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Identification and Characterization of Bacteriocin-Producing Lactic Acid Bacteria Isolated from Kimchi

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Abstract Lactic acid bacteria were isolated from Kimchi and screened for bacteriocin. A total of 99 strains showed antimicrobial activity when grown on solid media, yet only 10 showed antimicrobial activity in liquid media. Strain H-559, identified as *Lactococcus lactis* subsp. *lactis*, exhibited the strongest inhibitory activity and was active against pathogenic bacteria including *Listeria monocytogenes*, *Staphylococcus aureus*, and *Bacillus cereus* as well as other lactic acid bacteria. The antimicrobial substance produced by *L. lactis* subsp. *lactis* H-559 was confirmed to be a bacteriocin by the treatment of α-chymotrypsin, and protease type IX and XIV. The bacteriocin activity remained stable between pH 2.0 and pH 11.0 and during heating for 10 min at 100°C. The bacteriocin production started in the exponential phase and stopped in the stationary phase. *L. lactis* subsp. *lactis* H-559 showed the highest bacteriocin activity at a culture temperature of 25°C, and an inverse relationship between the bacteriocin productivity and mean growth rate at different culture temperatures was observed. The mean growth rate and bacteriocin productivity of *L. lactis* subsp. *lactis* H-559 increased as the initial pH of the media increased.

Key words: Bacteriocin, lactic acid bacteria, *Lactococcus lactis* subsp. *lactis*, Kimchi

Lactic acid bacteria comprising the genera of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and the newly recognized *Carnobacterium*, contribute to the flavor and color development, as well as the preservation of foods. Lactic acid bacteria also have the potential to inhibit the growth of pathogenic and spoilage bacteria, thereby improving the hygienic quality. It is now generally accepted that this antimicrobial activity is due to organic acids, hydrogen peroxide, diacetyl, carbon dioxide, and bacteriocins [21].

Bacteriocins are proteins or protein complexes with bactericidal activity towards closely related species to the producer bacteria [21]. Recently, bacteriocins have aroused great interest as candidates for food preservation. They avert consumers' hostility to chemical preservatives as they are sensitive to proteolytic enzymes in the digestive system; in addition, lactic acid bacteria and their products have been classified as GRAS (Generally Recognized As Safe). The possibility of genetic manipulation of genes encoding bacteriocin has been considered as major attracting concerns of bacteriocin research. It has also been reported that bacteriocins are produced by all genus of lactic acid bacteria, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Carnobacterium*, and *Bifidobacterium*.

Kimchi is prepared with various vegetables, and it becomes palatable and preservable through proper fermentation caused by lactic acid bacteria. Therefore, Kimchi is a unique source of lactic acid bacteria, especially originated from vegetables. However, the importance and industrial value of bacteriocins in Korea have been underestimated. The possibility of screening a new bacteriocin producer is one of the major interests of bacteriocin research with Kimchi [20, 5, 6]. This study was conducted to screen a new bacteriocin producer from lactic acid bacteria isolated from Kimchi. This paper described the isolation of lactic acid bacteria producing bacteriocin from Kimchi and the identification and physiological characterization of this novel bacteriocin producer.

Materials and Methods

Bacterial Strains and Media

The bacterial strains used as indicator microorganisms for the bacteriocin screening and evaluation of antimicrobial

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activities were obtained from the Korean Collection for Type Culture (KCTC) and American Type Culture Collection (ATCC). *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus* strains were propagated in MRS broth (Difco, Detroit, U.S.A.) at either 30°C or 37°C. *Bacillus* strains were propagated in nutrient broth (Difco) at 30°C. *Streptococcus thermophillus* was grown in tomato juice medium (tomato juice 100 ml/l, yeast extract 5 g/l, skim milk 100 g/l) at 37°C, whereas *Candida albicans* was grown in YEPD medium (yeast extract 10 g/l, peptone 20 g/l, glucose 20 g/l) at 30°C. *Lactococcus raffinolactis* and *Listeria monocytogenes* were propagated in blood agar base medium (Difco) at 30°C and BHI media (Difco) at 37°C, respectively. All other strains were grown in LB medium (Difco) at 37°C. MRS agar, phenylethyl alcohol sucrose (PES) agar [26] (trypticase pepton 5 g/l, yeast extract 0.5 g/l, sucrose 20 g/l, $MgSO_4 \cdot 7H_2O$ 0.244 g/l, $(NH_4)_2SO_4$ 2 g/l, KH_2PO_4 1 g/l, phenylethyl alcohol 2.5 g/l, agar 15 g/l), modified *Lactobacillus* selection (m-LBS) agar [27] (trypticase pepton 10 g/l, yeast extract 5 g/l, glucose 20 g/l, ammonium citrate 2 g/l, sodium acetate 15 g/l, $MgSO_4 \cdot 7H_2O$ 0.575 g/l, $MnSO_4$ 0.12 g/l, KH_2PO_4 6 g/l, $FeSO_4$ 0.034 g/l, sorbitan monooleate 1 g/l, agar 15 g/l), and M-*Enterococcus* agar [26] (trypticase pepton 15 g/l, phyton pepton 5 g/l, yeast extract 5 g/l, glucose 2 g/l, KH_2PO_4 4 g/l, agar 15 g/l) were used to isolate lactic acid bacteria from Kimchi. MRS agar plates containing only 0.2% glucose (MRS-0.2) were used for screening of the bacteriocin activity in order to exclude the antimicrobial activity caused by acid production.

