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Antibiosis and Bacteriocin Production of Lactic Acid Bacteria Isolated from Kimchi

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Abstract

In other to elucidate roles of lactic acid bacteria(LAB) for the antibiosis occurring in the fermenting environment of Kimchi, 2,052 strains of LAB were isolated from Kimchi. Fifty two strains which showed antagonistic effect against 4 indicator strains were finally selected and investigated. Based upon responses to protease treatment, antibiosis of the 52 strains of LAB were classified into 3 types. Type A antibiosis resulted from action of antibiotic-like substances which were not affected by protease treatment and which had broad action spectra against even natural inhabitants of Kimchi. Type B antibiosis was due to bacteriocin-like substances which were very sensitive to treatment of protease and more effective against foreign bacteria than original inhabitant microflora. Type C antibiosis was owing to proteinaceous compounds which were activated or induced by the presence of protease and then exerted antibacterial activities. Therefore, lactic acid bacteria appeared to contribute to antibiosis of Kimchi by the concerted action of these three different types of antibacterial compounds. As one of model system for type B bacteriocin, the antagonistic compound produced by LAB31-9 as well as the producer strain itself was further characterized. Strain LAB31-9 was identified as *L. casei*. Bacteriocin produced by LAB31-9 was proteinaceous and stable over wide range of pH and to various solvents, but very labile to heat treatment . Its mode of action was bactericidal. Based upon these data, bacteriocin produced by LAB31-9 was named as 'caseicin K319'. Genetic determinant for the bacteriocin production of LAB31-9 was located in the chromosome.

Key words: antibiosis, bacteriocin, lactic acid bacteria, Kimchi, caseicin K319, genetic determinant

INTRODUCTION

Lactic acid bacteria(LAB) are common microflora of fermented foods such as dairy products and processed vegetables, and play an important role on the quality of these foods. Substances produced by lactic acid bacteria used in these food fermentations would show inhibitory activities against putrefactive and pathogenic microorganisms in the food system. Inhibitory activities of lactic acid bacteria in these foods have been associated with major end products such as organic acids (1), diacetyls(2), hydrogen peroxide(3), ammonia and bacteriocins(4,5). Among them, bacteriocins are gaining increasing interest, which can be defined as anti microbial polypeptides that are ribosomally determined directly from their genes and usually inhibit closely related strains and species(4,6). Bacteriocins of LAB can be divided into three subclasses: (i) lantibiotics, small bacteriocins containing unusual amino acids such as dehydroalanine, lanthionine, and β-methyllanthionine, (ii) small hydrophobic heat-stable peptides(<13,000 daltons), and (iii) large heat labile proteins(>30,000 daltons) (7). In several cases it has been demonstrated that bacteriocin production is associated with plasmid DNA(8-10), whereas in other cases bacteriocin production was linked to chromosomal DNA(11). In recent years, a large number of different bacteriocins from LAB has been detected and purified, and some of the corresponding genes have been cloned and sequenced(12). *Lactobacilli* were especially known for their bacteriocinogenicity. Within the genus *Lactobacillus*, a number of bacteriocin has been reported in *L. acidophilus, L. helveticus, L. fermenti, L. plantarium,* and *L. sake*(4,11-14).

Even if bacteriocins have been proved not suitable for medicine due to their relatively large size of molecules that would cause problems in solubility and diffusibility in human circulatory system, they have been successful in establishing their importance as a new kind of bioregulator of microflora in various fermented food systems where obtaining of antibiotic resistance

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by ingestion of foods would be critical and where narrower spectra of antibacterial agents are more desirable for the specific inhibition of target microorganism among food ecosystem. Furthermore, the public's concern about the safety of chemical preservatives has been increased, and consumers prefer foods that have no preservative or contain natural preservatives such as bacteriocins.

Therefore, history-long ingestion of their end products as well as lactic acid bacteria themselves by fermented and dairy foods, and the wide inhibitory spectra of various bacteriocins against various food pathogens (15) have made it possible to use bacteriocins as bioregulators or biopreservatives. It is also suggested to use whole bacteria as starter cultures in order to provide the competitive dominants in a mixed fermentation environment(16).

The development of new bacteriocins in Korea is promising owing to the relative abundance in traditionally fermented foods such as Kimchi where bacteriocin may play an important role for competition in natural microflora during fermentation. However, importance and industrial value of bacteriocins in Korea have been under estimated, and only recently, research efforts have been concentrated on finding bacteriocin producers such as *Pediococcus* from Kimchi(17,18). Recently, Yoo reviewed the lactococcal bacteriocins isolated from *Lactococcus* sp.(19).

Therefore, in this study, we tried to characterize the roles of lactic acid bacteria for the antibiosis in the fermenting environment of Kimchi. We also tried to isolate effective bacteriocin producers from Kimchi, characterize antogonistic compounds and its mode of production, and finally locate the genetic determinants of the bacteriocin production.

MATERIALS AND METHODS

Isolation of lactic acid bacteria from Kimchi

Supernatant of Kimchi was added to sterile distilled water to obtain 1 : 10 dilution. Serial dilutions were made in sterile distilled water, spread onto MRS agar plates, and incubated anaerobically at 25°C or 37°C for 24 to 48hr until growth was evident, and then colonies were enumerated. After enumeration, colonies were randomly picked up with sterile tooth picks and streaked to fresh MRS agar plates in order to use them as master control plates. These plates were incubated anaerobically at 25°C or 37°C overnight, and stored at 4°C for future use. Primary authenticity of the isolates as lactic bacteria was tested by Gram-staining and catalase test. Anaerobic incubation(Anaerobic jar; Almore International Inc., Portland, U.S.A.) was used to rule out growth of aerobes and any inhibition due to hydrogen peroxide production. MRS or APT was used as the base agar, and 0.75% agar was used as soft overlay agar.

