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Isolation of a Nisin-Producing *Lactococcus lactis* Strain from Kimchi and Characterization of its *nisZ* Gene

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Abstract Bacteriocin-producing lactic acid bacteria were isolated from kimchi. One isolate producing the most efficient bacteriocin was identified and named *Lactococcus lactis* B2, based on the biochemical properties and 16S rDNA sequences. The B2 bacteriocin inhibited many different Gram positive bacteria including *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Streptococcus*, and *Staphylococcus*, but did not inhibit Gram-negative bacteria. The bacteriocin was maximally produced at temperatures between 25°C and 30°C and at the initial pH of 7.0. Ninety % of the activity remained after 10 min of heat treatment at 121°C, and 100% after 1 h exposure to organic solvents. The bacteriocin was purified from culture supernatant by ammonium sulfate precipitation, CM Sepharose column chromatography, ultrafiltration, and finally, by reverse-phase HPLC. A 1.58-kb fragment was amplified from B2 chromosome by using a primer set designed from the published *nisA* sequence. Sequencing result showed that the fragment contained the whole *nisZ* and 5' portion of *nisB*, whose gene product was involved in postmodification of nisin. The upstream sequence, however, was completely different from those of reported nisin genes.

Key words: Bacteriocin, *Lactococcus lactis*, kimchi, nisinZ

Lactic acid bacteria (LAB) are commonly isolated from dairy, meat, and vegetable products. They contribute to flavor development as well as preservation of foods. Many LAB produce bacteriocins, which are considered to be advantageous for producers when in the competition for foods and niches [23, 28, 32]. Extensive efforts to find bacteriocins with a broad inhibition spectrum and superior stabilities against heat treatment and pH variation have been made during the last decade [23, 32]. Since many fermented foods containing LAB have been consumed for thousands of years, bacteriocins from LAB or LAB producing bacteriocins are regarded as safe for human consumption. Therefore, bacteriocins from LAB are the prime candidates for developing safe food preservatives, which can replace chemical preservatives [6, 8, 9].

Kimchi is a traditional Korean fermented food and has been mostly prepared at home. In recent years, the portion of commercially produced kimchi is steadily increasing, necessitating the development of methods for mass production of high quality kimchi [18]. During kimchi fermentation, various LAB are involved at different stages of the ripening process. *Leuconostoc mesenteroides* is the most important organism and dominant during the early and middle stages. *Lc. mesenteroides* endows kimchi its desirable and characteristic refreshing flavor by producing organic acids and carbon dioxide [26]. *Lc. mesenteroides* cells, however, quickly disappear in the late stage because of accumulated acids. More acid resistant lactobacilli such as *Lb. plantarum* and *Lb. brevis* become the dominant flora and they accelerate the deterioration of kimchi by producing more acids. For the purpose of extending the shelf life of kimchi, many studies were carried out to develop acid resistant *Lc. mesenteroides* mutants and some mutants were reported [21, 22]. However, no detailed studies have been done on the nature of the acid resistance mechanism. LAB with bacteriocin activities against lactobacilli might be useful as starters for the commercial production of kimchi. With this objective in mind, we screened LAB from various

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kimchi to isolate bacteriocin producers. This paper reports on the isolation of a nisin-producing *Lactococcus lactis* B2 strain from kimchi and the characterization of its nisin gene.

MATERIALS AND METHODS

Isolation and Identification of a Strain Inhibiting *Lb. plantarum*

To isolate bacteriocin producers from kimchi, diverse kimchi samples were collected, serially diluted, spread on MRS plates, and incubated at 30°C [3, 7]. When colonies appeared, MRS top agar mixed with an indicator strain (100 µl of overnight culture per 3 ml top agar), *Lb. plantarum* KFRI00464, was overlaid and the plates were incubated at 30°C. Morphological and biochemical properties of the bacteriocin producers were examined according to the *Bergey's Manual of Determinative Bacteriology* [17]. These included the optimal growth temperature, growth pH range, Gram staining, mobility, catalase test, CO_2 production, acid production from sugars, and 16S rDNA sequence determination [14, 17].

Bacteriocin Activity in MRS Medium

Production of bacteriocin was examined by the spot-on-the lawn test [10, 30]. Supernatant from an overnight culture was obtained by centrifugation at 11,000 ×g for 20 min. Two ml supernatant was spotted on the MRS plates and overlaid with 3 ml top agar (0.7%, w/v) containing *Lactobacillus plantarum* cells (1×10^8 CFU). After overnight incubation at 30°C, the plates were examined for the presence of inhibition zones. Production of bacteriocin was confirmed by protease treatment of culture supernatant [36]. The bacteriocin activity, expressed as AU (activity unit) per milliliter, was defined as the reciprocal of the highest two-fold dilution showing inhibitory action towards the indicator organism.