Isolation of Lactic Acid Bacteria from Kimchi

Various kinds of Kimchi were used as the sources to isolate lactic acid bacteria. Homogenized Kimch was serially diluted l0-fold with saline solution, and plated on MRS agar and selection agar media. PES agar media were incubated at 20°C for 5 days and M-*Enterococcus* agar media were incubated at 37°C for 3 days. MRS and m-LBS agar media were incubated at 30°C for 3 days. Isolated lactic acid bacteria were maintained as frozen stock cultures at - 70°C in MRS broth with 20% glycerol.

Screening of Bacteriocin-Producing Lactic Acid Bacteria

For the detection of antimicrobial activity, the agar spot test was used as described by Harris [14] with several modifications. The lactic acid bacteria isolated from Kimchi were transferred twice in MRS broth before use. Overnight cultures of the strains were spotted onto the surface of a MRS-0.2 agar plate and incubated for 20 to 24 h at 30°C to allow colonies to develop. Five milliliters of the MRS soft agar (containing 0.7% agar) seeded with an overnight culture of the indicator strain at a level of 5×10^6- 5×10^7 CFU/ml was poured over the plate. After incubation for 24 h at appropriate temperatures, the plates were checked for inhibition zones. The well-diffusion assay, as described by Lyon and Glatz [24] with some modifications, was used to select the lactic acid bacteria that were excreting bacteriocin into the liquid media. The basal layer of MRS contained 2% agar and was 5 mm deep. Wells of 7 mm diameter were cut and the bottoms of the wells were sealed with a few drops of MRS agar. After the isolates were grown in MRS broth for 16 h at 30°C, the cells were removed by centrifugation (10,000×g, 15 min). Two hundred microliters of the supernatant fractions, adjusted to pH 7 with 1 N NaOH, were added to the wells and allowed to diffuse at 4°C. To examine the proteinaceous nature of the antimicrobial substances, proteolytic enzyme solutions were spotted just beside the wells. The proteolytic enzyme (all obtained from Sigma) solutions were α-chymotrypsin (10 mg/ml) in 50 mM Tris-HCl and 10 mM $CaCl_2$ (pH 8.0), trypsin (10 mg/ml) in 50 mM Tris-HCl (pH 8.0), protease type IX and protease type XIV (20 mg/ml) in Tris-HCl (pH 7.5). The supernatant fluid and buffer without enzyme were tested as control. The base agar was flipped into the Petri dish lid and overlayed with soft agar of MRS inoculated with the indicator strains at a level of 10^7 CFU/ml. After incubation for 20- 24 h at proper temperatures, the zones of inhibition and any partial loss were checked.

Characterization of Bacteriocin-Producing Strain

To identify the bacteriocin-producing strain, several morphological and cultural characteristics were examined. These tests included cell morphology, gram stain, spore formation, motility, growth in MRS broth at different temperatures and pH, and growth in MRS broth supplemented with various concentrations of NaCl. Several biochemical tests, as shown in Table 2, were performed according to the method of Cowan and Steel [7] and Macfaddin [25].

16S rDNA Sequencing and Phylogenetic Analysis

The 16S rDNA was amplified by PCR using two universal primers [35]. The PCR product was purified by using the QIA quick PCR purification kit (Qiagen). Sequencing was performed using a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) and a model 310 automatic DNA sequencer (Applied Biosystems). The 16S rDNA sequence of strain H-559 was aligned with 16S rRNA gene sequences of *Lactococcus* species and some other related taxa by using the CLUSTAL W software [33]. Gaps at the 5' and 3' ends of the alignment were omitted from further analysis. Evolutionary distance matrices were calculated by using the algorithm of Jukes and Cantor [19] with the DNADIST program within the PHYLIP package [12]. A phylogenetic tree was constructed by using the neighbor-joining method [30] as implemented within the NEIGHBOR program of the same package.

Bacteriocin Activity Assay

The antimicrobial activities of the culture supernatants and partially purified bacteriocin were determined using the serial dilution method. The cell-free-culture supernatant was adjusted to pH 7, and partially purified bacteriocin samples were prepared as follows. The bacteriocin producing strain was grown in MRS broth for 13 h at 30°C. The cell-free supernatant was made up to 75% saturation by the addition of ammonium sulfate. After overnight incubation at 4°C, the precipitate was collected, dialyzed using a Spectra-Por dialysis membrane (2,500 molecular weight cut-off, Spectrum Medical Industries, Inc., Ca, U.S.A.) against 10 mM sodium phosphate buffer (pH 6.0), and lyophilized. Next, the lyophilized samples were redissolved in 10 mM sodium phosphate buffer (pH 6.0). The antimicrobial activities of the serially 2-fold diluted samples (200 μl) were examined by the well diffusion method as described previously. The indicator strain was *Pediococcus acidilactis* KCTC 3101. The activity units (AU) per ml of bacteriocin sample were calculated from the reciprocal of the highest dilution that produced a detectable zone of inhibition.

