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Characterization of Paraplantaricin C7, a Novel Bacteriocin Produced by *Lactobacillus paraplantarum* C7 Isolated from *Kimchi*

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Abstract A *Lactobacillus paraplantarum* strain producing a bacteriocin was isolated from *kimchi* using the spot-on-the lawn method and named *L. paraplantarum* C7 [15]. The bacteriocin, paraplantaricin C7, was found to inhibit certain *Lactobacillus* strains, including *L. plantarum, L. pentosus,* and *L. delbrueckii* subsp. *lactis*. It also inhibited *Enterococcus faecalis*, yet did not inhibit most of the other LAB (lactic acid bacteria) tested. The maximum level of paraplantaricin C7 activity was observed under the culture conditions of 25°C and a constant pH of 4.5. Paraplantaricin C7 retained 90% of its activity after 10 min of treatment at 100°C and remained stable within a pH range of 2–8. Based on a culture supernatant, paraplantaricin C7 was purified by DEAE-Sephacel column chromatography and C_{18} reverse-phase HPLC. SDS-PAGE and activity staining were then conducted using the purified paraplantaricin C7, and its molecular mass determined to be about 3,800 Da. The 28 N-terminal amino acids from the purified paraplantaricin C7 were determined, and the structural gene encoding paraplantaricin C7, *ppnC7*, was cloned by PCR using degenerate primers based on the N-terminal amino acid sequence. The nucleotide sequences for *ppnC7* and other neighboring *orfs* exhibited a limited homology to the previously reported plantaricin operon genes. Paraplantaricin C7 is a novel type II bacteriocin containing a double glycine leader sequence.

Key words: *Lactobacillus paraplantarum*, bacteriocin, *kimchi*

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Many lactic acid bacteria (LAB) produce bacteriocins, which are supposedly advantageous for producers in the competition for foods and niches [19]. Thus, intensive efforts have been made over the last few decades to identify bacteriocins from LAB with a broad inhibitory spectrum and superior stabilities against heat treatments and pH variations [24]. Since foods fermented by LAB have been consumed for thousands of years, bacteriocins from LAB or LAB producing bacteriocins are generally regarded as safe for human consumption. Therefore, bacteriocins from LAB are promising as natural food preservatives, and may eventually replace chemical preservatives to allay safety concerns [6]. *Kimchi* is a traditional Korean fermented food that has a characteristic acid flavor. In the later stage of *kimchi* fermentation, excessive acids are produced by lactobacilli, and this causes rapid acid spoilage of *kimchi*. Thus, to extend the shelf-life of *kimchi* products, various approaches have been reported, including the addition of plant extracts to retard the growth of LAB, especially lactobacilli, thereby reducing the amount of acid produced [17, 21]. Another approach was the use of acid-tolerant *Leuconostoc mesenteroides* mutants as starters for *kimchi* fermentation [14], where keeping leuconostocs as the dominant flora for a longer period delays the acid production by lactobacilli. However, LAB producing bacteriocins that inhibit lactobacilli may be an effective alternative for extending the shelf life of *kimchi*. *Enterococcus* sp. K-25 producing a bacteriocin that inhibits *L. plantarum* was previously isolated from *kimchi* and applied to *kimchi* with/without chemicals (chitosan, fumaric acid) [20]. When applied together with

fumaric acid, *Enterococcus* sp. K-25 extended the shelf life for six days, which is promising, since many LAB from *kimchi* produce bacteriocins [6, 13, 16, 20]. However, more studies are needed if this approache is to be practical for extending the shelf life of *kimchi*, as bacteriocin production and inhibition are complex phenomena and the effects against lactobacilli can vary depending on the fermentation condition. *L. paraplantarum* C7, which produces a novel bacteriocin that strongly inhibits *L. plantarum*, was already isolated from *kimchi* [15]. Accordingly, this paper reports on the characterization, purification, and nucleotide sequence of paraplantaricin C7 produced by a *L. paraplantarum* C7.

MATERIALS AND METHODS

Measurement of Bacteriocin Activity

The bacteriocin production was examined using the spot-on-the lawn test [7, 13, 16], where 2 ml of an overnight culture in an MRS broth was spotted on MRS plates, and then overlaid with 3 ml of an MRS soft agar (0.7%, w/v) containing the indicator strain (1×10^8 CFU). After overnight incubation at 30°C, the presence of an inhibition zone was examined. The bacteriocin activity expressed as activity units (AUs) per ml was defined as the reciprocal of the highest two-fold dilution still showing inhibitory action towards the indicator organism. The growth of *L. paraplantarum* C7 and bacteriocin activity measurements were monitored at different incubation temperatures and pHs.

Inhibition Spectrum of *L. paraplantarum* C7

Two μl of a fully grown *L. paraplantarum* C7 culture was spotted on an MRS plate. After 5 h, the indicator strain was overlaid and the plate was incubated at a temperature optimal for the growth of each indicator organism for one day, after which the size of the inhibition zone was determined [7]. The LAB were growth in MRS, whereas the *Escherichia coli* and *Salmonella typhimurium* were grown in LB.

Stability of Paraplantaricin C7 to Heat, pH, Solvents, and Enzymes

The stability of paraplantaricin C7 against heat, pH variation, organic solvents, and enzyme treatments was examined [12]. For the heat treatment, partially purified paraplantaricin C7 (the fraction after a DEAE-Sephacel column) with an activity of 4,000 AU/ml was held at 100°C for 60 min and 121°C for 30 min, and then the remaining bacteriocin activity was examined. The stability of paraplantaricin C7 at various pHs (2–10, using a 50 mM concentration of the appropriate buffers) was also investigated. The stability against organic solvents was assessed by measuring the remaining activity after the partially purified bacteriocin was mixed with an equal volume of a solvent and the mixture kept for 1 h at 25°C. Finally, the partially purified paraplantaricin C7 was treated with hydrolyzing enzymes, including proteases, where the bacteriocin samples were digested by each enzyme at a concentration of 1 mg/ml for 1 h at 37°C.