Detection of antagonistic activity of LAB

Four replica MRS plates were made from master control plates, incubated anaerobically at 25°C or 37°C, depending on the initial isolation conditions. The plates were then overlayered with soft MRS agar containing 10^7CFU/ml of *Carnobacterium divergens*, *Leuconostoc gelidum* UAL187-13, *Lactobacillus delbrueckii lactis* ATCC4797 and *Lactococcus lactis* LM0230, respectively. After overnight incubation at appropriate temperature, colonies showing zones of inhibition were checked and their counterparts were picked up from the master control plate for the streak onto fresh MRS agar plates. After formation of homogeneous colonies, one of them was inoculated into MRS broth and incubated at 25°C or 37°C. These cultures were made into stock cultures by adding 30% glycerin.

Detection of bacteriocin production and activity assay

Bacteriocin production was determined by adaptations of spot on the lawn and deferred antagonism assay described previously(5,8). Spot on the lawn test was performed by adding 20μl of supernatant on the surface of an agar plate which had been overlayered with 7ml of soft agar containg 10^7CFU/ml of appropriate indicator culture. The plates were incubated at the optimal growth temperature of the indicator strain and checked for the formation of inhibition zones. For the deferred antagonism assay, overnight cultures were spotted onto MRS agar plates and incubated overnight at their optimal temperature, and then overlayered by 7ml of soft agar containing 10^7CFU/ml of appropriate indicator strain. The activity unit of the bacteriocin was assayed by critical dilution method(5). Indicator strains which were used as for detection of bacteriocin activity were listed in Table 1. All the stock cultures were maintained at 80°C in MRS or APT broth containing 30% glycerin.

Table 1. Indicator strains and their culture conditions

Stock No.	Indicator strains	Media and growth temperature(°C)
IS11	*Lactobacillus acidophilus* VPI11088	MRS, 37
IS13	*Lactobacillus delbrueckii-lactis* ATCC4797	MRS, 37
IS14	*Lactobacillus gasseri* ATCC33323	MRS, 37
IS15	*Lactobacillus gasseri* VPI11092	MRS, 37
	Lactobacillus brevis (LB50)	MRS. 37
IS21	*Lactococcus lactis* LM0230	M17, 30
IS41	*Pediococcus pentosaceus* FBB61-2	MRS/APT, 35
	Leuconostoc gelidum UAL187	MRS, 25
IS51	*Carnobacterium piscicola* LV17	APT, 25
	Carnobacterium piscicola UAL26	APT, 25
IS53	*Carnobacterium divergens* LV13	APT, 25
IS61	*Listeria monocytogenes* ATCC1911	APT, 37
IS71	*Staphylococcus aureues* ATCC25923	TSB, 37
IS81	*Enterococcus faecalis* ATCC19433	MRS. 37
IS82	*Enterococcus faecium* ATCC11576	MRS, 37
IS91	*Escherichia coli* ATCC25922	TSB, 37
IS101	*Salmonella typhimurium* ATCC14028	NB, 37
IS111	*Pseudomonas aeruginosa* ATCC27853	NB, 37
IS112	*Pseudomonas fluorescence* ATCC21541	NB, 26

Effect of protease treatment on the antagonism

In order to differentiate inhibition by bacteriocin from other inhibition mechanisms such as antibiotic compounds, either broth supernatants or colonies on the plates were treated by pronase at the final concentration of 1mg/ml. Substance which would lose its inhibitory activity on the treatment of pronase was considered as bacteriocin-like inhibitory substances(BLIS). Otherwise, the inhibitory substance was considered as antibiotic-like inhibitory substances(ALIS).

Strain identification of lactic acid bacteria isolated from Kimchi

Bacteriocin-producing strains were identified basically by Schillinger and Lucke's method(20) and API strip (50CHL, BioMerieux Vitek, Inc., U.S.A.). An overnight culture of the test organism grown in MRS broth was spot-inoculated on basal medium containing 1% proteose peptone #3, 0.1% beef extract, 0.5% sodium chloride, 20mg bromocresol purple and 0.5% of the filter sterilized carbohydrate in 1000ml. All plates were checked for acid production after incubation at 25°C or 37°C for 24, 48 and 72hr.

Gas production from glucose was performed in the inverted vials in MRS broth, with citrate omitted. NH_3 production from arginine was tested in MRS broth without beef extract, yeast extract, and glucose, but containing 0.3% arginine and 0.2% sodium citrate replacing ammonium citrate. Ammonia was detected using Nessler's reagent. Growth at different temperature was performed in MRS broth after incubation for 3 days at 15°C, for 5 days at 45°C, for 10 days at 4°C. Salt tolerance was tested in MRS broth added with 7% and 10% NaCl, respectively. The production of dextran from sucrose was determined on MRS agar in which the glucose had been replaced by 5% sucrose. The production of H_2S was tested on modified lead acetate agar containing 1.5% bacto peptone, 0.5% proteose peptone 0.1% bacto dextrose, 0.02% lead acetate, 0.008% sodium thiosulfate, and 1.5% agar. Growth in acetate agar was performed on acetate agar containing of 2g of sodium acetate, 0.1g of magnesium sulfate, 5g of sodium chloride, 1g of mono ammonium phosphate, 1g of dipotassium phosphate, 0.08g of bromo thymol blue, 20g of agar in 1000 ml. Voges-Proskauer test was performed in MR-VP broth containing 0.7% buffered peptone, 0.5% dipotassium phosphate and 5% dextrose.

Mode of bacteriocin production

MRS broths adjusted from pH 4.0 to 7.0 at 1.0 intervals were prepared with 10N NaCl and HCl. Each set of broth with different pH was inoculated with 1% of overnight culture of LAB 31-9, then incubated at 15°C, 25°C, 37°C, respectively. Bacterial growth(OD at 600nm), pH change and bacteriocin production of each culture were monitored periodically.