Effects of pH, Heat Treatment, and Enzymes on Bacteriocin Activity

The pH stability of bacteriocin was examined as described by Lyon and Glatz [23]. Partially purified bacteriocin was dissolved in 10 mM Na-phosphate buffers (pH 6.0) and individually dialyzed using a Spectra-Por dialysis membrane (2,500 molecular weight cut-off, Spectrum Medical Industries, Inc., Ca, U.S.A.) for 24 h against 2 liters of the following buffers: 0.05 M glycin-HCl buffer (pH 2.0, 3.0), 0.05 M acetate buffer (pH 4.0, 5.0), 0.05 M citrate buffer (pH 6.0), 0.05 M Tris-HCl buffer (pH 7.0, 8.0), 0.05 M glycin-NaOH buffer (pH 9.0, 10.0), 0.05 M phosphate-NaOH buffer (pH 11.0). Each buffer was changed three times during the dialysis. After dialysis, the bacteriocin solutions were assayed for activity. The effect of heat was determined as described by Lyon and Glatz [24]. Partially purified bacteriocin samples were boiled for 10, 20, and 30 min in a water bath, autoclaved (121°C) for 10 min and 20 min, cooled, and then assayed for residual activity. The following enzyme (all obtained from Sigma) solutions were used for the determination of bacteriocin stability to various enzymes: catalase (1,540 U/mg) in 10 mM potassium phosphate (pH 7.0); glucose oxidase (6,000 U/mg) in 50 mM sodium acetate (pH 5.0); phospholipase A_2 (7 U/mg) in 50 mM Tris-HCl (pH 8.0); lipase (900 U/mg) in 50 mM Tris-HCl and 10 mM $CaCl_2$ (pH 7.0); peroxidase (78 U/mg) in 50 mM sodium acetate (pH 6.0); α-amylase (200 U/mg) in 50 mM sodium acetate (pH 6.0); β-amylase (80 U/mg) in 30 mM sodium acetate (pH 5.0); pepsin (2100 U/mg) in 10 mM citrate (pH 2.0); trypsin (17,600 U/mg) in 50 mM Tris-HCl (pH 8.0); protease type IX (0.7 U/mg), protease type III (0.5 U/mg) and protease type XIV (4.8 U/mg) in 50 mM Tris-HCl (pH 7.5); α-chymotrypsin (46 U/mg) in 50 mM Tris-HCl and 10 mM $CaCl_2$ (pH 8.0); subtilisin (15 U/mg) in 50 mM Tris-HCl (pH 7.5); carboxy peptidase A (920 U/mg) in 50 mM Tris-HCl (pH 7.5). The partially purified bacteriocin was dissolved in buffers recommended by the supplier and incubated with each enzyme solution at a final concentration of 1 mg/ml for 2 h at 37°C, except for catalase, trypsin, α-chymotrypsin, and carboxypeptidase A, which were incubated at 25°C. Samples containing peroxidase and α-amylase were incubated at 20°C. Separate aliquots, with bovine serum albumin instead of enzymes, were included as controls. After incubation, samples were boiled for 3 min and the residual activities were determined.

Antimicrobial Spectrum

The inhibitory activity of partially purified bacteriocin was tested against several Gram-positive and Gram-negative strains, including food spoilage and pathogenic organisms, by the well-diffusion method [24]. Indicator strains were subcultured in the appropriate medium and then inoculated in the soft agar medium.

Influence of pH and Temperature on Growth and Bacteriocin Production

MRS broth inoculated with an overnight culture of strain H-559 at a level of 1% (v/v) was incubated at 25, 30, and 37°C. The influence of pH was studied using MRS broth adjusted to pH 5.0, 5.7, 6.1, 6.5, 7.0, 7.7, 8.0, and 9.0 with 2 N NaOH and 2 N HCl at 30°C. Cultures were also carried out in a 1.5 liter working volume fermenter (Biostat M, B. Braun Biotech. Allentown, PA, U.S.A.). The temperature was maintained at 30°C, and the culture was agitated at 50 rpm. The pH of the growth media was maintained at 7.0 by a pH controller. Cell growth was monitored spectrophotometrically and by plate counts. The bacteriocin activity of the culture broth was evaluated by the method described previously.

Results and Discussion

Screening of Bacteriocin-Producing Lactic Acid Bacteria

About 4,000 lactic acid bacteria were isolated from various kinds of Kimchi using a variety of isolation media (MRS, PES, m-LBS, and M-*Enterococcus* agar media). These isolates were tested for their antimicrobial activity towards 25 indicator strains, using the spot-on-the lawn method. In this test, a total of 99 strains showed antimicrobial activity to some of the indicator strains. However, only 10 strains produced inhibition zones when the cell-free supernatant of these strains were checked with the well-diffusion assay. The antagonistic activities of 10 strains were sensitive to proteolytic enzymes, indicating that these activities were

due to bacteriocins. The inhibitory spectrum of strain H-559 was broader than that of any other isolates. Therefore, strain H-559 was selected for further study. The bacteriocin produced by strain H-559 was purified and confirmed as a novel bacteriocin through an analysis of mass determination, amino acid composition, and amino acid sequencing (unpublished data).