Production of Paraplantaricin C7 Under Constant pH

The effect of pH on paraplantaricin C7 production was examined using a 5-l lab fermentor (KoBioTech, KF-5L, Seoul, Korea). MRS broth (3 l) was inoculated with 1% (30 ml) of an overnight culture of *L. paraplantarum* C7 and cultured at 25°C for 48 h at a constant pH (pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5). The bacteriocin activities were then examined using a spot-on-the lawn test.

SDS-PAGE of the Partially Purified Paraplantaricin C7

The partially purified bacteriocin sample (fraction after DEAE-Sephacel column) was examined by SDS-PAGE with a discontinuous urea-SDS-PAGE system, as described by Anderson *et al.* [2], using a 16.5% acrylamide gel and a Mini-Protein II (BioRad) electrophoresis system. After the gel electrophoresis, the gel was stained with coomassie brilliant blue R-250, and a bioassay of the antimicrobial peptides in the polyacrylamide gel performed, as described by Bhunia *et al.* [5]. The slab gels were first washed with sterile ultra-pure water for 4 h, and when the SDS concentration had decreased to a nontoxic level, the gels were placed on MRS plates and covered with a lawn of a soft agar (0.7%, w/v) containing an exponentially growing culture of the indicator strain *L. plantanum* (1×10^8 CFU). After 16 h of incubation at 37°C, the plate was examined for the growth of an inhibition zone.

Purification of Paraplantaricin C7

The supernatant from an overnight culture of *L. paraplantarum* C7 was obtained by centrifugation at 10,000 ×*g* for 15 min, and filtration through a 0.22-μm-pore sized membrane filter. The culture supernatant was then concentrated by freeze-drying without ammonium sulfate precipitation [11]. For ion-exchange chromatography, the freeze-dried material was resuspended in 50 mM Tris-HCl (pH 8.3) and loaded onto a DEAE-Sephacel column (2.5× 30 cm) equilibrated with the same buffer. After washing the column with the same buffer, a linear NaCl gradient (0–1.0 M) was applied to elute the bound proteins. The flow rate was adjusted to 0.5 ml/min, and 5 ml of each fraction was collected. The fractions exhibiting bacteriocin activity were pooled and freeze-dried. Reverse phase HPLC using a C_{18} column was also employed for further purification. After washing the HPLC column (μBondapak C_{18} 3.9× 300 mm column, Waters Corp) with 0.1% TFA (trifluoroacetic

acid), an acetonitrile gradient (0–60%) was used to elute the bacteriocin [3, 11]. The antimicrobial activity of each fraction (1 ml) was determined using the spot-on-the lawn method. A second HPLC was also executed to further purify the bacteriocin (data not shown). The N-terminal peptide sequencing of paraplantaricin C7 was performed by Edman degradation [3, 4] at the Korea Basic Science Institute in Seoul.

DNA Sequencing of Paraplantaricin C7 Gene

The degenerate primer (5'-ACN GTN ACN AAR GGN CAY ATG-3') was designed based on the determined amino acid sequence of paraplantaricin C7, and Southern blotting was carried out to locate the gene encoding paraplantaricin C7, *ppnC7*. The primer was labeled using T4 polynuceotide kinase and γ-P^{32}-ATP, and the hybridization carried out at 45°C [23]. The undigested plasmid and chromosomal DNA, plus the EcoRI- and HindIII-digested plasmid and chromosomal DNA, were separated on an agarose gel by electrophoresis, transferred to a zeta-probe membrane (BioRad), and then subjected to hybridization. For the cloning of *ppnC7* and the surrounding *orfs*, a 2.06-kb fragment was PCR amplified using a primer pair [foward primer (5'-CGTACTATTCACTCGACTTAACG-3') and reverse primer (5'-CTTAGCGCAGCGCTAAAGAA-3')], and the PCR carried out under the following conditions: denaturation, 95°C, 30 s; annealing, 50°C, 30 s; extension, 72°C, 3 min, 30 cycles. The fragment was inserted into a T-easy vector (Promega) and the DNA prepared from the white transformant on an LBAp (100 μg/ml) plate containing X-gal. DNA sequencing of the fragment was performed, and the primer walking method employed to

Table 1. Inhibition spectrum of paraplantaricin C7.

Indicator	Antimicrobial activity*
Lactococcus lactis MG 1614	-
Lactococcus lactis MG 1363	-
Lactococcus lactis IL 1403	-
Lactococcus lactis LM 0230	-
Leuconostoc mesenteroides ATCC 10830	-
Leuconostoc mesenteroides subsp. *mesenteroides* KFRI 00666	-
Leuconostoc mesenteroides KFRI 00817	-
Leuconostoc mesenteroides subsp. *mesenteroides* KFRI 00820	-
Leuconostoc mesenteroides subsp. *dextranicum* KFRI 00155	-
Leuconostoc mesenteroides KFRI 00465	-
Leuconostoc mesenteroides B-512	-
Leuconostoc mesenteroides ATCC 9135	++
Leuconostoc mesenteroides	++
Lactobacillus acidophilus IAM 1084	-
Lactobacillus acidophilus KFRI 2161	-
Lactobacillus bulgaricus CH 2	-
Lactobacillus delbrueakii lactis KFRI 347	++
Lactobacillus delbrueakii lactis ATCC 4797	-
Lactobacillus delbrueakii lactis	-
Lactobacillsu brevis IL 2014	-
Lactobacillus casei YIT 9018	-
Lactobacillus pentosus KFRI 481	++
Lactobacillus plantarum	++
Staphylococcus epidermidis	-
Staphylococcus aureus	-
Staphylococcus carnosum	-
Bacillus subtilis DB 104	-
Bacillus cereus	-
Streptococcus thermophilus	-
Streptococcus bovis	-
Eenterococcus fecalis	++
Listeria monocytogenes	-
Escherichia coli K-12	-
Salmonella typhimurium	-

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

determine the whole sequence. The nucleotide sequence for *ppnC7* and the neighboring *orfs* was deposited in the GenBank under the accession number AF420260.