Preparation of partially purified bacteriocin

One liter of MRS broth was inoculated with 10ml of an overnight culture of LAB31-9, and incubated overnight at 25°C. Cells were removed by centrifugation (8,000rpm, 15min, 4°C), and the supernatant was filtered through 0.22μm filters(HA Type, Millipore) to remove cellular debris. Culture supernatant was concentrated by ammonium sulfate precipitation up to 25% saturation cut. After centrifugation at 8,000rpm, 15min, 4°C, supernatant was treated with ammonium sulfate up to 50% saturation cut. After centrifugation at 8,000 rpm, 15min, 4°C, pellet was melted with 10ml of sterilized Milli-Q water and dialyzed(molecular weight cut-off 12,000, Sigma) against the 10mM sodium phosphate buffer(pH 7.0) at 4°C, 24hr with three changes of buffers. Then the dialysate was lyophilized and resuspended in 3ml of sterilized Milli-Q water.

Molecular stability of the bacteriocin

Sensitivity of bacteriocin to heat treatment was determined by heating 100μl of partially purified bacteriocin(3,200AU/ml) to 60°C, 100°C and 125°C. Sensitivity of bacteriocin to enzyme treatment was tested using enzymes at the final concentration of 1mg/ml as follows; trypsin(EC 3.4.21.4; Sigma) in 50mM tris-HCl(pH 8.0), pronase(EC 3.4.24.4; Boeringer Mannheim) in distilled water, β-amylase(EC 3.2.1.2, type II-B; Sigma), lipase (EC 3.1.1.3 type VII from *Candida cylindracea*; Sigma) in 50mM tris-HCl containing 10mM $CaCl_2$(pH 8.0), pepsin(Ishizu Pharmaceutical Co., LTD.) in 10mM citrate (pH 6.0), ribonuclease-A(type I-AS; Sigma), lysozyme (Sigma) in distilled water. Mixtures of bacteriocin and enzyme were incubated at 37°C for 1hr.

To test stability of bacteriocin over wide range of pH, crude bacteriocin was dialyzed in 2 liter of 10mM citrate buffer(pH 3.0, 4.0 and 5.0), 10mM sodium phosphate buffer(pH 6.0 and 7.0) and 10mM tris-HCl(pH 8.0 and 9.0). Three changes of each buffer were made during dialysis. After dialysis, the bacteriocin solutions were assayed for the residual activity.

To test the stability of bacteriocin over organic solvents, bacteriocin was suspended in the following organic solvents: 50% ethanol, 50% acetonitrile, 50% acetone, 50% methanol, chloroform, toluene, ethyl acetate. The mixture was incubated at 25°C for 1hr, and then tested for the residual bacteriocin activity.

Mode of bacteriocin action

To elucidate the effect of the bacteriocin treatment on the sensitive cells, 100AU or 200AU of partially purified bacteriocin was added to suspensions of 10^7CFU/ml of *L. delbrueckii-lactis* ATCC 4797 in the phosphate buffer(50mM, pH 7.0) by using the procedures described by Ahn and Stiles(8). Indicator cells without bacteriocin treatment and those with pronase E treatment were used as experimental controls. Pronase E(1mg/ml) was also added to cells treated with 100AU of bacteriocin per ml after 3hr of reaction time.

Plasmid isolation

Cells were grown to the late exponential phase in MRS broth, and plasmid DNA was extracted by the method of Ahn and Stiles(8). Plasmid DNA was visualized by horizontal gel electrophoresis in 0.7% agarose gel in TAE buffer(40mM Tris, 20mM acetic acid, 1mM EDTA, pH 8.0). Electrophoresis was performed at 70V for 2hr. Gels were then stained with ethidium bromide and photographed. Plasmid sizes were estimated by using *E. coli* V517 which contains plasmids of known sizes(21).

Curing of residential plasmid

To determine whether genetic determinants of bacteriocin production reside chromosome or plasmid, plasmid was forced to be removed by applying protoplast-induced curing method(8) and acridine orange treatment. Mutants were tested for the bacteriocinogenicity by the deferred method.

RESULTS

Isolation of lactic acid bacteria from Kimchi

Lactic acid bacteria were successfully isolated by spreading aqueous phase of traditionally fermented Kimchi onto the MRS or APT agar plates, and by incubating them at 37°C or 25°C. The average plate count of lactic acid bacteria with four independent trials on MRS agar was 5.0×10^7CFU/ml of Kimchi. 2,052 colonies were randomly picked up, and streaked onto the pair of fresh MRS plates. All the colonies showed primary authenticity as lactic acid bacteria on Gram-staining(positive), catalase test(negative), and morphology(non-sporeforming, white or yellow colony with raised smooth and round surface).

Table 2. Antibiosis of lactic acid bacteria from Kimchi on the solid agar surface

	IS13[1)]	IS11	IS15	IS16	IS21	IS41	IS51	IS53	IS54	IS61	IS71	IS81	IS82	IS91	IS101	IS111	IS112
2-1																	
5-4																	
5-11																	
8-6																	
15-7																	
16-12																	
19-11																	
117-6																	
117-9																	
118-9																	
159-1																	
159-5																	
159-12																	
160-7																	
160-8																	
161-4																	
162-4																	
162-8																	
163-8																	
22-9																	
23-1																	
23-2																	
25-2 T																	
27-2																	
27-3																	
27-4																	
28-1																	
28-4																	
31-9																	
78-8																	
145-3																	
145-3A																	
149-6																	
150-4																	
150-7																	
150-8																	
164-4																	
164-5																	
164-8																	
166-5																	
166-11																	
166-12																	
167-3																	
169-2																	
169-3																	
169-8																	
169-10																	
170-8																	
170-11																	
170-12																	
171-5																	
171-9																	

[1)]Stock number as illustrated in Table 1

Medium shaded area: Producer strain effective against indicator strain on the solid medium

Dark shaded area: Ammonium sulfate-precipitated supernatant of culture of producer effective against indicator strain

Antibiosis of lactic acid bacteria

All the lactic acid bacteria were initially screened for their ability to form inhibitory clear zones against *Carnobacterium divergens*, *Leuconostoc gelidum* UAL187-13, *Lactobacillus delbrueckii-lactis* ATCC4797 and *Lactococcus lactis* LM0230 using deferred method. Among 192 isolates which exhibited immediate inhibition activity against those 4 indicator strains, 52 isolates with clearer inhibitory zones were selected and tested for antagonism against 17 indicator strains as listed in Table 1. Inhibitory spectra of the 52 isolates are summarized in Table 2.

