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Identification of Bacteriocin-Producing *Lactobacillus paraplantarum* First Isolated from Kimchi

LEE, JONG-HOON[1], MIJIN KIM[1], DO-WON JEONG[2], MINJUNG KIM[2], JEONG HWAN KIM[3], HAE CHOON CHANG[4], DAE KYUN CHUNG[5], HAE-YEONG KIM[5], KYOUNG HEON KIM[6], AND HYONG JOO LEE[2]*

[1]*Department of Food Science and Biotechnology, Kyonggi University, Suwon 442-760, Korea*
[2]*Department of Food Science and Technology and School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea*
[3]*Division of Applied Life Science, Graduate School, Gyeongsang National University, Jinju 660-701, Korea*
[4]*Department of Food and Nutrition, Chosun University, Kwangju 501-759, Korea*
[5]*School of Biotechnology and Institute of Life Science and Resources, Kyung Hee University, Suwon 449-701, Korea*
[6]*Division of Food Science, Korea University, Seoul 136-701, Korea*

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Abstract A bacteriocin-producing lactic acid bacterium with inhibitory activity against the growth of *Lactobacillus plantarum* was isolated from kimchi, a traditional Korean fermented vegetable. For the identification of the isolate, its 16S rDNA was sequenced. As a result, the sequence showed 99% homology with those from *Lactobacillus paraplantarum*, *Lb. plantarum*, and *Lactobacillus pentosus*. For further identification of the isolate, the sequence of its 16S/23S rDNA spacer region was determined, and the sequence matched perfectly with that of *Lb. paraplantarum*. SDS-PAGE fingerprinting of whole-cell proteins of the isolate was almost identical with that of *Lb. paraplantarum*. The isolation and identification of *Lb. paraplantarum* suggest that *Lb. paraplantarum* is one of the lactic acid bacteria involved in kimchi fermentation.

Key words: Bacteriocin, *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, 16S/23S rDNA spacer region, SDS-PAGE fingerprinting, kimchi

Bacteriocins are antimicrobial compounds that are ribosomally synthesized by many different bacterial species including lactic acid bacteria (LAB). LAB that grow as the adventitious microflora of foods or that are added to foods as cultures are generally considered to be harmless or even advantageous for human health. Accordingly, they are awarded GRAS (Generally Recognized as Safe) status in many countries including the United States. Because of these facts, there have been extensive investigations for more than decades to find bacteriocins from LAB with broad inhibition spectra and superior stabilities against heat treatments and pH variations [15, 21, 35, 37]. Such efforts have led to the isolation of a range of different bacteriocin-producing strains, many of which have strong potential in food applications [36].

Kimchi represents one group of traditionally fermented vegetables originating from Korea, and is known to be the product of a mixed-fermentation process carried out principally by naturally occurring LAB [25]. Since kimchi has been considered as a good resource for bacteriocin-producing LAB, there have been substantial efforts in isolating novel bacteriocin-producing LAB from kimchi and the characterization of their bacteriocins to find bacteriocins with broad inhibition spectra and superior stabilities against heat treatments and pH variations [1, 5, 6, 8, 12, 16, 22–24, 27, 31, 38].

Recently, the market of commercially produced kimchi is steadily expanding, which requires effective mass production methods of quality-controlled kimchi in the kimchi industry [13]. To effectively control the method for fermentation and preservation, some food microbiologists are turning their attention to the screening of starter strains that can control the fermentation of kimchi [11, 14, 18–20].

In previous studies on kimchi, *Leuconostoc* species were found to be the major LAB in the initial stage of kimchi fermentation [7, 26, 30, 33, 39]. After the maximum growth of *Leuconostoc* species, as pH decreased, the number of *Leuconostoc* species decreased and the number of lactobacilli that have a strong pH tolerance continued to increase until the last stage of fermentation. For these

*Corresponding author
Phone: 82-2-880-4853; Fax: 82-2-873-5095;
E-mail: leehyjo@snu.ac.kr

reasons, *Leuconostoc* and *Lactobacillus* species are generally considered to be responsible for the good taste and acidification of kimchi, respectively. *Lactobacillus plantarum* was shown to appear when the *Leuconostoc* species begins to decrease, and later it becomes dominant, thereby being considered as the main acidifying organism in kimchi fermentation [26, 33]. Based on these findings, the isolation of *Leuconostoc* strains, which are favored in kimchi fermentation, from kimchi and the development of acid-resistant strains have been carried out in the hope of possible application as a starter of kimchi [14, 18-20].