Inhibition Spectrum of the Bacteriocin

Two µl overnight culture of *L. lactis* B2 was spotted on the MRS plate. After 5 h of incubation at 30°C, the indicator strain was overlaid and the plate was incubated for 1 day at the temperature optimal for the growth of each indicator organism tested [2, 15, 34]. Lactic acid bacteria were grown in MRS, and *Escherichia coli* and *Salmonella typhimurium* in LB.

Optimal Production of the Bacteriocin

The effect of initial pH of MRS medium on the bacteriocin production was investigated. Overnight culture of *L. lactis* B2 at 1% ratio was inoculated into fresh MRS broth adjusted to pH 4, 5, 6, 7, 8, and 9, respectively. Also, the effect of incubation temperature was examined. *L. lactis* B2 cells were grown in MRS media at 20, 25, 30, and 37°C, and the bacteriocin activity of each culture was determined.

Stability of the Bacteriocin

Stabilities of the bacteriocin against heat, organic solvents, and hydrolytic enzymes were examined [19]. After overnight cultivation in MRS broth, *L. lactis* B2 culture supernatant was obtained by centrifugation at 11,000 ×g for 20 min. Ammonium sulfate was added to the supernatant to the final concentration of 50%, and pellet was obtained by centrifugation at 11,000 ×g for 30 min. Pellet was dissolved in a small volume of water and dialyzed using Spectrapor CE membrane (Spectrum Medical Industries, Inc., Houston, Texas, MW cutoff: 1,000) against distilled water for 12 h at 4°C. Dialyzate was lyophilized and designated as partially purified bacteriocin. Partially purified bacteriocin, stored at −76°C, was used for stability tests after being redissolved in buffer or MRS broth at appropriate concentrations. Bacteriocin concentration used for the stability tests was 2,000 AU/ml. The purified bacteriocin was held at 100°C for 60 min, or 121°C for 30 min, and remaining activities were examined by the spot-on-the lawn method [10]. Stabilities of bacteriocin against pH variation (2–10) were investigated by measuring activities after resuspending the bacteriocin in different pH buffers. Stabilities against organic solvents were assessed by measuring the remaining activities after the bacteriocin was mixed with an equal volume of the solvent and the mixture was kept for 1 h at 25°C. Partially purified bacteriocin sample was also treated with hydrolyzing enzymes, including proteases, for 1 h at 37°C at the concentration of 1 mg enzyme/ml.

Mode of Action and Growth Inhibition of the Bacteriocin against *Lb. plantarum* and *Listeria monocytogenes*

Cells of *Lb. plantarum* KFRI00464 and *L. monocytogenes* ScottA in log-phase growth were washed and resuspended in 5 ml of 50 mM phosphate buffer (pH 6.0) or 50 mM Tris-HCl buffer (pH 9.0) to the final concentration of 8×10^9 CFU/ml and 3×10^9 CFU/ml, respectively. The partially purified bacteriocin was added to the final concentration of 2,000 AU/ml and cells were held at 37°C. Aliquots were taken at the predetermined time intervals for viable cell counting [1, 29]. Growth inhibition of the bacteriocin against *Lb. plantarum* and *L. monocytogenes* was also studied [3]. The bacteriocin was dialyzed in Spectrapor CE membrane (Spectrum Inc., Houston, MW Cutoff: 1,000) against 50 mM phosphate buffer (pH 6.0) or 50 mM Tris-HCl buffer (pH 9.0) at 4°C for 1 day, respectively. Overnight cultures of *Lb. plantarum* and *L. monocytogenes* were inoculated at 1% into 15 ml MRS media containing the bacteriocin (final concentration, 2,000 AU/ml), and the OD_{600} was measured [27].

Purification of the Bacteriocin

Partially purified bacteriocin was further purified. Freeze-dried bacteriocin preparation was resuspended in 10 ml of 20 mM sodium phosphate buffer (pH 6.0), filtered (0.20

μm syringe filter, Whatman), and loaded onto a CM Sepharose fast flow (Sigma) column (bed vol, 75 ml) equilibrated with the same buffer. After washing the column with the same buffer, a linear NaCl gradient (0–0.5 M) was applied at the flow rate of 1 ml/min. Each fraction (5 ml) was collected and monitored for protein content by measuring absorbance at 280 nm. Bacteriocin activity was measured by the spot-on-the lawn method. Fractions with bacteriocin activity were pooled and subjected to ultrafilteration (Centriplus 10, MWCO 10,000, Amicon). Reversed phase HPLC was employed as the final purification step. After the HPLC column (Delta Pak, C18 5 μm, Waters, Japan) was equilibrated with 0.1% TFA solution, an acetonitrile gradient (0–100%) was applied to elute the bacteriocin [4]. Each fraction (1 ml) was monitored by measuring absorbance at 220 nm, and the antimicrobial activity was determined by the spot-on-lawn method. Protein concentration was measured according to the Bradford method using bovine serum albumin as a standard [5].