Characteristics of inhibition by lactic acid bacteria exhibited in Table 2 were their wide range of antagonism against non-lactic acid bacteria including food pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Enterococcus* species. Most of them also showed inhibitory activity against Gram-negative bacteria on the solid surface of media. Ammonium sulfate-precipitate of culture supernatants of 9 LAB showed inhibitory activity effective on *Pseudomonas* species.

Effects of protease treatment on the antagonistic activities of LAB

In order to know whether the antagonistic activity of lactic acid bacteria from Kimchi was due to bacteriocin or other inhibitory substances such as antibiotics, 10μl of pronase(1mg/ml) was applied near the colony of lactic acid bacterium which had been overlayered by soft agar containing *L. delbrueckii-lactis* ATCC4797. Disappearence of clear zone by the protease treatment would indicate proteinaceousness of the antagonistic substance, identifying it as bacteriocin- like inhibitory substance(BLIS). Otherwise, it would be classified as antibiotic-like inhibitory substance(ALIS).

Application of protease to the 52 LAB strains, however, showed that there were three types of responses rather than two, as illustrated in Fig. 1 and Table 3. Fig. 1-A shows the first type of antibiosis(type A) which was not affected by the presence of protease. Therefore, cause of type A antibiosis by lactic acid bacteria from Kimchi can be attributed to ALIS. Fig. 1-B exhibits the second type of antibiosis(type B) whose inhibitory activity was lost by the protease treatment,

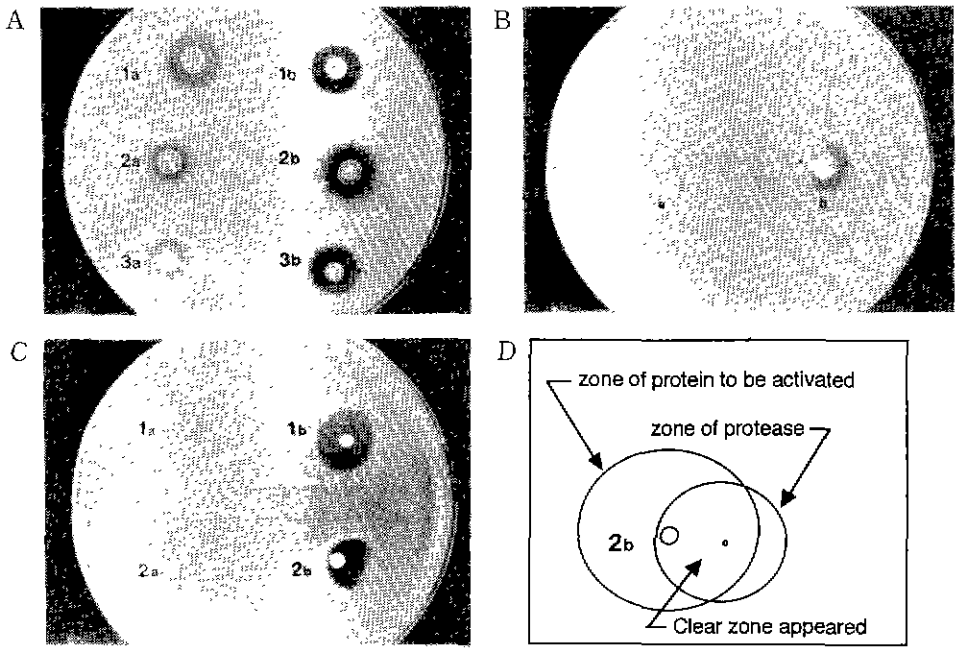

Fig. 1. Effect of protease treatment on the antagonism of the inhibitory substances produced by lactic acid bacteria *isolated from Kimchi*.

Panel A: Inhibitory substances not affected by protease treatment(colony 1: LAB5-4, colony 2: LAB15-7, colony 3: LAB19-11), Panel B: inhibitory substance affected by protease treatment(colony from LAB31-9), Panel C: inhibitory substances induced or activated by protease treatment(colony 1: LAB16-12, colony 2: LAB5-11). Panel D: schematic diagram on how halfmoon-shaped clear zone was formed at colony 2b in Panel C. a: colonies not treated by protease, b: colonies treated by protease. Where protease was applied is indicated as black spots.

Table 3. Effect of protease treatment on antibacterial activity of inhibitory substances produced by lactic acid bacteria from Kimchi against selected indicators

Producer \ Indicator	*L. delbrueckii*		*Listeria*		*Salmonella*	
	−P[1]	+P	−P	+P	−P	+P
5-4	−[2]	−	−	−	+20	+20
15-7	−	−	+25	+23	+20	+20
19-11	−	−	+20	+20	+20	+20
31-9	+18[3]	−	+18	−	+20	−
145-3A	+16	−	−	−	+20	−
160-8	+16	−	+12	−	+20	−
5-11	−	+15	+20	+22	+20	+20
16-12	+17	+32	+15	+28	+20	+20
28-4	−	+16	+15	+20	+20	+20

[1] −P: Not treated by protease, +P: Treated by protease
[2] +: Effective against indicator
−: Not effective against indicator
[3] Numerical values: diameter of clear zone in mm

identifying the cause of antibiosis as bacteriocin(BLIS).