Identification of Isolate H-559

The phenotypic characteristics of strain H-559 are summarized in Table 1. The isolate was Gram-positive, nonmotile, and catalase-negative. The cells were coccus (Fig. 1), homofermentative, negative to growth at 45°C, and hydrolyzed arginine and esculin. Most characteristics of strain H-559 coincided with the description of

Table 1. Comparison of morphological and physiological characteristics of strain H-559 with *Lactococcus lactis* subsp. *lactis* [7, 8].

Characteristics		*Lactococcus lactis* subsp. *lactis*	Strain H-559
Morphology			
Shape		cocci	cocci
Cell size (μm)		0.5~1.2×0.5~1.5	0.55~0.6×0.7~1.25
Spores		-	-
Motility		-	-
Acid-fast straining		-	-
Culture characteristics			
Growth in air		+	+
Growth anaerobically		+	+
Growth at 10°C		+	-
15°C		W	+
30°C		+	+
37°C		+	+
40°C		+	+
42°C		W	+
45°C		NT	-
Growth in broth at	pH 4.2	NT	-
	pH 7.5	NT	+
	pH 8.5	NT	+
	pH 9.2	+	+
Growth in broth with	3% NaCl	+	+
	4% NaCl	+	+
	6% NaCl	NT	-
	6.5% NaCl	NT	-
	8% NaCl	NT	-
	18% NaCl	NT	-
Physiological characteristics			
Catalase		-	-
Oxidase		-	-
O/F		F	F
Growth in 0.3% methylene blue in milk			
Gas from glucose		+	+
Acid from:			
Glucose		+	+
Dextrin		-	d
Rhamnose		-	-
Rhaffinose		-	-
Ribose		+	NT
Sorbitol		-	-
Lactose		+	+
Sucrose		d	d
Mannitol		(-)	d
Maltose		+	+
Arginine hydrolysis		+	+
Esculin hydrolysis		NT	+
TTC (2,3,5-triphenyl tetrazolium chloride)		NT	+(red)

Symbols: +, 90% or more of strains positive; -, 90% or more of strains negative; d, Different reactions in different strains; F, Fermentation; O, Oxidation; W, Weak reaction; NT, Not testable.

Lactococcus lactis subsp. *lactis* based on Bergey's Manual of Systematic Bacteriology [31] and Bergey's Manual of Determinative Bacteriology [13]. The 16S rDNA sequence of strain H-559 determined were 1502 bp long which corresponded to the regions between positions 28 and 1524 by comparison with the 16S rDNA of *Escherichia coli*. The phylogenetic tree (Fig. 2) shows that strain H-559 forms its phylogenetic position within the radiation enclosed by the genus *Lactococcus*. Strain H-559 had a 16S rDNA sequence identical with that of *Lactococcus lactis* subsp. *lactis* ATCC19435^T. Therefore, this strain was identified as *Lactococcus lactis* subsp. *lactis*, and the isolate was tentatively named as *Lactococcus lactis* subsp. *lactis* H-559.

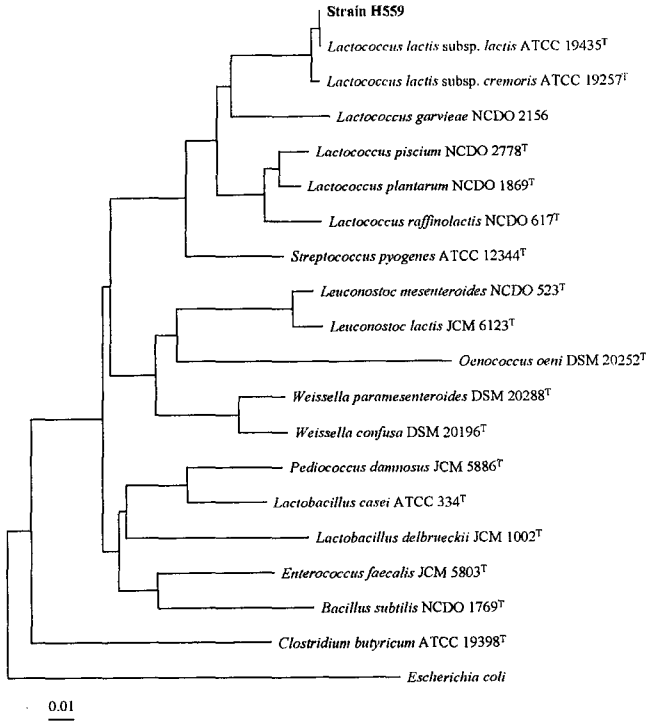

Fig. 1. Phylogenetic tree based on 16S rDNA sequences showing the position of strain H-559, *Lactococcus* species, and representatives of some related taxa. The scale bar represents 0.01 substitution per nucleotide position.