Sequencing of the Bacteriocin Gene and Surrounding Genes

PCR reaction (denaturation, 95°C, 30 s; annealing, 55°C, 30 s; extension, 72°C, 2 min) was carried out with primer 1 (5'-AATAATGGGCATATCGGGTTTAAA-3') and primer 2 (5'-GCAAAGCGTTACAGTTTCACAAATATC-3'). Primers were designed based on the published *nisA* sequences [7, 15, 24]. Fifty ng of chromosomal DNA was used as a template with *ex-Taq* DNA polymerase (Takara). DNA sequences were determined by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/ primer cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CT, U.S.A.). Primers for sequencing were synthesized at Bioneer (Seoul, Korea). The primer walking method was used to determine the complete sequence of the PCR fragment [11, 12, 33]. Nucleotide sequences of the bacteriocin gene and adjacent genes from *L. lactis* B2 were deposited into GenBank under the accession number AF420259. Homology of the deduced amino acid sequence was analyzed by Blast program at NCBI (National Center for Biotechnology Information, Bethesda, MD, U.S.A.).

Plasmid Preparation

Plasmid DNA from *L. lactis* B2 was prepared according to the method of O'Sullivan and Klaenhammer [31].

Results and Discussion

Isolation and Identification of a *L. lactis* Strain Inhibiting *Lb. plantarum*

Many bacteriocin-producing organisms were isolated from kimchi and one isolate (B2) among them inhibited the growth of *Lb. plantarum* most effectively. Bacteriocin production in B2 was confirmed by testing pH-neutralized and catalase-treated supernatant for inhibition of the indicator bacterium. These treatments did not abolish the inhibiting activity of the supernatant but protease treatment abolished, indicating a proteinous nature of the inhibiting activity. B2 was a Gram-positive, nonmotile, catalase-negative, and facultatively anaerobic coccus. It produced acid from sugars but did not produce gas. It grew at 10°C but not at 45°C. These properties (summarized in Table 1) were in agreement with the reported characteristics of *Lactococcus lactis*. The 16S rDNA sequence of B2 had 98% homology with those of other *L. lactis* strains (results not shown). The subspecies status of B2, however, was not clear. B2 was accordingly named as *L. lactis* B2.

Table 1. Some physiological characteristics of *Lactococcus lactis* B2.

Test items	Result
Gram staining	+
Cell form	cocci
Colony color	white
Mobility	-
Catalase	-
Gas production	
Growth at 10°C	+
Growth at 45°C	-
NaCl tolerance	<4.0%
Growth at 10% EtOH	-
Initial pH of MRS broth where growth occur	4.8-9.6
Acid formation from	
D(+) cellobiose	+
α-L-rhamnose	-
Salicin	+
Melibiose	-
D(+) trehalose	+
D(-) ribose	-
D(+) raffinose	-
D(-) arabinose	-
D(+) mannose	+
Starch	-
Maltose	+
D(+) xylose	-
D(-) fructose	+
D(+) galactose	+
α-Lactose	+
D-mannitol	-
Sucrose	+

symbols: +, positive; –, negative.

Inhibition Spectrum of the Bacteriocin

Various Gram-positive and Gram-negative bacteria were tested for their susceptibilities to the B2 bacteriocin and the results are shown in Table 2. The bacteriocin inhibited most strains of *Lactococcus*, *Leuconostoc*, *Lactobacillus*,

Table 2. Inhibition spectrum of the bacteriocin produced by *L. lactis* B2.

Indicator	Antimicrobial activity*
Lactococcus lactis MG 1614	-
L. lactis MG 1363	+++
L. lactis IL 1403	+
L. lactis LM 0230	++
Leuconostoc mesenteroides ATCC 10830	++
Lc. mesenteroides. subsp. *mesenteroides* KFRI 00666	+++
Lc. mesenteroides. KFRI 00817	+++
Lc. mesenteroides. subsp. *mesenteroides* KFRI 00820	+++
Lc. mesenteroides. subsp. *dextranicum* KFRI 00155	+++
Lc. mesenteroides KFRI 00465	+++
Lc. mesenteroides B-512	+++
Lc. mesenteroides ATCC 9135	+++
Lc. mesenteroides	+++
Lactobacillus acidophilus	++
Lb. acidophillus IAM 1084	+++
Lb. acidophillus KFRI 2161	+++
Lb. bulgaricus CH2	+++
Lb. delbrueckii lactis	++
Lb. delbrueckii lactis ATCC 4797	++
Lb. delbrueckii lactis KFRI 347	+++
Lb. casei YIT 9018	+++
Lb. brevis IL 2014	+++
Lb. pentosus KFRI 481	+++
Lb. plantarum KFRI00464	+++
Listeria monocytogenes ScottA	++
Staphylococcus epidermidis	++
Sta. aureus	+++
Sta. carnosum	-
Bacillus subtilis DB 104	++
B. cereus	++
Streptococcus thermophillus	++
Str. bovis	+++
Enterococcus faecalis	++
Salmonella typhimurium TA98	++
Escherichia coli K-12	-

*The radius of the inhibition zone was indicated by the following : -, negative; +, below 1.5 mm; ++, 1.5-3 mm; +++, above 3 mm.