The third type of antibiosis(type C) as shown in Fig. 1-C was unexpected one, which has not been reported elsewhere. Protease treatment was rather inducing more production of inhibitory substances as shown in LAB 16-12, or activating inhibitory substances as shown in LAB 5-11 in Fig. 1-C. Activation of the inhibitory substances by protease in the case of LAB 5-11(colony 2b in Fig. 1-C) was shown by a schematic diagram in Fig. 1-D. Halfmoon-shaped clear zone appeared at the overlapped region between the zone of protein to be activated and the zone of protease to diffuse. Therefore, the activated substance seemed proteinaceous in nature, and the inhibition was not due to the elevated sensitivity of indicator strain to the protein.

The effects of protease treatment on the three types of antibiosis were enumerated by measuring changes of clear zone on the protease treatment, using strains of *Lactobacillus*, *Listeria*, and *Salmonella* as indicators, as summarized in Table 3. Substances which caused antibiosis in LAB 5-4, 15-7, and 19-11 were not affected at all by protease, while those substances produced by LAB 31-9, 145-3A and 160-8 appeared as protease-sensitive bacteriocins. Inhibitory substances of LAB 5-11 and 28-4 was activated by protease treatment while that of LAB 16-12 was induced to more production. However, anti-*Salmonella* activity of LAB5-11, 16-12 and 28-4 seemed due to acid effect of culture media. Therefore, lactic acid bacteria apparently contribute to

Table 4. Classification of 52 LAB exhibiting antibiosis in Kimchi

Species	No	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)	4 °C	8 °C	15 °C	45 °C
Lb. casei sp.	1	−[1]	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	+	+
Lb. collinoides	1	+	−	−	+	+	+	−	−	−	−	−	−	+	−	−	+	−	−	+	−	−	+	+	+	+
Lb. divergens	1	−	+	−	+	+	+	+	−	+	−	+	+	−	−	−	+	+	+	+	+	−	+	+	+	+
Lb. fructosus	15	33[2]	−	−	+	60	87	−	−	−	−	27	13	7	−	−	53	−	−	53	13	−	+	+	+	+
Lb. halotolerans	1	−	−	−	+	+	+	−	−	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	+	+
Lb. kandleri	1	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	+	+	+
Lb. maltaromicus	1	−	+	−	+	+	+	−	−	−	+	+	+	+	−	−	+	+	+	+	+	−	+	+	+	+
Lb. minor	1	+	+	−	+	+	+	−	−	+	−	+	+	−	−	−	+	−	+	−	+	−	+	+	+	+
Lb. plantarium	2	+	+	−	+	+	+	−	−	−	+	+	+	+	+	−	+	+	−	+	+	+	+	+	+	+
Lb. sake	3	+	+	−	+	+	+	−	−	−	+	+	+	+	67	33	+	+	67	+	+	−	+	+	+	+
Lb. sanfrancisco	22	5	−	−	5	23	36	−	−	−	−	9	−	−	−	−	5	−	−	−	−	−	82	86	+	+
Leu. gelidum	1	+	+	−	+	−	+	−	−	−	−	+	−	−	−	−	−	+	−	−	−	+	+	+	+	+
Leu. carnosum	1	−	+	−	+	−	+	−	−	−	−	+	−	−	−	−	+	+	−	−	+	−	+	+	+	+
Lc. morbillonum	1	−	−	−	−	−	+	+	−	−	−	−	−	+	−	−	+	−	−	−	−	−	+	+	+	+

(1) Arabinose (2) Cellobiose (3) Dulcitol (4) Fructose (5) Galactose (6) Glucose (7) Glycerol (8) Inositol (9) Inulin (10) Lactose (11) Maltose (13) Mannitol (14) Melibiose (15) Raffinose (16) Rhamnose (17) Ribose (18) Salicin (19) Sorbitol (20) Trehalose (21) Xylose
[1] +: 90% or most strains positive, −: 90% or most strains negative
[2] Numerical values: Percentage of LAB showing positive fermentation
Lb: *Lactobacillus*. Leu: *Leuconostoc*, Lc: *Lactococcus*

antibiosis in Kimchi by the concerted action of three different types of antibacterial substances.

Strain identification of lactic acid bacteria isolated from Kimchi

Based upon Schillinger and Lucke's classifying criteria(20), 52 strains of LAB showing at least one kind of antibiosis were tentatively identified as 11 species of *Lactobacillus*, 2 species of *Leuconostoc*, and one species of *Lactococcus* as summarized in Table 4. *L. sanfrancisco* and *L. fructosus* were the major population which participate in the antibiosis in the fermenting environment of Kimchi. Some of the strains were further characterized by API 50CHL strips.

Spectrum of the antagonistic substance produced by LAB31-9

Strain LAB31-9 produced bacteriocin as shown in Fig. 1-B and Table 2. To develop the substance and the strain as a model system in the study of antibiosis of Kimchi, they were characterized at the biochemical and molecular level.

The strain LAB31-9 could be classified as a strain belonging to *Lactobacillus casei* based upon Schillinger and Lucke's classifying criteria(20) including growth capabilities at different temperatures, salt resistance, patterns of carbohydrate fermentation, and plasmid profiles. The result was also confirmed by API identification system(50CHL). According to data obtained from both approaches, it was tentatively named as *L. casei* LAB31-9.

Antagonistic effect of LAB31-9 on various Gram-positive and Gram-negative bacteria was tested by deferred test, and the results were summarized in Table 5. Its spectra of activity were broad on the solid media, while ammonium sulfate-precipitated bacteriocin preparation exhibited inhibition against *L. delbrueckii-lactis* ATCC4797 and *E. faecalis*. This narrow spectrum of bacteriocin activity could be exploited as food preservative which is specific to a certain pathogen in the food.