Despite the importance of *Lb. plantarum* in kimchi as an acidifying strain, there have been only a few studies on that microorganism, which include our recent report of the isolation of nisin-producing *Lactococcus lactis* from kimchi, its inhibition on the growth of *Lb. plantarum*, and the characterization of the *nisZ* gene and its product of *Lc. lactis* strain [28]. In the present study, we first isolated and identified a bacteriocin-producing *Lactobacillus paraplantarum* from kimchi, which inhibits the growth of *Lb. plantarum*.

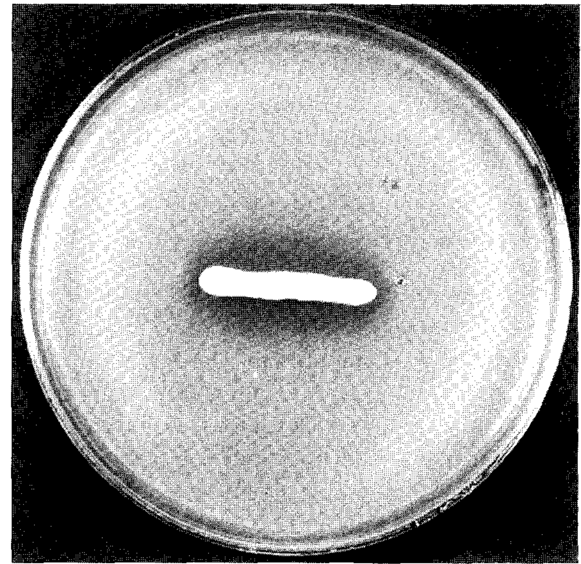

Fig. 1. Bioassay for bacteriocin production.
Lb. paraplantarum C7 was streaked onto plates containing *Lb. plantarum* KFRI 00464, a bacteriocin-sensitive strain, as the indicator strain.

Isolation of Bacteriocin-Producing LAB from Kimchi

To isolate bacteriocin-producing LAB from kimchi, serially-diluted kimchi samples were plated on MRS agar (Difco, U.S.A.) plates. When colonies appeared, MRS top agar mixed with the indicator strain, *Lb. plantarum* KFRI 00464, was overlaid and incubated at 30°C. Colonies with inhibition zone were further examined for the production of bacteriocin by the spot-on-the lawn test [28].

Several bacteriocin-producing strains were isolated from kimchi; among them, one isolate with the highest inhibitory activity on the growth of *Lb. plantarum* was named C7 (Fig. 1). When the supernatant of C7 was treated with catalase and neutralized to eliminate possible inhibition by hydrogen peroxide and lactic acid, the supernatant still exhibited growth inhibition. However, treatment of the supernatant with protease resulted in a loss of the inhibitory activity, indicating the proteinaceous nature of the inhibitory substance.