Northern Blot Experiment

L. paraplantarum C7 cells were grown until the mid-exponential growth phase (A_{600}≈0.7) in an MRS broth. The total RNA was extracted from 15 ml of the culture using a FastRNA Pro Blue Kit (Q-biogene, Montreal, Canada) according to the protocol provided by the manufacturer. First, the cells were disrupted using a Mini-Beadbeater-8 Cell Disrupter (BioSpec, Bartlesville, U.S.A.), then the RNA molecules were separated on a 1.2% agarose-formaldehyde gel, transferred to a Hybond-XL nylon membrane (Amersham, Uppsala, Sweden), and hybridized at 65°C with a ^{32}P-labeled probe [22]. Next, the probe was PCR amplified using a primer set of lcck-F (5'-TGAAC-TGACTGCAATCACTG-3') and lccK-R (5'-TTGAAGA-GTGCATATGTCCC-3') in a total volume of 50 μl: 10 ng of the template DNA; 1 mM of each primer; 0.2 mM of each dNTP (dATP, dGTP, and dTTP); 2 μM of dCTP; 5 μl of 10 mCi/ml [α-^{32}P]dCTP; 2 U of Ex-*Taq* DNA polymerase (Takara, Japan); and 1× Ex-*Taq* DNA polymerase buffer. The PCR amplification was carried out under the following conditions: predenaturation for 5 min at 95°C, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and an extension at 72°C for 20 sec, plus a final extension of 7 min at 72°C. The probe was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, U.S.A.), and then the radioactivity was checked using a liquid scintillation counter (LS3000, Beckman Instruments, Inc., Palo Alto, CA, U.S.A.).

Results and Discussion

Inhibition Spectrum of *L. paraplantarum* C7

The inhibition spectrum of *L. paraplantarum* C7 was examined by testing its growth inhibition abilities against various Gram-positive bacteria and two Gram-negative bacteria (*E. coli* and *S. typhimurium*). As a result, *L. paraplantarum* C7 was found to inhibit strains of *L. plantarum*, *L. pentosus*, *L. delbrueckii* ssp. *lactis*, and *Enterococcus faecalis*, yet not the other species tested, including *Listeria monocytogenes* (Table 1), or the Gram-negative bacteria. As such, this indicates that paraplantaricin C7 is a bacteriocin with a narrow inhibition spectrum, which can be desirable under some circumstances; for example, when a bacteriocin is needed to control a specific organism without affecting other organisms. Thus, paraplantaricin C7 may be useful for extending the shelf-life of *kimchi* based on inhibiting *L. plantarum* and *L. pentosus*, the organisms responsible for excessive acid production during *kimchi* fermentation.

Stability of Paraplantaricin C7 with Heat, pH, Solvents, and Enzymes

Although paraplantaricin C7 was unaffected by heat treatment at 80°C for 20 min (results not shown), its activity was reduced by half after 30 min at 121°C and by 20% after 60 min at 100°C. pH variation between 2 and 8 had no effect, yet the activity was reduced at pH 9 and 10. Paraplantaricin C7 was completely inactivated by proteinase K and protease (from *Streptomyces griseus*, type XIV, Sigma), yet unaffected by lysozyme, catalase, RNaseA, β-amylase, trypsin, and pepsin. Proteinase K and protease are both highly nonspecific enzymes, thus they inactivated paraplantaricin C7, whereas trypsin and pepsin failed to inactivate paraplantaricin C7. Finally, paraplantaricin C7 was unaffected by exposure to 50% concentrations of organic solvents under the conditions described in Materials and Methods (Table 2). Consequently, all these results indicate that paraplantaricin C7 has a considerable degree of stability against heat and organic solvents, a common characteristic of class II bacteriocins, and a desirable property when the bacteriocin is intended for incorporation into food to extend the shelf life.

Table 2. Stability of paraplantaricin C7 against various treatments.

Treatment	Residual activity (AU/ml)
Control (in Tris-Cl buffer)	4,000
Enzyme treatments:	
β-Amylase	4,000
Lysozyme	4,000
Proteinase K	0
Pepsin	4,000
RNaseA	4,000
Trypsin	4,000
Catalase	4,000
Protease	0
Solvent treatments:	
Ethanol	4,000
Methanol	4,000
Acetonitrile	4,000
Acetone	4,000
Chloroform	4,000
pH changes:	
pH 2 to 8	4,000
pH 9	3,200
pH 10	2,800
Heat treatments:	
100°C, 10 min	3,600
100°C, 20 min	3,200
100°C, 30 min	2,800
100°C, 60 min	800
121°C, 10 min	3,600
121°C, 20 min	2,800
121°C, 30 min	2,000