Mode of bacteriocin production in LAB31-9

Effect of temperature and initial pH of media on the bacteriocin production was monitored every 3hr after inoculation of LAB31-9 in each set of MRS media as shown in Fig. 2. The optimal production of bacteriocin was achieved at 37°C and at the initial pH of 7.0 as exhibited in Fig. 2-B. Maximal production of bacteriocin was 3,200AU/ml at the optimal condition which was twice amount of bacteriocin produced at 15°C and pH 7.0(Fig. 2-A).

Table 5. Activity spectrum of LAB 31-9 on the solid agar surface

Indicator strains	Activity
Lactobacillus delbrueckii lactis ATCC4797	+
L. acidophilus VPI11088	−
L. gasseri ATCC33323	+
L. gasseri VPI11092	+
L. brevis (LB50)	−
Lactococcus lactis LM0230	+
Pediococcus pentosaceus FBB61-2	−
Leuconostoc gelidum UAL 187	+
Carnobacterium piscicola LV17	+
C piscicola UAL26	+
C. divergens LV13	+
Listeria monocytogens ATCC1911	+
Staphylococcus aureus ATCC25923	+
Enterococcus faecalis ATCC19433	+
E. faecium ATCC11576	+
Escherichia coli ATCC25922	+
Salmonella typhimurium ATCC14028	+
Pseudomonas aeruginosa ATCC27853	+
P. fluorescence ATCC21541	+

+: effective against indicator strain
−: not effective against indicator strain

Bacteriocin production by LAB31-9 was first detected after 6hr of incubation as phase of the bacterial population growth entered to logarithmic growth phase. At the temperature of 15°C, it was detected after 15 hr as illustrated in Fig. 2-A. However, production was coincided with beginning of population growth as it was at 37°C. Inhibitory substance was not detected at pH 4 and at pH 5(data not shown).

Stability of bacteriocin was rapidly decreased after 15hr of incubation as exhibited in Fig. 2-B. Daba et al. described that mesenteroidecin production by *L. mesenteroid* ul5 decreased by more than 90% after 24hr of incubation(22), and lactacin B production by *Lactobacillus acidophilus* sharply decreased after stationary phase. It was reported that decrease of inhibitory substance was due to proteolytic exoenzyme(s) of the producing strains, or due to pH variation during growth(22).

Molecular stability of bacteriocin produced by LAB31-9

Stability of bacteriocin molecule over treatment by

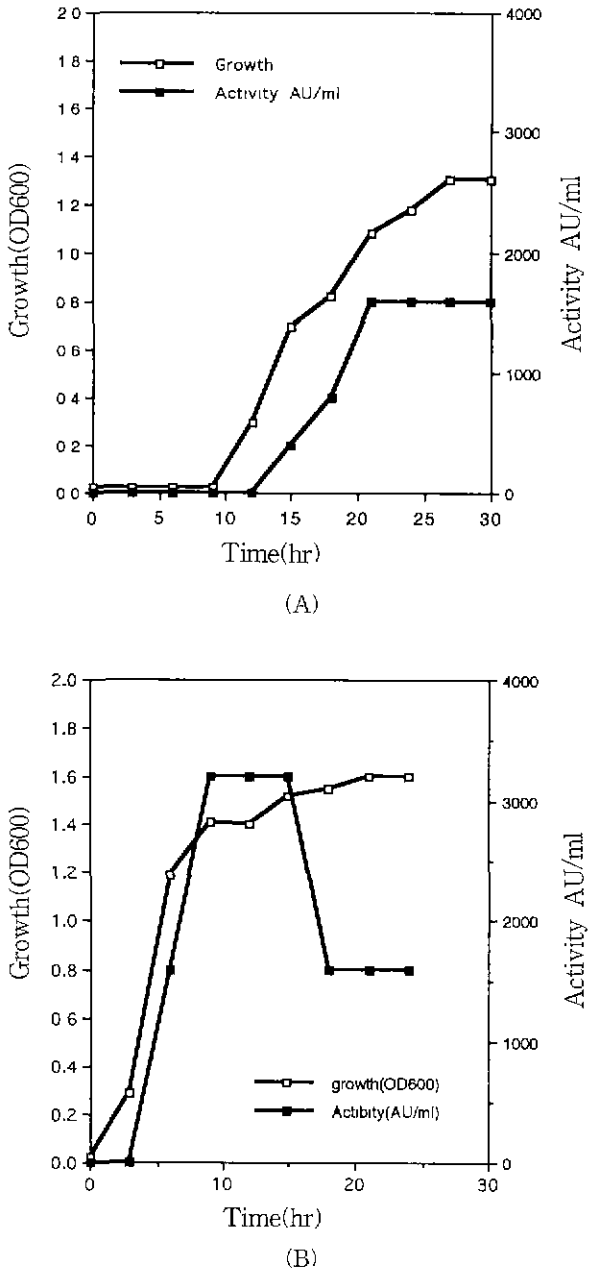

Fig. 2. Mode of bacteriocin production by LAB31-9. Bacteriocin production at 15°C with initial pH 7.0 (Panel A), and at 37°C with initial pH 7.0(Panel B) are illustrated.

hydrolytic enzymes, changes of pH, and heat treatment is summarized in Table 6. As presented in the Table 6, partially purified bacteriocin was found to be sensitive to treatment of protease including pronase, pepsin and trypsin. Inactivation of bacteriocin in its native structural conformation by pepsin was incomplete, which may be attributed to the proteolytic requirement of pepsin for low pH of buffer solution. Bacteriocin activity was

Table 6. Sensitivity of bacteriocin produced by LAB31-9 to various environmental factors

Treatment	Residual activity(AU/ml)[1)]
Control(in phosphate buffer)	3200
Pronase	0
Pepsin	800
Trypsin	0
Lysozyme	3200
β-Amylase	3200
Lipase	3200
RNase I	3200
Ethanol	1600
Acetone	200
Methanol	1600
Acetonitrile	0
Chloroform	3200
Toluene	3200
Ethylacetate	1600
pH 3 to 4	1600
pH 5 to 9	3200
60°C, 5min	200
60°C, 15min	100
60°C, 30min	0

[1)]AU(Activity Unit) determined against *Lactobacillus delbruekii-lactis* ATCC4797

not affected by treatment with lysozyme, β-amylase, lipase, and RNase I, suggesting the inhibitory substance is an authentic protein. Buffer or enzyme preparations alone had no effect on the indicator lawns.