16S rDNA Sequence Determination of the Bacteriocin-Producing Isolate

The 16S rDNA fragment of total DNA from C7 was amplified by an UNOII Thermocycler (Biometra, Germany). In the PCR solution, template DNA, 0.5 μM each of primer, 1 U of *Taq* polymerase (TaKaRa, Japan), 2.5 mM $MgCl_2$, 100 mM dNTPs, and 1× PCR buffer, supplied by the manufacturer, were included. The total DNA was extracted from C7, using a Dneasy Tissue Kit (Qiagen, Germany). Samples were preheated for 5 min at 95°C, and then amplified for 30 cycles of the following conditions: 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C. Oligonucleotide primers were synthesized by TaKaRa (Korea), and their sequences are presented in Table 1. Lab1 and Lab2 primers chosen for the amplification of 16S rRNA gene were selected from the conserved region of various LAB. The amplified PCR product was cloned into pGEM-T Easy vector (Promega, U.S.A.), and DNA sequencing was conducted at TaKaRa (Korea). DNA sequence data analyses were performed using the DNASIS program (Hitachi, Japan). The homology searches of DNA sequences were done by the BLAST sever maintained at National Center for Biotechnology Information, Bethesda, U.S.A. (http://www.nibi.nlm.nih.gov/recipon/blast-search.html), using the BLASTN program. Multiple sequence alignments of DNA sequences were performed using CLUSTAL W.

After the 16S rDNA of the isolate was amplified by PCR, using primers Lab1 and Lab2, the 1,349-bp nucleotide

Table 1. Sequences of the oligonucleotide primers used in PCR amplification and sequencing.

Primer	Location	Oligonucleotide sequence (5'→3')	Specificity	Reference
Lab1	Conserved region of 16S rRNA gene, forward	GCGGCGTGCCTAATACATGCAAGTCG	16S rRNA gene	This study
Lab2	Conserved region of 16S rRNA gene, reversed	GACCCGGGAACGTATTCACCGCCGCCGC	16S rRNA gene	This study
16	16S rRNA gene, 3' end, forward	GCTGGATCACCTCCTTTC	16S rRNA gene	[3]
23	23S rRNA gene, 5' end, reversed	AGTGCCAAGGCATCCACC	23S rRNA gene	[3]
Lpl	16S/23S spacer region of *Lb. plantarum* DNA, reversed	ATGAGGTATTCAACTTATG	*Lb. plantarum*	[3]

sequence was determined. It was found that the 16S rDNA of the isolate has 99% identity with those from *Lb. paraplantarum* DSM 10667T (accession number: AJ306297, identity: 1346/1349), *Lb. plantarum* WCFS1 (AL935258, 1343/1349), and *Lb. pentosus* (D79211, 1343/1349).

Sequence Determination of 16S/23S rDNA Spacer Region

The species *Lb. plantarum* is described as a taxon, exhibiting heterogeneity in its phenotype and genotype. Some *Lb. plantarum* are regrouped into two other species: *Lb. pentosus* [40] and *Lb. paraplantarum* [10], where *Lb. pentosus* can be distinguished from *Lb. plantarum* by its ability to produce acid from D-xylose and glycerol; however, strains which do not ferment D-xylose have also been described [4], therefore, this physiological basis does not appear to be sufficient to distinguish *Lb. plantarum* from *Lb. pentosus*.

Oligonucleotide DNA probes that mainly target variable regions of 16S or 23S rRNA genes and rRNA sequences have been widely used for the identification and detection of bacterial species. However, rRNA probes or rRNA sequences are not useful anymore for closely related species whose rRNA sequences show more than 99% homology [9]. To overcome this obstacle, a PCR-based method to amplify 16S/23S rDNA spacer regions (SRs) has recently been developed for distinguishing *Lb. plantarum, Lb. pentosus*, and *Lb. paraplantarum* [3].

For the amplification of the 16S/23S rDNA spacer regions (SRs), primer pair 16/23, which was designed from the flanking terminal sequences of the 16S and 23S

Table 2. Reference strains used in this study.

Reference strain	Strain No.
Lactobacillus confusus	KFRI 227
Lactobacillus hilgardii	KFRI 229
Lactobacillus paraplantarum	ATCC 700211
Lactobacillus pentosus	KCCM 35472 (IFO 12011)
Lactobacillus pentosus	KCTC 3120 (ATCC 8041)
Lactobacillus plantarum	KFRI 819[a]
Lactobacillus plantarum	KFRI 821[a]
Lactobacillus plantarum	KCTC 3104 (ATCC 10241)
Lactobacillus plantarum	KCTC 3105 (ATCC 10012)
Lactobacillus plantarum	KCTC 3108 (ATCC 10830)
Lactobacillus sake	KCTC 3598 (ATCC 31063)
Leuconostoc mesenteroides	KCTC 3505 (ATCC 8293)
Weissella kimchii	KCTC 3746 (KCCM 41287)
Weissella paramesenteroides	KCTC 3531 (ATCC 33313)

The reference strains used in this study were purchased from Korea Food Research Institute (KFRI), Korean Collections for Type Cultures (KCTC), Korean Culture Center for Microorganisms (KCCM), and American Type Culture Collection (ATCC). They were grown in MRS broth at 30°C in a facultative-anaerobic condition.