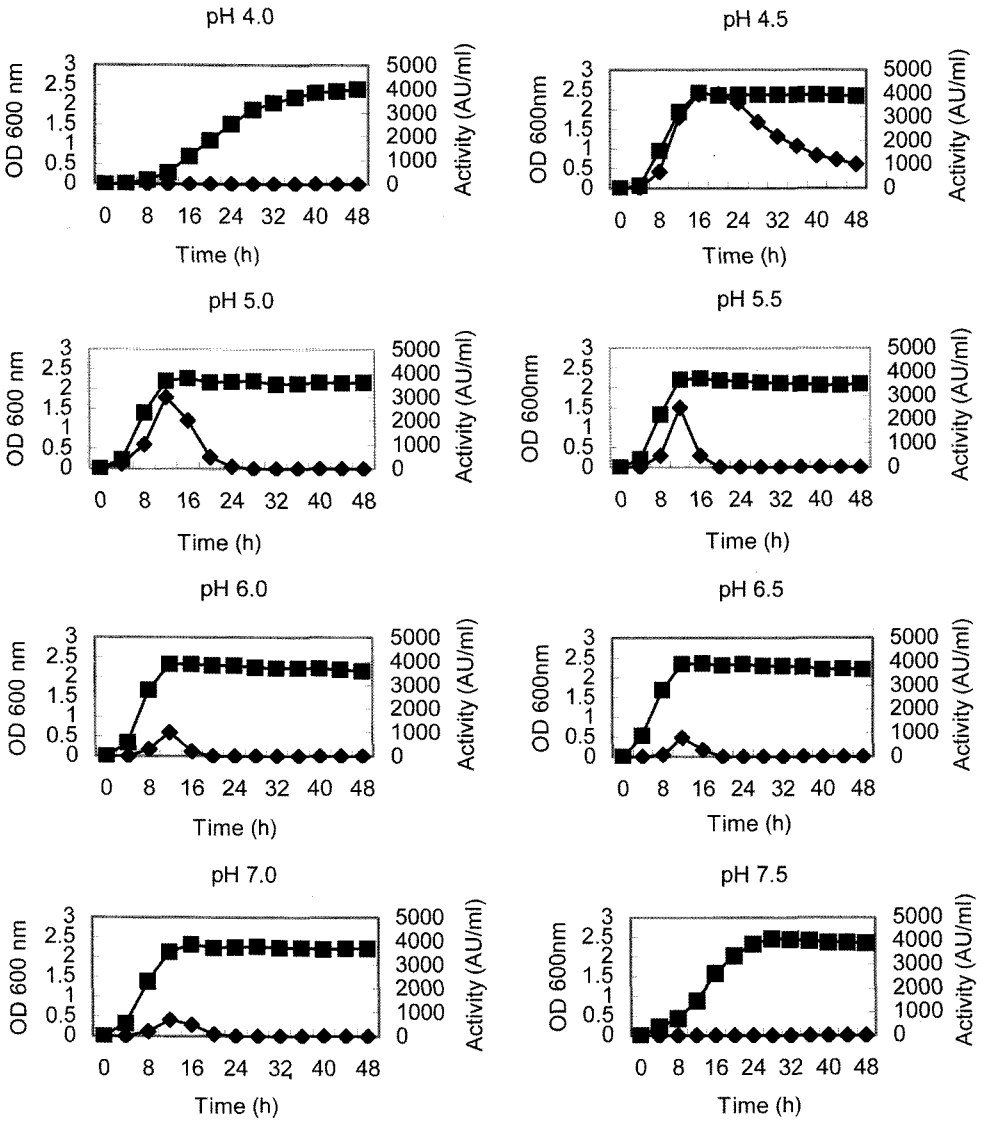

Fig. 1. Growth and bacteriocin production of *L. paraplantarum* C7 at different pHs.
L. paraplantarum C7 was grown at 25°C in a 5-l fermenter in which the pH was constantly maintained at 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, or 7.5. -■-, $OD_{600\ nm}$; -◆-, activity (AU/ml).

Production of Paraplantaricin C7 at Different pHs

The paraplantaricin C7 activity was examined at a constant pH of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5 at 25°C, and the maximum activity was observed at pH 4.5 (Fig. 1) where the maximum level, 4,000 AU/ml, was reached after about 16 h of cultivation. *L. paraplantarum* C7 did not grow at a pH below 4.0. At pH 4.0, the *L. paraplantarum* C7 cells grew very slowly and no bacteriocin activity was detected in the culture supernatant, even after 72 h of cultivation. The *L. paraplantarum* C7 cells grew rapidly at pH 6.0 and 6.5, although the bacteriocin activities were much lower than that at pH 4.5. The highest growth rate for the *L. paraplantarum* C7 cells was at pH 7.5, yet no bacteriocin activity was detected (Fig. 1). Fig. 1 also shows that the production of paraplantaricin C7 occurred during the growth phase of *L. paraplantarum* C7.

Purification and Amino Acid Sequence of Paraplantaricin C7

The paraplantaricin C7 was further purified from the freeze-dried culture supernatant by sequential passage through DEAE-Sephacel (Fig. 2A) and C_{18} reverse phase columns (Fig. 2B). The molecular mass of the partially purified paraplantaricin C7 (fractions obtained from DEAE-Sephacel column chromatography) was estimated to be 3,500–4,000 Da when analyzed by SDS-PAGE (Fig. 3B). When a bioassay for antimicrobial peptides was performed using a polyacrylamide gel under the same conditions, a growth inhibition zone of the indicator strain *L. plantarum*

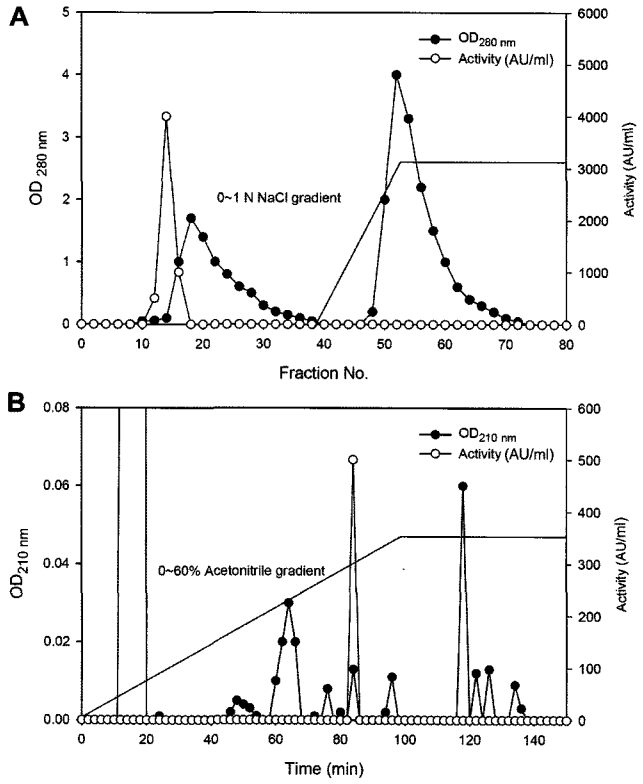

Fig. 2. Elution profile of partially purified paraplantaricin C7.
A. DEAE-Sephacel column equilibrated with 50 mM Tris-HCl buffer (pH 8.3). The unbound paraplantaricin C7 was eluted with a washing buffer (50 mM Tris-HCl, pH 8.3), and the bacteriocin activity of each fraction (5 ml) assayed using the spot-on-the lawn method; -●-, OD (280 nm); -▲-, activity (AU/ml). **B.** Reverse phase HPLC using C_{18} column (μBondapak C_{18} 3.9×300 mm, Waters Corp). The paraplantaricin C7 was eluted with 0.1% TFA, and an acetonitrile gradient (0–60%) used to elute the bacteriocin. The bacteriocin activity of each fraction (1 ml) was determined using the spot-on-the lawn method; -●-, OD (210 nm); -▲-, activity (AU/ml).