Bacteriocin of LAB31-9 was very sensitive to heat treatment. After subjecting bacteriocin(3200AU/ml) to 60°C for 15min, bacteriocin activity was reduced to 100 AU/ml. Sensitivity of bacteriocin to heat and proteolytic enzymes demonstrated that the antimicrobial compound has proteinaceous nature. A class of *Lactobacillus* bacteriocins include heat-labile proteins of large molecular weight(4). Up to date only helveticin J(37,000 daltons) has been purified and characterized at the genetic level (7,11). It is a heat-sensitive protein(inactivated at 100°C within 30min.) which does retain activity after treatment with various dissociating agents. Acidophilucin A, lacticin A/B, and casicin 80 appear to be large proteins since they are inactivated within 10~15 min at 60°C(23, 24). Noting their size and heat-lability, the bactericidal activities of these proteins are likely to be affected by changes in tertiary structure on heat treatment. Activity of bacteriocin appeared stable over wide range of pH 3.0 to 9.0. However, there was partial loss of activity (1600AU/ml) at pH 3.0 to 4.0.

Mode of action

To investigate whether bacteriocin of LAB31-9 works on the sensitive cell *L. delbrueckii-lactis* ATCC4797 in the bactericidal or bacteriostatic mode, partially purified bacteriocin was added to the sensitive cells in the phosphate buffer, and the viable count was periodically monitored. Bacteriocin of LAB 31-9 exhibited bactericidal mode of action against *L. delbrueckii lactis* in phosphate buffer as shown in Fig. 3. However, killing was not immediate. Protease treatment of the reaction mixture after 3hr interaction rescued cells from bactericidal effect of the bacteriocin.

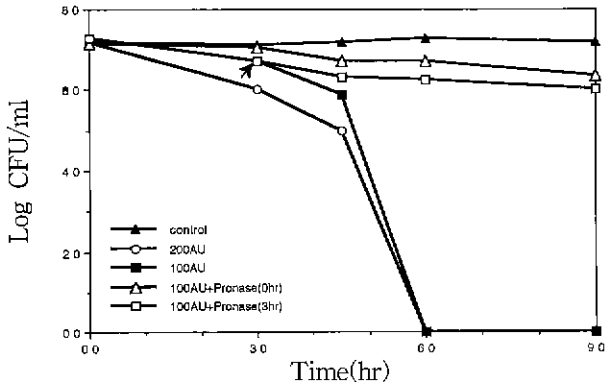

Fig. 3. Mode of inhibitory action by LAB31-9.
The effect of bacteriocin from LAB31-9 on the survival rate of *L. delbrueckii-lactis* ATCC4797 was analyzed by adding 100, 200AU/ml of crude bacteriocin into the cell suspension in 50mM phosphate buffer(pH 7.0), and monitored on the CFU of surviving cells by 3hr.

Plasmid profiles of LAB from Kimchi and location of the genetic determinant of bacteriocin production in LAB31-9

In order to find location of genetic determinants of bacteriocin production in LAB from Kimchi, plasmids were isolated, and their profiles were compared, as illustrated in Fig. 4. As for LAB31-9, plasmid was cured of the cell, and then plasmid-free mutant was compared with wild type regarding bacteriocinogenicity as well as other fermentation properties.

What was first conspicuous from plasmid profiles of LAB as in Fig. 4 was the diversity of residential plasmids in LAB, which might reflect very competitive interaction between microorganisms in ecosystem of Kimchi. Secondly, plasmid profiles could be used as molecular criteria to classify lactic acid bacteria. LAB 5-4 and LAB 19-11 have identical plasmid profile, so they were considered as the same species. Thirdly, diversity of residential plasmids in lactic acid bacteria from Kimchi would provide good reservoir to meet needs of various vector plasmid sources for genetic manipulation of lactic acid bacteria. For example, plasmids of LAB23-1 and LAB27-4 have low molecular weight and high copy number which can be immediately exploited to develop as vector specific to lactic acid bacteria. In case of LAB 27-3 which has no residential plasmid, it could be developed as a recipient for genes of known bacteriocin production such as nisin.