Staphylococcus, *Enterococcus*, and *Streptococcus*. However, *Lactococcus lactis* MG 1614, *Staphylococcus carnosum*, *Escherichia coli*, and *Salmonella typhimurium* were insensitive. The results indicate that the B2 bacteriocin had a broad inhibition spectrum, similar to plantaricin [34].

Optimal Production of the Bacteriocin

L. lactis B2 produced the bacteriocin maximally when grown in MRS medium adjusted to initial pH of 7.0 and at temperatures between 25°C and 30°C (Fig. 1). Higher inhibitory activities were observed in cells grown at 25°C (2,000 AU/ml) or 30°C (2,000 AU/ml) than that grown at 20°C (1,600 AU/ml) or 37°C (600 AU/ml) at initial pH of 7.0. Higher activity was observed in cells grown at 30°C and initial pH of 7 (2,000 AU/ml) than cells at initial pH of 4 (0 AU/ml), 5 (200 AU/ml), 6 (1,800 AU/ml), 8 (1,800 AU/ml), or 9 (1,600 AU/ml). When *L. lactis* B2 was inoculated into MRS medium of initial pH 4.0, the activity was not detected even at 25°C or 30°C (Fig. 1).

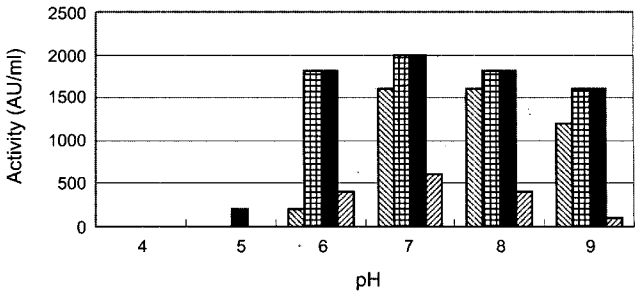


Fig. 1. Production of bacteriocin by *L. lactis* B2 under different cultivation conditions.
-▨-, 20°C; -▦-, 25°C; -■-, 30°C; -▨-, 37°C.

Stability of the Bacteriocin

Inhibitory activity of the bacteriocin was not affected by heat treatment at 80°C for 10 min (data not shown). However, the activity was reduced to half after 20 min of exposure at

Table 3. Stability of bacteriocin against various treatments.

Treatment	Residual activity (AU/ml)
Control	2,000 AU
Enzymes	
β-amylase	2,000 AU
Lysozyme	2,000 AU
Proteinase K	2,000 AU
Pepsin	2,000 AU
RNaseA	2,000 AU
Trypsin	2,000 AU
Catalase	2,000 AU
Protease (5 mg/ml)	0 AU
Solvents	
Ethanol	2,000 AU
Methanol	2,000 AU
Acetonitrile	2,000 AU
Acetone	2,000 AU
Chloroform	2,000 AU
pH change	
pH 2 to 7	2,000 AU
pH 8	60 AU
pH 9 to 10	0 AU
Heat treatment	
100°C, 10 min	1,800 AU
100°C, 20 min	1,600 AU
100°C, 30 min	1,400 AU
100°C, 60 min	200 AU
121°C, 10 min	1,800 AU
121°C, 20 min	1,000 AU
121°C, 30 min	600 AU

121°C (Table 3). Ten % of the initial activity was detected after 60 min of exposure at 100°C. Variation of pH between 2 and 7 did not affect the activity, but the activity was rapidly reduced at pHs 8, 9, and 10. The activity was completey abolished by protease treatment (5 mg/ml), while full activity remained after treatments with lysozyme, catalase, RNaseA, β-amylase, trypsin, pepsin, and proteinase K. The activity was not affected by exposure to 50% organic solvent under the conditions described in Materials and Methods. These results indicate that the B2 bacteriocin is significantly stable, a desirable property, when incorporated to foods.