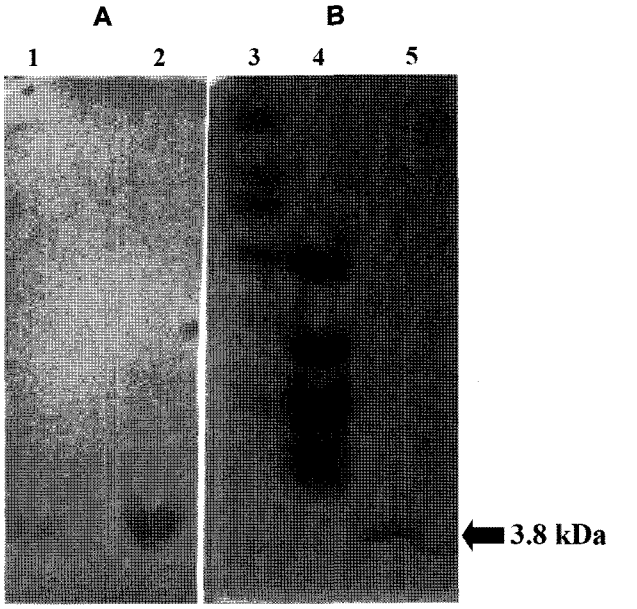

Fig. 3. Polyacrylamide gel (16.5%) electrophoresis and activity staining of partially purified paraplantaricin C7 using Anderson's discontinuous urea-SDS-PAGE system.
A. Activity-stained gel. Lane 1, 1 μg of purified paraplantaricin C7; lane 2, 2 μg of purified paraplantaricin C7. One of the twin gels was overlaid with the indicator strain *L. plantarum* to confirm the bacteriocin band. **B.** One of twin gels was stained with Coomassie brilliant blue R-250. Lane 3: low-range molecular weight standards (Sigma); lane 4, ultra-low range molecular weight standards (Sigma); lane 5, purified paraplantaricin C7.

appeared at the same position (Fig. 3A) where the band of purified paraplantaricin C7 had migrated. The paraplantaricin C7 was further purified by reverse phase HPLC and subjected to Edman degradation. Up to 28 amino acids were determined and the results were as follows: Thr (Asp?, Asn)-Phe(Pro)-Val(Lys)-Gly(His)-Tyr(Pro)-Tyr(Ile)-Leu(Lys)-Gly(Ser)-X(Gln)-Phe-Leu-Ala-Ser-Ala-Thr-His-Tyr(Ser?)-Tyr-Gly-Lys-Thr-Val-Thr(Ser?)-Lys-Gly-His-Met-Ser?(His?). In the case where a specific amino acid was not definitely identified, an alternative amino acid is shown in parenthesis. Low concentrations of contaminating proteins may have been the reason for the uncertainties. The seven underlined amino acids were then used to design the degenerate primers to clone the structural gene of paraplantaricin C7.

Gene Location and DNA Sequence of Paraplantaricin C7 Gene

The degenerate primers (5'-**ACN GTN ACN AAR GGN CAY ATG**-3') were designed based on the determined amino acid sequence of paraplantaricin C7, where threonine at the 21st position was used as the first amino acid, and methionine at the 27th position was used as the last. Southern blot results indicated that the structural gene encoding paraplantaricin C7 was located in the chromosome rather than the plasmid (result not shown). A PCR product of 2.06 kb was amplified using a forward and reverse primer pair, and then the nucleotide sequence of the fragment was determined after being subcloned into a pGEM-T-vector (Promega). *ppnC7* was 156 bp in size, and encoded a 52-amino-acid peptide with a calculated molecular mass of 5,828 Da. A putative ribosome binding site (RBS), GGAGGG, was located seven nt (nucleotides) upstream of the start codon and marked as a shadowed box (Fig. 4). Putative -35 and -10 promoter sequences were also found upstream of the RBS and underlined. A sequence analysis of the *ppnC7* gene showed that the mature active bacteriocin likely consisted of 34 amino acids and had a molecular mass of 3,809 Da, which agreed well with the SDS-PAGE results for the purified paraplantaricin C7 (Fig. 3B). A double glycine motif, known as a signal peptide cleavage site, was also present in the signal peptide sequence, which was confirmed by the N-terminal amino acid sequencing results for the purified bacteriocin. Although the first amino acid was mistakenly recognized as Thr, Asn, or Asp, the subsequent amino acids were correctly