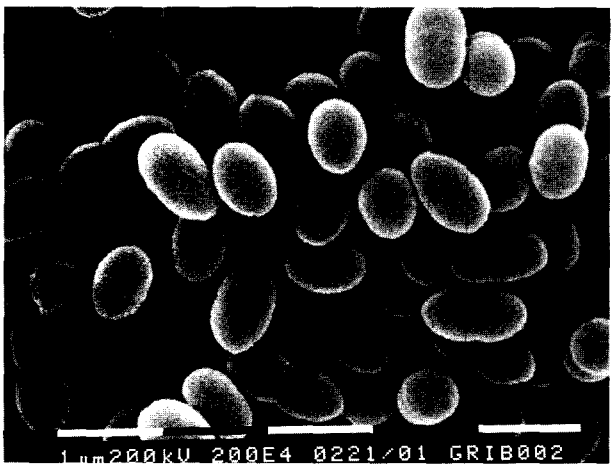

Fig. 2. Scanning electron micrograph (SEM) of the isolate *Lactococcus lactis* subsp. *lactis* H-559.

Antimicrobial Spectrum

The inhibitory activity of the partially purified bacteriocin by ammonium sulfate precipitation was tested against various gram-negative and gram-positive bacteria, including other lactic acid bacteria and several pathogenic strains. The results are shown in Table 2. All three species of *Pediococcus* tested were sensitive to the bacteriocin. Seven

Table 2. Antimicrobial spectrum of the bacteriocin produced by *Lactococcus lactis* subsp. *lactis* H-559.

Indicator strain	Antimicrobial activity
Lactobacillus brevis subsp. *brevis* KCTC 3498	++
Lactobacillus plantarum KCTC 3099	+++
Lactobacillus delbrueckii-lactis ATCC 4797	+++
Lactobacillus viridescens KCTC 3504	++
Lactobacillus hilgaridi KCTC 3500	+++
Lactobacillus parabuchneri KCTC 3503	++
Lactobacillus animalis KCTC 3501	+
Lactobacillus hilgardii KCTC 3500	-
Lactobacillus vaginalis KCTC 3515	-
Lactobacillus delbruckii-lactis KCTC 1058	-
Lactobacillus confusus KCTC 3499	-
Lactobacillus paracasei subsp. *paracasei* KCTC 3510	-
Lactobacillus delbrueckii-lactis KCTC 1058	-
Leuconostoc carnosum KCTC 3525	+
Leuconostoc lactis KCTC 3528	++
Leuconostoc mesenteroides KCTC 3100	++
Leuconostoc mesenteroides subsp. *mesenteroides* KCTC 3505	+
Leuconostoc mesenteroides subsp. *cremoris* KCTC 3529	++
Leuconostoc mesenteroides subsp. *dextranicum* KCTC 3530	+++
Leuconostoc paramesenteroides KCTC 3531	++
Leuconostoc amelibiosum KCTC 3524	-
Leuconostoc citreum KCTC 3526	-
Leuconostoc pseudomesenteroides KCTC 3532	-
Lactococcus raffinolactis KCTC 3509	-
Pediococcus acidilactici KCTC 3101	+++
Pediococcus dextrinicus KCTC 3506	+++
Pediococcus pentosaceus KCTC 3507	+
Listeria monocytogenes KCTC 3444	++
Enterococcus faecium KCTC 3095	-
Staphylococcus aureus 209 KCTC 1916	+
Staphylococcus aureus R-209 KCTC 1928	+
Candida albicans IFO 1594 KCTC 1940	-
Escherichia coli BE 1186 KCTC 1924	-
Salmonella typhimorium SL 1102 KCTC 1926	-
Streptococcus thermophilus KCTC 2185	-
Bacillus subtilis KCTC 1023	-
Bacillus cereus KCTC 1013	+

-, no inhibition zone; +, radius of inhibition zone <3 mm; ++, radius of inhibition zone 3 to 6 mm; +++, radius of inhibition zone >6 mm.

species of *Lactobacillus* and 7 species of *Leuconostoc* tested were inhibited. Among the pathogenic and food-spoilage bacteria tested, *Listeria monocytogenes, Staphylococcus aureus,* and *Bacillus cereus* were inhibited. However, the inhibitory activity was not observed against *Candida albicans* and *Bacillus subtilis*. The bacteriocin was not active against gram-negative bacteria, *Escherichia coli* and *Salmonella typhimurium*. Accordingly, from its inhibitory spectrum, the bacteriocin produced by *Lactococcus lactis* subsp. *lactis* H-559 appeared to take an intermediate position between lantibiotic nisin, which inhibits most gram-positive bacteria [3] and several bacteriocins from *Lactobacillus* sp. such as lactacin B [2], helveticin [18], and caseicin 80 [28], whose activity spectrums are rather narrow and include only strains belonging to the same genus.

Effects of pH, Heat Treatment, and Enzymes on Bacteriocin Activity

The partially purified bacteriocin by ammonium sulfate precipitation was stable within a wide pH range from 2 to 11 (data not shown). Its inhibitory activity was unaffected by heat treatment at moderate temperatures and at 100°C for 10 min. However, its inhibitory activity was reduced during subsequent heating, although not completely inactivated even at 121°C for 20 min (Table 3). This heat stability also rules out the possibility of the inhibitory action being due to bacteriophage. The heat stability could be due to the formation of small globular structures and the occurrence of strongly hydrophobic regions, stable cross-linkage, and a high glycine content [9]. Heat stability is very useful if bacteriocin is to be used as a food preservative, because many processing procedures involve a heating stage. Therefore, the bacteriocin produced by *Lactococcus lactis* subsp. *lactis* H-559 shows the potential as a food preservative for pasteurized products or canned foods. Furthermore, the bacteriocin stability at neutral and basic pH constitutes an advantage over nisin. The maximal solubility and stability of nisin are at pH 2, and these parameters decrease significantly as the pH increases, which is a considerable disadvantage for use as an additive in non-acidic foods [11]. The effects of various enzymes on bacteriocin activity are shown in Table 4. The antibacterial activity of the bacteriocin was not inactivated in the presence of catalase, which excluded an inhibition by hydrogen peroxide. Treatment with protease type III, IX, XIV, and α-chymotrypsin caused a complete loss of bacteriocin activity. When the partially purified bacteriocin was treated with lipase and amylases, the activity was not changed. These results confirm the proteinaceous nature of the antimicrobial substance and suggest that neither lipid nor carbohydrate moieties are essential to the bacteriocin acitivity.