Mode of Action and Growth Inhibition of the Bacteriocin

To determine whether the B2 bacteriocin had bactericidal or bacteriostatic effects against sensitive strains, the partially purified bacteriocin (2,000 AU/ml) was added to *Lb. plantarum* KFRI00464 and *L. monocytogenes* ScottA cells suspended in 5 ml of 50 mM phosphate buffer (pH 6.0) and 50 mM Tris-HCl buffer (pH 9.0), respectively. Viable cells were counted, using the standard plate counting method, at specific time intervals. As shown in Fig. 2, the number of viable cells decreased rapidly during the first 8 h of exposure at pH 6.0 and then gradually decreased. Approximately 3.9-log scale reduction in the viable cell number occurred during the 48-h period for *L. monocytogenes* and 5-log scale reduction for *Lb. plantarum*. No reduction was observed at pH 9.0. The results showed that the *L. lactis* B2 bacteriocin had bactericidal effects and the bacteriocin was unstable at higher pH. Growth inhibition of these cells was examined after inclusion of the bacteriocin in the broth (Fig. 2). For the first 32-h period, *L. monocytogenes* and *Lb. plantarum* did not grow. After 32 h, bacteriocin resistant cells appeared and the numbers increased as incubation continued. At 48 h, the number of the *Lb. plantarum* cells exposed to the bacteriocin reached to almost the same level of the control culture.

Purification of the Bacteriocin

The purification steps of the bacteriocin are summarized in Table 4. The bacteriocin was purified from the culture supernatant by serially employing ammonium sulfate precipitation, ion-exchange chromatography, ultrafilteration, and reversed-phase HPLC. When a NaCl gradient (from 0 to 0.5 M) was applied to the CM Sepharose column, the bacteriocin was eluted at about 0.2 to 0.3 M NaCl concentration and the fraction contained 1,000 AU/ml of the bacteriocin activity (Fig. 3). The HPLC elution profile is shown in Fig. 4. The bacteriocin was eluted in a single peak with 55–60% (v/v) acetonitrile concentration. After the HPLC purification step, the specific activity of the bacteriocin preparation was 22,241 AU/mg protein, corresponding to a 1,912-times increase in purity from the starting culture supernatant and 503-times from the partially purified

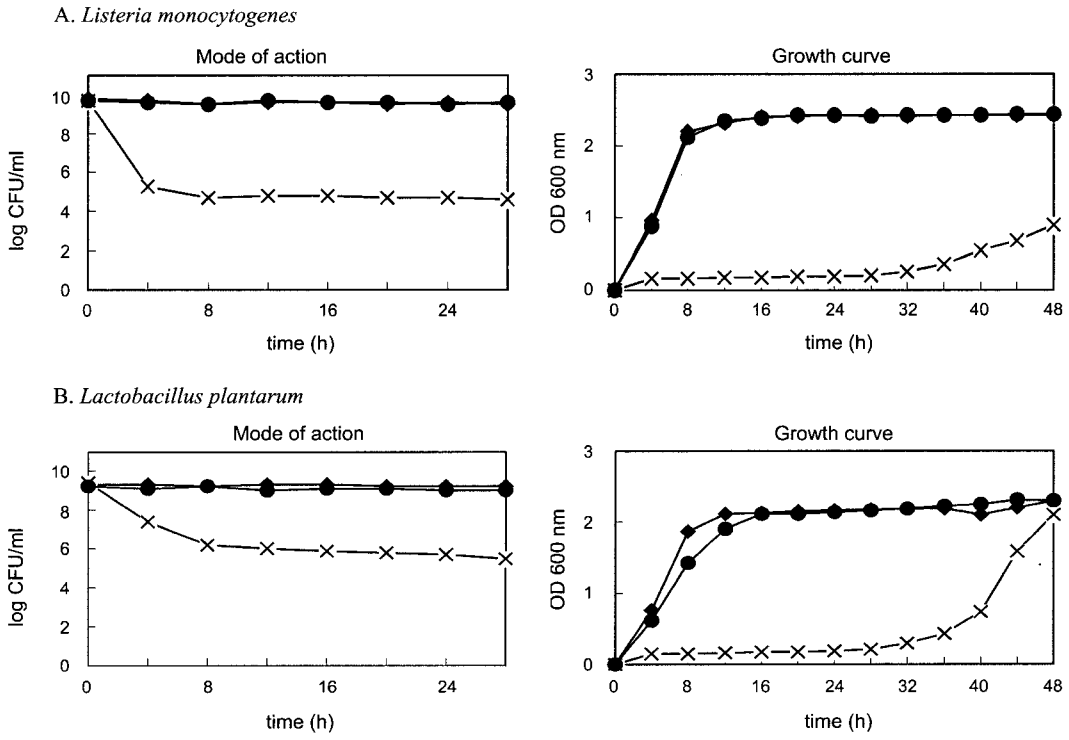


Fig. 2. Mode of action and growth inhibition of the bacteriocin against *Lactobacillus plantarum* and *Listeria monocytogenes*.
-◆-, control; -×-, pH 6.0; -●-, pH 9.0.

Table 4. Purification of the bacteriocin produced by *L. lactis* B2.