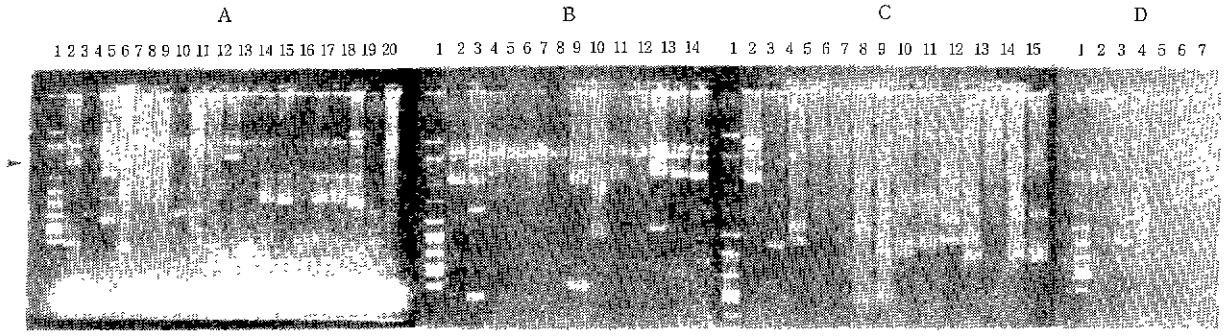

Fig. 4. Plasmid profiles of lactic acid bacteria with more than one kind of antibiosis mechanism.
Lane 1 of each panel is molecular weight markers from *E. coli* V517. Strains are as follows: Panel A(lane 2; LAB2-1, 3; LAB5-4, 4; LAB5-11, 5; LAB8-6, 6; LAB15-7, 7; LAB16-12, 8; LAB19-11, 9; LAB117-6, 10; LAB117-9, 11; LAB118-9, 12; LAB159-1, 13, LAB 159-9, 14; LAB159-12, 15; LAB160-7, 16; LAB160-8, 17, LAB161-4, 18, LAB162-4, 19; LAB162-8, 20; 163- 8), Panel B(lane 2; LAB22-9, 3; LAB23-1, 4; LAB23-2, 5; LAB25-2T, 6; LAB27-2, 7; LAB27-3, 8; LAB27-4, 9; LAB28-1, 10; LAB28-4, 11; LAB31-9, 12; LAB78-8, 13, LAB145-3, 14; LAB145-3A), Panel C(lane 2; LAB 149-6, 3; LAB150-4, 4; LAB150-7, 5; LAB150-8, 6; LAB164-4, 7; LAB164-5, 8; LAB164-8, 9; LAB166-5, 10; LAB166-11, 11; LAB166-12, 12; LAB167-3, 13; LAB169-2, 14; LAB169-3, 15; LAB169-8), Panel D(lane 2; LAB 169-10, 3; LAB170-8, 4; LAB170-11, 5; LAB170-12, 6; LAB171-5, 7; LAB171-9). Position of chromosomal DNA is marked as an arrow

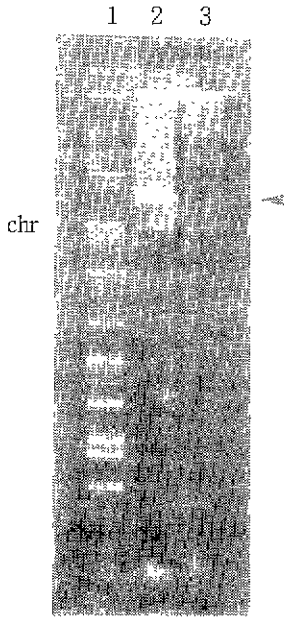

Fig. 5. Plasmid profile of wild type and plasmid-cured mutant strain of LAB31-9.
Lane 1: molecular weight standard from *E. coli* V517, lane 2: wild type strain, lane 3: plasmid-cured mutant strain. Position of chromosomal DNA was marked as chr, and 41kb plasmid was marked as an arrow.

Strain LAB31-9 has one residential plasmid as exhibited in Fig. 5. Molecular size of the plasmid was calibrated as 41kb. When the 41kb plasmid was cured by acridine orange treatment as shown in Fig. 5, the resultant plasmid-free mutant showed unchanged production of bacteriocin, suggesting that the genetic determinant of bacteriocin production is located in the chromosome.

DISCUSSION

It has long been suggested that lactic acid bacteria would have played an important role in antibiosis as well as fermentation of Kimchi. In this study, we showed that lactic acid bacteria, in fact, are actively controlling microflora in ecosystem of Kimchi. Based upon responses to protease treatment, antibiosis of lactic acid bacteria in the fermenting environment of Kimchi could be classified as 3 types. Type A antibiosis resulted from action of antibiotic-like substances which were not affected by protease treatment and had broad action spectrum against even natural inhabitants of Kimchi. Type B antibiosis was due to bacteriocin-like substances which were very sensitive to the treatment of protease and more effective against foreign bacteria than original inhabitant microflora. Type C antibiosis was due to proteinaceous compounds of which antibacterial activities are increased or induced by the presence of protease.

Therefore, lactic acid bacteria contribute to antibiosis of Kimchi by the concerted action of three different types of antibacterial compounds. Especially, activation or induction of bacteriocin by protease makes sense in the fermenting environments of Kimchi where protease would be secreted into liquid media by many bacteria competing to dominate the complicated ecosystem. McCormick et al.(25) and Eijsink et al.(26) reported induction of bacteriocin production by small peptides produced by the producer microorganisms themselves. However, even in those cases, relationship between protease treatment and bacteriocin activation/induction has not been referred. So, to our knowledge, protease-activated bacteriocin production(type C) which was reported in this study is the first one in the bacteriocin studies. Furthermore, this kind of bacteriocin meets well the industrial needs where resistance to proteolysis becomes a good asset of bacteriocins.

As one of model system for future study of type B bacteriocin, the antagonistic compound produced by LAB31-9 as well as the producer strain itself was further characterized. Strain LAB31-9 was identified as *L. casei*. The antagonistic substance was proteinaceous and stable over wide range of pH, but very labile to heat treatment. Its mode of action was bactericidal. Molecular weight of the substance was calibrated as 38 kdal by SDS PAGE(data not shown), suggesting its similarity to class III bacteriocin(4). Therefore, the antagonistic substance deserves the name of bacteriocin, and we propose 'caseicin K319' for the bacteriocin.

Plasmid profiles of lactic acid bacteria isolated from Kimchi showed possibility of plasmid-mediation in the production of the antagonistic compounds. Diversity of residential plasmids in lactic acid bacteria immediately suggests availability of those plasmids as molecular sources of vector development in lactic acid bacteria. Curing experiments for LAB31-9 located genetic determinants of caseicin production to chromosome.

Bacteriocin has attracted attention of food industry as potential natural food preservatives against several spoilage bacteria and pathogens(6,15,27,28). Future study should be targeted for further characterization of bacteriocins from Kimchi at the molecular and genetic levels.

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