Table 3. Effect of heat treatment on bacteriocin activity.

Heat treatment	Residual activity (AU/ml)
100°C 10 min	1,280
20 min	640
30 min	640
121°C 10 min	640
20 min	320
Control	1,280

Table 4. Susceptibility of bacteriocin activity to various enzymes.

Enzyme	Residual activity (AU/ml)
Catalase	1,280
Glucose oxidase	1,280
Phospholipase A_2	1,280
Lipase	1,280
Peroxidase	1,280
α-amylase	1,280
β-amylase	1,280
Pepsin	1,280
Trypsin	1,280
Protease type IX	0
Protease type III	0
Protease type XIV	0
α-chymotrypsin	0
Subtilisin	1,280
Carboxypeptidase	1,280
Control	1,280

Influence of Temperature on Growth and Bacteriocin Production

Figure 3 shows the cell growth, bacteriocin production, and pH change of the culture broth when the cells were cultured at 25, 30, and 37°C. In all cases, the biosynthesis of the bacteriocin and its excretion into the medium took place during the exponential growth phase. The highest activity was found at the onset of the stationary phase; after that, the biosynthesis of the bacteriocin stopped. Consequently, the bacteriocin was only produced during the active growth phase. Almost all bacteriocins of lactic acid bacteria are produced during the active growth phase. Furthermore, the lantibiotics Rep 5 [29], epidermin [16], and gallidermin [15] are also synthesized during the growth phase of the producer strain. Growth at 25°C was poor, yet the maximal bacteriocin activity (160 AU/ml) was higher than that at any other temperature. Although the strain had the highest mean growth rate in cultures grown at 37°C, bacteriocin production was much lower at 40 AU/ml. The results are summarized in Table 5. There was an inverse relationship between the value of the mean growth rate at different temperatures and bacteriocin productivity (maximal bacteriocin activity divided by the time for maximal bacteriocin activity). This fact was in accordance with the results of Sudirman *et al.* [32] who reported that *Leuconostoc mesenteroides* subsp. *mesenteroides* FR 52

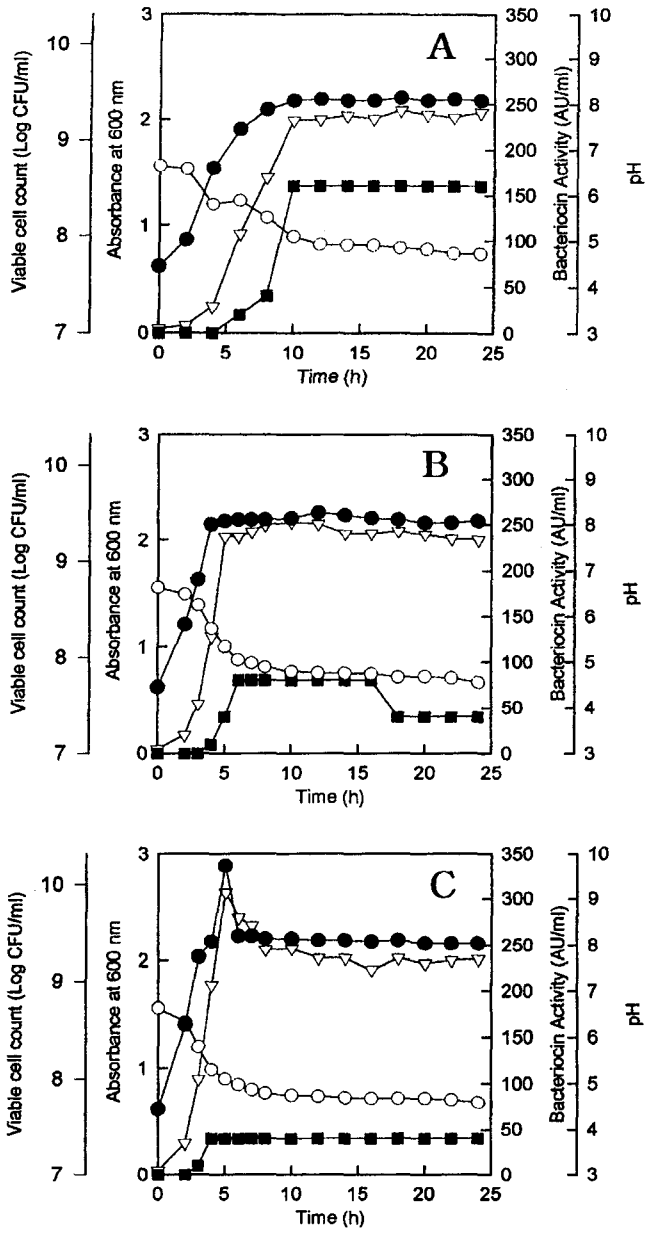

Fig. 3. Batch culture profiles of *Lactococcus lactis* subsp. *lactis* H-559 cell growth and bacteriocin production at different temperatures.
A, 25°C; B, 30°C; C, 37°C. ●, viable cell count (Log CFU/ml); ▽, absorbance at 600 nm; ■, bacteriocin activity (AU/ml); ○, pH.