Step	Total activity (AU)[a]	Total protein (mg)	Specific activity (AU/mg)[b]	Fold of purity
Culture supernatant	200,000	17.2	11,628	1
Ammonium sulfate precipitation	188,000	4.25	44,235	3.8
CM-Sepharose column chromatography	129,800	0.51	254,510	21.9
Ultrafilteration	98,000	0.22	445,455	38.3
Reversed-phase HPLC	64,500	0.0029	22,241,379	1,912.7

[a]Total activity is the activity unit multiplied by the volume in milliliters.
[b]Specific activity is AU divided by total protein.

bacteriocin preparation. Purified bacteriocin had the same antibacterial activity against sensitive microorganisms and stability against heat treatments as the partially purified bacteriocin had (results not shown).

Sequence Comparison of the Bacteriocin Gene and Related Genes

Since the bacteriocin was produced by a *L. lactis* strain and the characteristics of the bacteriocin were similar to those of nisin, the B2 bacteriocin might likely be nisin. To prove this, the bacteriocin structural gene was cloned by PCR. The cloning strategy was that if the bacteriocin were nisin, the nucleotide sequence of the gene would be identical or almost identical to those of *nisA* or *nisZ* genes, thus making PCR cloning possible. Therefore, primers 1 and 2 were designed based on the published *nisA* gene sequence [13, 24]. When Southern blot was done using a *nisA* probe, a positive signal was detected on the chromosomal DNA from *L. lactis* B2, indicating that the bacteriocin gene located on the chromosome rather than the plasmid (data not shown). Subsequently, a 1.58-kb fragment was amplified and subcloned into pBSKSII (+) (Stratagen, U.S.A.), and the sequence was determined by the primer walking method. Seven primers (primer 1, 2, 3, 4, 5, 6, and 7 [12]) were used and their positions are marked in Fig. 5A. Blast analysis of the sequence (Fig. 5B) confirmed that the bacteriocin gene of *L. lactis* B2 was identical with *nisZ*. *nisZ* encodes NisZ, a lantibiotic antimicrobial peptide and a natural variant of NisA. *nisZ* of *L. lactis* B2 can encode a preprotein of 57 amino acids and the nucleotide sequence is completely identical with the known *nisZ* genes (accession numbers A30280, Z18947, X61144). In addition to the *nisZ* gene, a truncated gene homologous to *nisB* was located downstream of *nisZ*. The gene product of *nisB* is a cell membrane-associated protein and involved in postmodification of NisA or NisZ [13, 24]. Karakas *et al.* [20] recently showed that NisB was involved in the dehydration of serine, the 33rd amino acid in mature nisin. NisB is a protein consisting of 993 amino acids, and only the first 183 amino acids are shown in Fig. 5. Homology of the *nisZ* and truncated *nisB* genes to the corresponding homologous genes is 100% and 99%, respectively. Upstream sequence of *nisZ* had low homology to other known genes. In particular, upstream 52 bp (198–250) was completely different from other *nis* genes in the data library.

Others have also reported isolation of nisin or nisin-like bacteriocin-producing organisms from kimchi [7, 25]. In one case [25], the bacteriocins were not characterized enough to confirm the exact nature. Kwak *et al.* [25] isolated a strain, *L. lactis* subsp. *lactis* J105, from kimchi and purified the bacteriocin. Amino acid sequence showed that bacteriocin J105 was in fact identical with NisA. NisA and

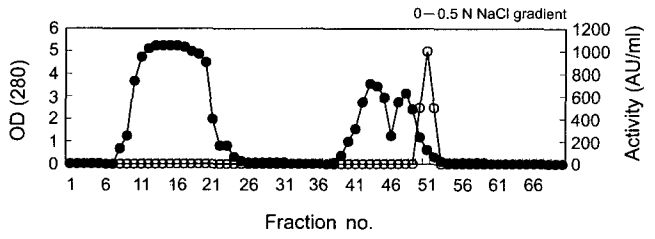


Fig. 3. Elution profile of bacteriocin from a CM sepharose fast flow column equilibrated with 20 mM sodium phosphate buffer (pH 6.0).
A linear NaCl gradient (from 0 to 0.5 M) was applied and the bacteriocin was eluted at the 0.2 to 0.3 M NaCl concentration. The bacteriocin activity of each fraction (5 ml) was assayed by the spot-on-the lawn method; -●-, OD (280 nm); -○-, Activity (AU/ml).