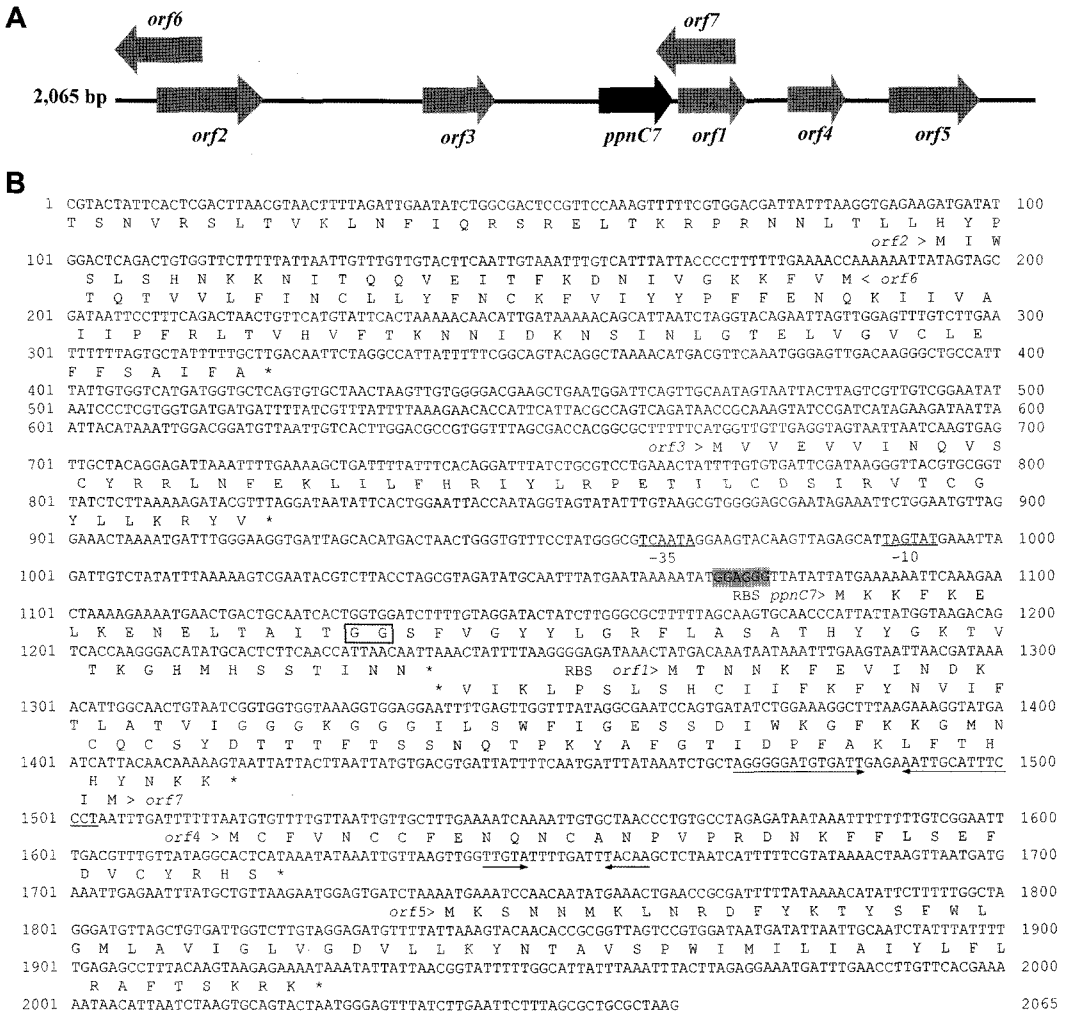

Fig. 4. A. Genetic organization of *ppnC7* gene locus. Arrows indicate the location and direction of the *ppnC7* and neighboring *orfs*. *ppnC7* is the structural gene for paraplantaricin C7 prepeptide. **B.** DNA and deduced amino acid sequences for *ppnC7* and neighboring *orfs*. Boxed letters are the double glycines at the prepeptide cleavage site.
Posssible -35 and -10 promoter sequences are underlined, whereas putative ribosomal binding site (RBS) for *ppnC7* is shadowed. The convergent arrows indicate dyad symmetries for possible transcription termination sites.

identified. Most of the amino acids determined using the Edman method were identical with the amino acids determined from the DNA sequence (26 out of 28 amino acids). The amino acid sequence indicated that the *ppnC7* gene encoded a prepeptide that only became an active peptide when cleaved by the double glycine motif. The *orf1* was located immediately downstream of *ppnC7*, and when considering the gene size (156 bp) and presence of double glycines at the prepeptide cleavage site, *orf1* was also suspected to be a gene encoding another bacteriocin or inducer for a bacteriocin, although the protein product was not confirmed. The homology search results showed that the region starting at nt 1,420 and ending at 2,057 (638 nts in length) had a 96% identity with the plantaricin operon from *L. plantarum* C11 [8, 9]. This region, immediately downstream of *orf1*, also contained *orf4* and *orf5* (Fig. 4). The homologous region in *L. plantarum* C11 corresponds to an intergenic space between *plnP* (encoding an immunity protein) and *plnA* (encoding a plantaricin precursor), encompassing *orf1* encoding a protein of 62 amino acids with an unknown function. Furthermore, a 391 nt (1,672–2,062 nt) region was also found to be 98% identical with a region in *L. plantarum* J51, containing *plnQ* (encoding a plantaricin biosynthesis protein) (DQ340868), whereas a 347 nt (1–347 nt) region showed a 98% identity with a region containing *orf2* (unknown function) in *L. plantarum* J51. In *L. plantarum* J51, *orf2* is located immediately upstream of *plnQ*. Thus, from these results, the genetic organization of *ppnC7* and the neighboring *orfs* seemed to be somewhat similar to that of the plantaricin operon genes from *L. plantarum* strains, although the number and orientation of genes vary depending on the strain. The Northern blot

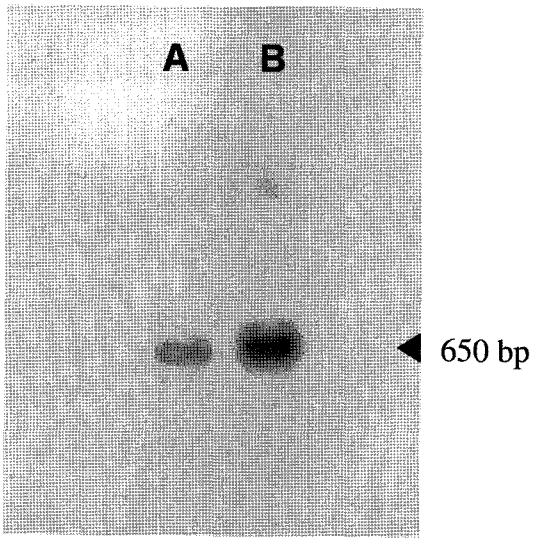

Fig. 5. Northern blot results for detecting *ppnC7* transcript.
Five mg (lane A) and 10 μg (lane B) of total RNA were applied to an agarose gel, size fractionated, and transferred onto a Hybond-XL nylon membrane. The probe was PCR amplified with a primer pair: ppnC7-F (TGAACTGACTGCAATCACTG) and ppn C7-R (TTGAAGAGTGCATATGTCCC). α-^{32}P-dCTP and a random prime labeling kit were used to label the PCR product. The size of the *ppnC7* transcript was determined by measuring the migration distance of the hybridization signal from the well on an agarose gel. RNA size markers (Promega, # G3191) were run in parallel to estimate the size of the RNA molecules.