showed an inverse relationship between the value of the mean growth rate at different growth temperatures and the final specific productivity (total activity of the culture versus the total biomass) of bacteriocin. The result that a lower temperature coincided with a higher maximum bacteriocin production under uncontrolled pH conditions, was also obtained by De Vuyst *et al.* [10] with the bacteriocin from *Lactobacillus amylovorus*, Vignolo *et al.* [34] with lactocin 705, Krier *et al.* [22] with mesenterocin 52A, and Ivanova *et al.* [17] with the bacteriocin from *Streptococcus thermophilus* 81. They supposed that bacteriocin production was stimulated by temperatures unfavorable for growth, particularly the low temperatures. The differential rate of bacteriocin synthesis seemed to increase when the overall protein biosynthetic rate in the cell slowed relatively at lower temperatures [27].

Influence of Initial pH of Media on Growth and Bacteriocin Production

The effect of the initial pH of the media on bacteriocin production was examined (Fig. 4). The bacteriocin was produced in the active growth phase. The highest bacteriocin activity was reached at the end of the exponential phase, and corresponded with the maximal biomass, regardless of the initial pH. As the initial pH of MRS broth increased, the values of the mean growth rate, the maximal bacteriocin activity, and bacteriocin productivity increased. The maximum bacteriocin activity was produced at pH 8.0. These results are summarized in Table 6. Figure 5 represents a batch culture profile of *Lactococcus lactis* subsp. *lactis* H-559 at a controlled pH of 7.0. The neutralization of lactic acid by the addition of 3 N NaOH accelerated the cell growth which had already ceased after 6 h of culture. The bacteriocin activity increased almost proportionally with growth and reached its maximum at the end of the exponential growth phase. After reaching their peak values, the bacteriocin activity and biomass levels (assumed by measuring the OD at 600 nm) both dropped sharply with a prolonged culture time. Similar patterns were obtained for several other bacteriocins, for instance, lacticin DP1 production by *L. lactis* subsp. *lactis* [27], thermophillin T production by *Streptococcus thermophilus* ACA-DC 0040 [1], and nisin production by *L. lactis* subsp.

Table 5. Relationship between the mean growth rate of *Lactococcus lactis* subsp. *lactis* H-559 and the productivity of the bacteriocin at different temperatures.

Temperature (°C)	Mean growth rate, μ (1/h)[a]	Time of exponential growth (h)[b]	Max. bacteriocin activity (AU/ml)[c]	Time for max. bacteriocin activity (h)[d]	Productivity (AU/ml/h)[e]
25	0.53	0-6	160	10	16
30	0.81	0-5	80	6	13.3
37	0.97	0-4	40	4	10

[a]mean growth rate during growth phase; [b]culture time to give exponential growth; [c]maximum bacteriocin activity; [d]minimum culture time for maximum bacteriocin activity; [e]c/d.