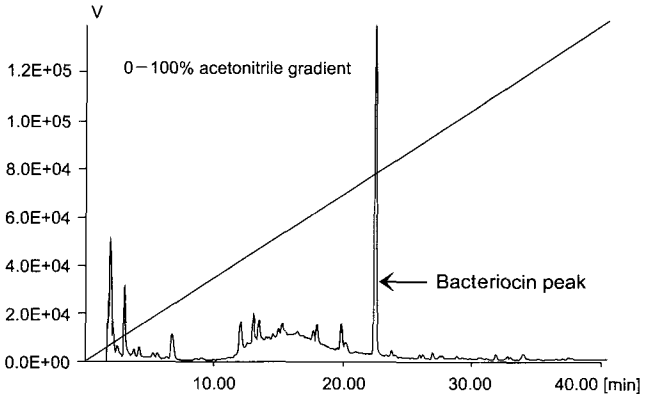
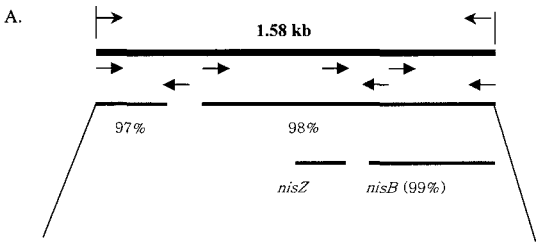


Fig. 4. Elution profile of bacteriocin from a reverse-phase HPLC.
A linear acetonitrile gradient (from 0 to 100%) was applied and the bacteriocin was eluted with 55–60% concentration. The bacteriocin activity of each fraction (1 ml) was determined by the spot-on-the lawn method.



B.