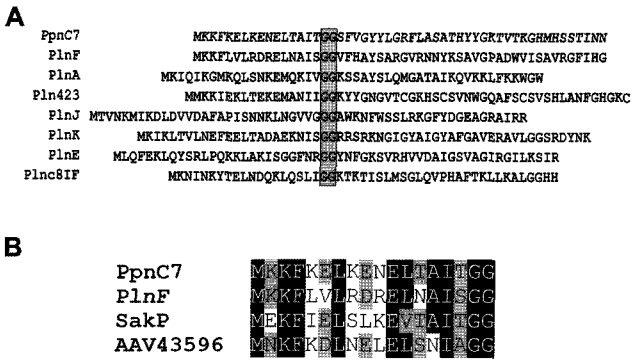

Fig. 6. Comparison of amino acid sequence for paraplantaricin C7 with those of other bacteriocins.
A. Comparision of paraplantaricin C7 with plantaricins. Double glycine motifs are marked as a shadowed box. PlnF, PlnJ, PlnK, and PlnE are the bacteriocins produced by *L. plantarum* C11 [10]. PlnA (inducer) is also produced by *L. plantarum* C11 [8], whereas Pln423 is a bacteriocin from *L. plantarum* 423 [26] and Plnc81F a bacteriocin from *L. plantarum* NC8 [18]. **B.** Alignment of signal peptide from paraplantaricin C7 with those from three bacteriocins with similar signal sequences. SakP is a bacteriocin from *L. sake* LTH 673 [25], whereas AAV43596 is a hypothetical protein from *L. acidophilus* NCFM [1]. Identical amino acids are marked as black boxes, and similar amino acids marked as gray boxes.

results indicated that the size of the *ppnC7* transcript was large, at around 650 nts (Fig. 5), indicating that *ppnC7* was transcribed as part of an operon consisting of *ppnC7*, *orf1*, and probably *orf4*. A region of dyad symmetry that could form a stem-loop structure was also located downstream of *orf1* (1,472–1,503 nt), as marked by convergent arrows under the sequence, and may function as a *rho*-independent terminator, in which case, the expected size of the transcript would be ca 500 nts, when starting the transcription from the promoter sequences mentioned above. Notwithstanding, another possible terminator was located downstream of *orf4* (1,645–1,662 nt), and if this site worked as a terminator, the size of the transcript would be 650 nts, matching the Northern blot results. When the amino acid sequence for paraplantaricin C7 was aligned with those for plantaricins (Fig. 6A), paraplantaricin C7 was found to have the same number of amino acids as plantaricin F (52 aa), and all the bacteriocins included double glycine motifs. In Fig. 6B, the signal sequence for paraplantaricin C7 was aligned with those for three bacteriocins from *Lactobacillus* species: PlnF from *L. plantarum* C11, SakP from *L. sake* LTH 673, and AAV43596 from *L. acidophilus* NCFM. All the signal sequences consisted of 18 amino acids and most of the amino acid residues were conserved. Thus, all the results show that paraplantaricin C7 is a novel bacteriocin belonging to class II. However, more studies are needed to elucidate the functions of the *orfs* near *ppnC7* along with the identity and location of the gene(s) responsible for immunity against paraplantaricin C7.

Inclusion of *L. paraplantarum* C7 into *Kimchi*

Preliminary work was conducted on the effectiveness of *L. paraplantarum* C7 as a starter for *kimchi*. Thus, 10 ml of an overnight culture of *L. paraplantarum* C7 in an MRS broth was centrifuged, the cells resuspended in cold water, and added to 1 kg of freshly prepared *kimchi* (3.3×10^{10} cells/kg *kimchi*). The viable cell count and pH of the *kimchi* were then examined during up to two weeks of fermentation at 10°C. The *kimchi* fortified with *L. paraplantarum* C7 cells exhibited lower pH values and higher viable cell counts when compared with the control *kimchi*. The pH of the fortified *kimchi* was 4.0 on day 7 and 3.6 on day 14, whereas the pH of the control *kimchi* was 5.3 and 4.6 at the same time points, respectively. The presumtive LAB producing acid were counted by using MRS plates containing $CaCO_3$, and the cell number for the fortified *kimchi* was 5.6×10^{10}/ml on day 7 and 7.9×10^{9}/ml on day 14, whereas the cell number for the control *kimchi* was 1.5×10^{10}/ml and 7.9×10^{9}/ml at the same time points, respectively. However, the *kimchi* fortified with *L. paraplantarum* C7 cells developed a unique acid flavor, which may or may not be desirable or undesirable, depending on individual taste. This result was understandable, since a significant number of cells were artificially introduced at the beginning of *kimchi* fermentation, and the normal *kimchi* flora development seemed to be severely affected when judged from the above pH and cell count results. The *L. paraplantarum* C7 interfered with the normal development

of *kimchi* flora, especially by inhibiting *Leuconostos mesenteroides*. Accordingly, *Leuconostoc mesenteroides* cells producing bacteriocins that inhibit lactobacilli would be the best choice. Thus, more studies are required on selecting the starter strain, determining the number of starter cells, and the method of addition to *kimchi*.