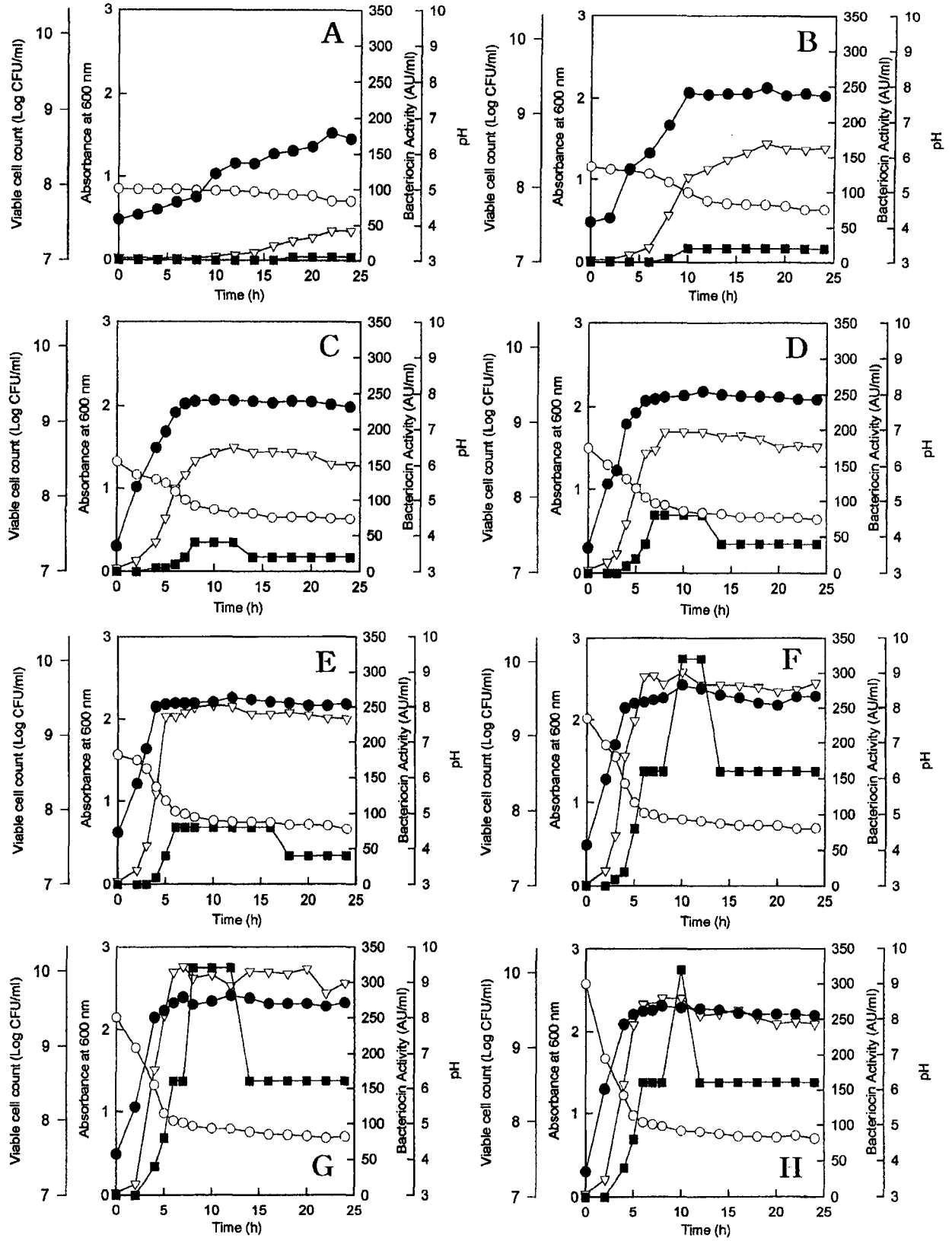

Fig. 4. Batch culture profiles of *Lactococcus lactis* subsp. *lactis* H-559 cell growth and bacteriocin production at different initial pHs. A, pH 5.0; B, pH 5.7; C, pH 6.1; D, pH 6.5; E, pH 7.0; F, pH 7.7; G, pH 8.0; H, pH 9.0. ● , viable cell count (Log CFU/ml); ▽ , absorbance at 600nm; □ , bacteriocin activity (AU/ml); ○ , pH.

Table 6. Relationship between the mean growth rate of *Lactococcus lactis* subsp. *lactis* H-559 and the productivity of the bacteriocin at different initial pHs.

Initial pH	Mean growth rate, (1/h)[a]	Time of exponential growth (h)[b]	Max. bacteriocin activity (AU/ml)[c]	Time for max. bacteriocin activity (h)[d]	Productivity (AU/ml/h)[e]
5.0	0.24	8-18	5	18	0.28
5.7	0.45	2-10	20	10	2
6.1	0.55	0-6	40	8	5
6.5	0.63	0-6	80	7	11.43
7.0	0.81	0-5	80	6	13.33
7.7	0.99	0-4	320	10	32
8.0	0.98	0-4	320	8	40
9.0	0.87	0-3	320	10	32

[a]mean growth rate during growth phase; [b]culture time to give exponential growth; [c]maximum bacteriocin activity; [d]minimum culture time for maximum bacteriocin activity; [e]c/d

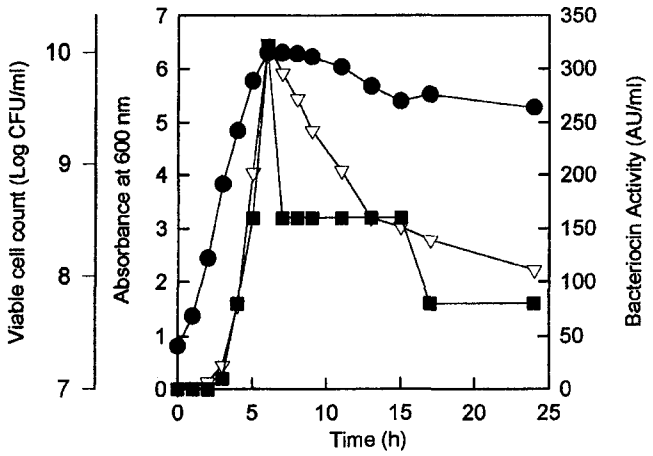

Fig. 5. Batch culture profile of *Lactococcus lactis* subsp. *lactis* H-559 cell growth and bacteriocin production at controlled pH 7.0.

●, viable cell count (Log CFU/ml); ▽, absorbance at 600 nm; ■, bacteriocin activity (AU/ml).

lactis NIZO 22186 [8]. A possible explanation for the decrease in bacteriocin activitiy is the release of non-specific proteolytic enzymes during cell lysis or adsorption by the producer cells [2, 4]. Consequently, the bacteriocin production may be proportionally related to the growth rate and biomass yield at different initial pHs.

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