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   1 caataatggg catatcgggt ttaaaaatac tatgtgttta aaagaatctc tcatgagttt   60
  61 gacgccaata acttagatta aaatcaccgt caccttattt ttaggcacgt tcggcagtaa  120
 121 ccttatcaaa ggtatctcag tcattaagtt tcatgatagt atttactatt ttgatggttt  180
 181 ttgttattat ccaatcgtta aaatgacaaa aacaaataga taaatagata aatagataaa  240
 241 tagataaata tttatgggga ggataagtga acttatcatg attaattgta aacgattgag  300
 301 ttctgaatgt ttcaaattat gaggaacaac aggagttgga ctattcttta aacgcctcga  360
 361 cgataccatc actcttcatt agcctaaaat taacaagtta aaatcattag aataatctct  420
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 481 ttgattttct agttcctgaa taatatagag ataggtttat tgagtcttag acatacttga  540
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 601 attctatctt gagaaagtat tggtaataat attattgtcg ataacgcgag cataataaac  660
 661 ggctctgatt aaattctgaa gtttgttaga tacaatgatt tcgttcgaag gaactacaaa  720
 721 ataaattata aggaggcact caaa atg agt aca aaa gat ttt aac ttg gat ttg gta tct  780
                  nisZ >  M   S   T   K   D   F   N   L   D   L   V   S
 781 gtt tcg aag aaa gat tca ggt gca tca cca cgc att aca agt att tcg cta tgt aca ccc  840
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 841 ggt tgt aaa aca gga gct ctg atg ggt tgt aac atg aaa aca gca act tgt aat tgt agt  900
      G   C   K   T   G   A   L   M   G   C   N   M   K   T   A   T   C   N   C   S
 901 att cac gta agc aaa taa ccaaatcaaa ggatagtatt ttgttagttc agacatggat ac  960
      I   H   V   S   K   *
 961 tatcctattt ttataagtta tttagggttg ctaaatagct tataaaaata aagagaggaa 1020
1021 aaaac atg ata aaa agt tca ttt aaa gct caa ccg ttt tta gta aga aat aca att tta 1079
      nisB >  M   I   K   S   S   F   K   A   Q   P   F   L   V   R   N   T   I   L
1080 tct cca aac gat aaa cgg agt ttt act gaa tat act caa gtc att gag act gta agt aaa 1139
      S   P   N   D   K   R   S   F   T   E   Y   T   Q   V   I   E   T   V   S   K
1140 aat aaa gtt ttt ttg gaa cag tta cta cta gct aat cct aaa ctc tat gat gtt atg cag 1199
      N   K   V   F   L   E   Q   L   L   L   A   N   P   K   L   Y   D   V   M   Q
1200 aaa tat aat gct ggt ctg tta aag aag aaa agg gtt aaa aaa tta ttt gaa tct att tac 1259
      K   Y   N   A   G   L   L   K   K   K   R   V   K   K   L   F   E   S   I   Y
1260 aag tat tat aag aga agt tat tta cga tca act cca ttt gga tta ttt agt gaa act tca 1319
      K   Y   Y   K   R   S   Y   L   R   S   T   P   F   G   L   F   S   E   T   S
1320 att ggt gtt ttt tcg aaa agt tca cag tac aag tta atg gga aag act aca aag ggt ata 1379
      I   G   V   F   S   K   S   S   Q   Y   K   L   M   G   K   T   T   K   G   I
1380 aga ttg gat act cag tgg ttg att cgc cta gtt cat aaa atg gaa gta gat ttc tca aaa 1439
      R   L   D   T   Q   W   L   I   R   L   V   H   K   M   E   V   D   F   S   K
1440 aag tta tca ttt act aga aat aat gca aat tat aag ttt gga gat cga gtt ttt caa gtt 1499
      K   L   S   F   T   R   N   N   A   N   Y   K   F   G   D   R   V   F   Q   V
1500 tat acc ata aat agt agt gag ctt gaa gaa gta aat att aaa tat acg aat gtt tat caa 1559
      Y   T   I   N   S   S   E   L   E   E   V   N   I   K   Y   T   N   V   Y   Q
1560 att att tct gaa ttt t
      I   I   S   E   F
```

Fig. 5. (A) Genomic organization of the bacteriocin gene locus. Arrows indicate the locations of the primers used for sequencing. Numbers in % are the homology values of the genes to the corresponding homologous genes. (B) Sequence of the bacteriocin and adjacent genes. Capital letters shown under the *nisZ* and *nisB* nucleotide sequences are amino acid sequences translated from the nucleotide sequences. The putative ribosomal binding site (RBS) for *nisZ* is underlined. The shaded letters correspond to the translation start and stop codons of *nisZ* and *nisB*, respectively.

NisZ are two natural variants of nisin, differing from each other in one amino acid (27th aa): NisA contains histidine and NisZ asparagine. Particular lactococcus producer strains only make one nisin variant. In addition to different variant types, the bacteriocin J105 was inactivated by pepsin treatment but B2 bacteriocin was not. It is not clear whether the difference reflects different structures or are simply due to different experimental conditions. The above results indicate that the nisin genes, *nisA* and *nisZ*, are probably distributed widely among *L. lactis* strains found in kimchi. Future studies on the distribution of nisin genes among *L. lactis* strains found in kimchi would help understanding the roles of these *L. lactis* strains during kimchi fermentation and utilizing them as starters for kimchi preparation. *L. lactis* B2 may be useful as a starter for kimchi fermentation by inhibiting the growth of undesirable pathogens such as *L. monocytogenes*. Also, the strain may slow down acidification of kimchi by inhibiting the outgrowth of lactobacilli, thus contributing to the extension of the shelf-life of kimchi.

Plasmid Profile of *L. lactis* B2

Plasmid DNA of *L. lactis* B2 was prepared according to the method of O'Sullivan and Klaenhammer [31], and the result is shown in Fig. 6. *L. lactis* B2 had one large plasmid, about 50 kb in size. The plasmid profile was different from those of other nisin producers. For example, *L. lactis* subsp. *lactis* 7962 harbors 5 plasmids [35], whereas plasmid DNA was not detected in *L. lactis* subsp. *lactis* NCK400, a nisin-producer isolated from fermenting sauerkraut [16]. *L. lactis* subsp. *lactis* NCK318-2 and NCK318-3, nisin producers and derivatives of another isolate from sauerkraut, harbored two (38 and 55 kb) and three plasmids (38, 55, and 75 kb), respectively [16]. Harris *et al.* [16] showed that the isolates were different from *L. lactis* subsp. *lactis* ATCC11454 and *L. lactis* subsp. *lactis*

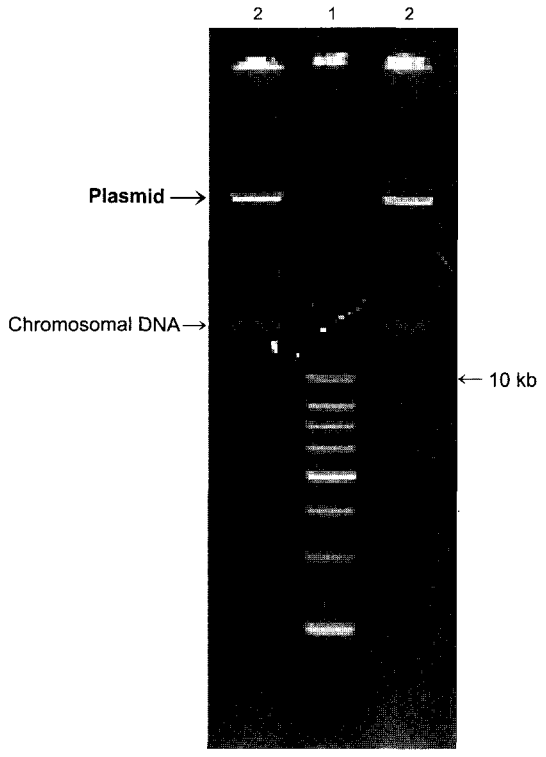

Fig. 6. Plasmid profile of *L. lactis* B2.
1. DNA size marker (1 kb ladder. Jeil. Biotech. Inc.); 2. plasmid prep from *L. lactis* B2.

ATCC7962 in terms of nisin gene organization. The results presented in this paper confirm that unique nisin-producing *L. lactis* strains are also present in kimchi.

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