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REFERENCES

1. Altermann, E., W. M. Russell, M. A. Azcarate-Peril, R. Barrangou, B. L. Buck, O. McAuliffe, N. Souther, A. Dobson, T. Duong, M. Callanan, S. Lick, A. Hamrick, R. Cano, and T. R. Klaenhammer. 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. *Proc. Natl. Acad. Sci. USA* **102:** 3906–3912.
2. Anderson, B. L., R. W. Berry, and A. Telser. 1983. A sodium dodecyl sulfate polyacrylamide gel electrophoresis system that separates peptides and proteins in the molecular weight range of 2,500 to 90,000. *Anal. Biochem.* **132:** 365–375.
3. Atrih, A., N. Rekhif, A. J. G. Moir, A. Lebrihi, and G. Lefebvre. 2001. Mode of action, purification and amino acid sequence of plantaricin C19, an anti-*Listeria* bacteriocin produced by *Lactobacillus plantarum* C19. *Int. J. Food Microbiol.* **68:** 93–104.
4. Benoit, V., A. Lebrihi, J.-B. Milliere, and G. Lefebvre . 1997. Purification and partial amino acid sequence of brevicin 27 a bacteriocin produced by *Lactobacillus brevis* SB27. *Curr. Microbiol.* **34:** 173–179.
5. Bhunia, A. K., M. C. Johnson, and B. Ray. 1987. Direct detection of an antimicrobial peptide of *Pediococcus acidilactici* in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Ind. Microbiol.* **2:** 319–322.
6. Choi, Y.-O. and C. Ahn. 1997. Plasmid-associated bacteriocin production by *Leuconostoc* sp. LAB145-3A isolated from *kimchi*. *J. Microbiol. Biotechnol.* **7:** 409–416.
7. Daeschel, M. 1992. Procedures to detect antimicrobial activities of microorganisms, pp. 57–80. *In* B. Ray and M. Daeschel (eds.), *Food Biopreservatives of Microbial Origin.* CRC Press, Boca Raton, Florida, U.S.A.
8. Diep, D. B., L. S. Håvarstein, J. Nissen-Meyer, and I. F. Nes. 1994. The gene encoding plantaricin A, a bacteriocin from *Lactobacillus plantarum* C11, is located on the same transcription unit as an *agr*-like regulatory system. *Appl. Environ. Microbiol.* **60:** 160–166.
9. Diep, D. B., L. S. Håvarstein, and I. F. Nes. 1996. Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. *J. Bacteriol.* **178:** 4472–4483.
10. Erlend, L. A., D. B. Diep, I. F. Nes, V. G. H. Eijsink, and J. Nissen-Meyer. 1999. Antagonistic activity of *Lactobacillus plantarum* C11: Two new two-peptide bacteriocins, plantaricins EF and JK, and the induction factor plantaricin A. *Appl. Environ. Microbiol.* **64:** 2269–2272.
11. Guyonnet, D., C. Fremaux, Y. Cenatiempo, and J. M. Berjeaud. 2000. Method for rapid purification of class IIa bateriocins and comparison of their activities. *Appl. Environ. Microbiol.* **66:** 1744–1748.
12. Ivanova, I., V. Miteva, T. Stefanova, A. Pantev, I. Budakov, S. Danova, P. Moncheva, I. Nikolova, X. Dousset, and P. Boyaval. 1998. Characterization of a bacteriocin produced by *Streptococcus thermophilus* 81. *Int. J. Food Microbiol.* **42:** 147–158.
13. Kim, H. T., J.-Y. Park, G. G. Lee, and J. H. Kim. 2003. Isolation of a bacteriocin producing *Lactobacillus plantarum* strain from *kimchi*. *Food Sci. Biotechnol.* **12:** 166–170.
14. Kim, Y.-C., E.-Y. Jung, E. H. Kim, D.-H. Jung, S.-H. Jung, D.-H. Yi, T.-J. Kwon, and S.-M. Kang. 1998. Properties of acid tolerance of acid-resistant mutant *Leuconostoc mesenteroides* which was improved as *kimchi* starter. *Kor. J. Appl. Microbiol. Biotechnol.* **26:** 102–109.
15. Lee, J. H., M. J. Kim, D. W. Jeong, M. J. Kim, J. H. Kim, H. C. Chang, D. K. Chung, H. Y. Kim, K. H. Kim, and H. J. Lee. 2005. Identification of bacteriocin-producing *Lactobacillus paraplantarum* first isolated from *kimchi*. *J. Microbiol. Biotechnol.* **15:** 428–433.
16. Lee, K.-H., G.-S. Moon, J.-Y. An, H.-J. Lee, H. C. Chang, D. K. Chung, J.-H. Lee, and J. H. Kim. 2002. Isolation of a nisin-producing *Lactococcus lactis* strain from *kimchi* and characterization of its *nisZ* gene. *J. Microbiol. Biotechnol.* **12:** 389–397.
17. Lee, S.-H., J.-S. Choi, K. N. Park, Y. S. Im, and W.-J. Choi. 2002. Effects of *Prunus mume sie.* Extract on growth of lactic acid bacteria isolated from *kimchi* and preservation of *kimchi*. *Kor. J. Food Preser.* **9:** 292–297.
18. Maldonado, A., R. Jiménez-Díaz, and J. L. Ruiz-Barba. 2004. Induction of plantaricin production in *Lactobacillus plantarum* NC8 after coculture with specific Gram-positive bacteria is mediated by an autoinduction mechanism. *J. Bacteriol.* **186:** 1556–1564.
19. Moll, G. N., W. N. Konings, and A. J. M. Driessen. 1999. Bacteriocins: Mechanism of membrane insertion and pore formation. *Antonie Van Leewenhoek* **76:** 185–198.
20. Moon, G.-S., C.-H. Kang, Y.-R. Pyun, and W. J. Kim. 2004. Isolation, identification, and characterization of a bacteriocin-producing *Enterococcus* sp. from *kimchi* and its application to *kimchi* fermentation. *J. Microbiol. Biotechnol.* **14:** 924–931.
21. Moon, Y.-J., S. Park, and C.- K. Sung. 2003. Effects of ethanolic extract of *Schizandra chinensis* for the delayed ripening *kimchi* preparation. *Kor. J. Food Nutr.* **16:** 7–14.

22. Park, J. Y., J. S. Park, J. H. Kim, S. J. Jeong, J. Y. Chun, J. H. Lee, and J. H. Kim. 2005. Characterization of the catabolite control protein (CcpA) gene from *Leuconostoc mesenteroides* SY1. *J. Microbiol. Biotechnol.* **15:** 749–755.
23. Park, J. Y., S. J. Jeong, J. Y. Chun, J. H. Lee, D. K. Chung, and J. H. Kim. 2006. Characterization of *ptsHI* operon from *Leuconostoc mesenteroides* SY1, a strain isolated from *kimchi*. *J. Microbiol. Biotechnol.* **16:** 988–992.
24. Ross, R. P., M. Galvin, O. McAuliffe, S. M. Morgan, M. P. Ryan, D. P. Twomey, W. J. Meaney, and C. Hill. 1999. Developing applications for lactococcal bacteriocins. *Antonie Van Leewenhoek* **76:** 337–346.
25. Tichaczek, P. S., R. F. Vogel, and W. P. Hammes. 1994. Cloning and sequencing of *sakP* encoding sakacin P, the bacteriocin produced by *Lactobacillus sake* LTH 673. *Microbiology* **140:** 361–367.
26. van Reenen, C. A., M. L. Chikindas, W. H. Van Zyl, and L. M. T. Dicks. 2003. Characterization and heterologous expression of a class IIa bacteriocin, plantaricin 423 from *Lactobacillus plantarum* 423, in *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* **81:** 29–40.