surface or reduction of effective concentration of bacteriocin in the media. However, the intrinsic nature of the killing or rescuing effect needs more investigation.

## Calibration of apparent molecular weight of bacteriocin

The apparent molecular weight of the partially purified bacteriocin was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

As illustrated in Fig. 6, partially purified bacteriocin preparation still contained other contaminating proteins than bacteriocin. However, when the activity-stained band was compared to the molecular weight standard in the Coomassie brilliant blue-stained gel, molecular weight of bacteriocin from LAB3113 was calibrated as approximately 10.5kdal.

Based upon data described above, the antagonistic substance which was produced by *L. lactis* LAB3113 was proved to authentic bacteriocin. Therefore, it was

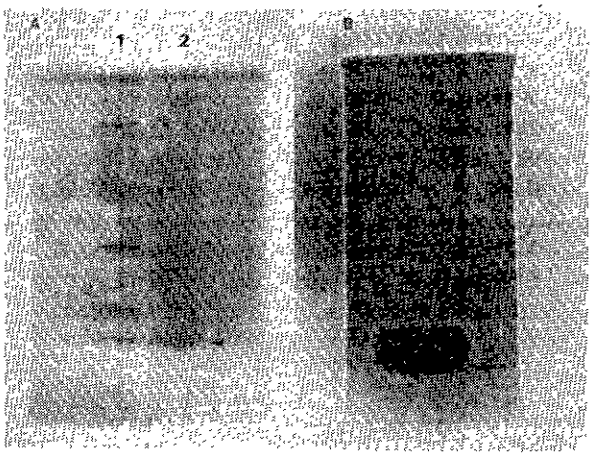


**Fig. 6. Polyacrylamide gel electrophoresis of partially purified lactococcin K3113.**
Panel A shows Coomassie brilliant blue stained pattern of bacteriocin. Panel B exhibits activity-stained pattern of the same gel. Low molecular weight marker(Bio-Rad) was used.

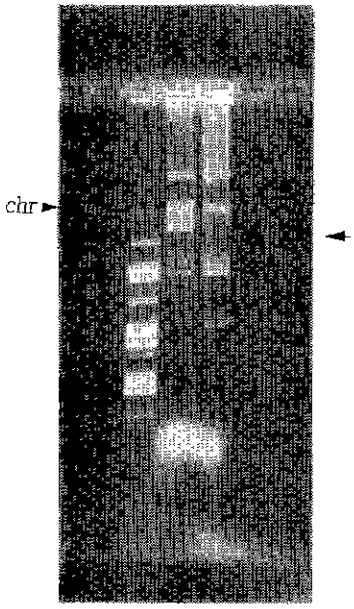


**Fig. 7. Plasmid profile of wild type strain of *Lactococcus lactis* LAB3113 and its Bac⁻ mutants.**
Lane 1: Molecular size marker of *E. coli* V517, Lane 2: Wild type strain, Lane 3: Bac⁻ mutant strain. Chromosomal fragment DNA marked as 'chr', and 15.5kb bacteriocin plasmid marked as arrow.

tentatively named as lactococcin K3113.

### Plasmid analysis for location of genetic determinants of lactococcin K3113

On isolation of residential plasmids, *L. lactis* LAB3113 exhibited 4 residential plasmids of 3.7kb, 11.2kb, 15.5kb, and 48kb in molecular sizes, respectively, as shown in Fig. 7. Plasmid profile of Bac⁻ mutant which lost capability to produce lactococcin K3113 during acridine orange treatment showed loss of 15.5kb plasmid, as evident in Fig. 7. This loss of 15.5kb plasmid was coincided with loss of bacteriocinogenecity(Fig. 8) and loss of immunity to the bacteriocin. Therefore, production of lactococcin K3113 in *L. lactis* LAB3113 and its immunity to its own bacteriocin appeared to be mediated by the residential plasmid of 15.5kb.


**Fig. 8. Loss of bacteriocin production in Bac⁻ mutant.**
a: Wild type strain
b: Bac⁻ mutant which also lost 15.5kb plasmid.

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