[a]The strains KFRI 819 and KFRI 821 were reidentified as *Lactobacillus plantarum* in this study.

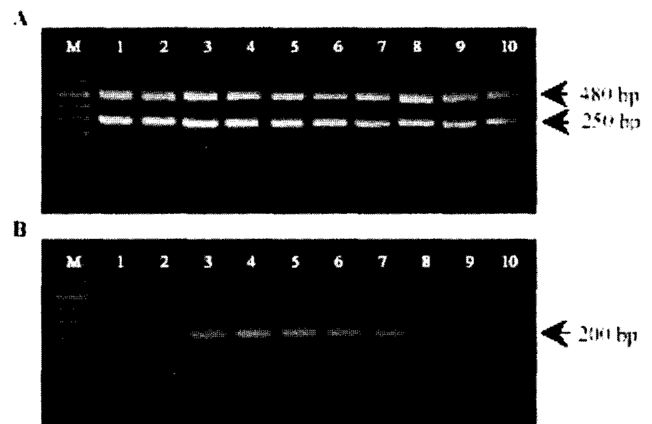

Fig. 2. PCR amplifications of genomic DNAs from reference strains and the isolate with 16/23 (A) and 16/Lpl (B) primer pairs.

Lanes: M, 50- 1,000 bp DNA size marker; 1, *Lb. confusus* KFRI 227; 2, *Lb. hilgardii* KFRI 229; 3, *Lb. plantarum* KCTC 3104; 4, *Lb. plantarum* KCTC 3105; 5, *Lb. plantarum* KCTC 3108; 6, strain KFRI 819; 7, strain KFRI 821; 8, *Lb. pentosus* KCTC 3120; 9, *Lb. pentosus* KCCM 35472; 10, the isolate C7.

rRNA genes that are conserved among various bacteria, was introduced [3]. For the species-specific detection of *Lb. plantarum*, the SR primer Lpl was also introduced [3]. For the amplification of SRs and the specific detection of *Lb. plantarum*, the annealing temperature of PCR was switched to 50°C and 52°C, respectively. The PCR products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide.

When genomic DNAs from the isolate and reference lactobacilli, listed in Table 2, were amplified by PCR by using primers 16 and 23, two SR fragments with sizes of approximately 250 bp and 480 bp, which were named small and large, respectively, were detected (Fig. 2A). The large SR fragment (480 bp) was found to contain coding sequences for $tRNA^{Ile}$ and $tRNA^{Ala}$ which are found in mostly Gram-negative bacteria and only lactobacilli among LAB [3, 34]. When *Lb. plantarum*-specific PCR amplifications were performed with primer pair 16 and Lpl, 200-bp PCR products were identified from *Lb. plantarum* together with strains KFRI 819 and KFRI 821 which were identified as *Lb. pentosus* by partial 16S rDNA sequence [29] (Fig. 2B). To confirm these results on identification of the isolate and KFRI 819 and KFRI 821, the large SRs of the three strains were amplified, and their DNA sequences were determined (Fig. 3). According to Berthier and Ehrlich [3], the large fragment exhibits interspecies sequence variations, but does not exhibit intraspecies variation that would allow the typing of *Lb. plantarum, Lb. pentosus*, and *Lb. paraplantarum*. The SR sequences of strain KFRI 819 and KFRI 821 agreed well with that of *Lb. plantarum*, and the sequences of the isolate perfectly matched with that of *Lb. paraplantarum*. These results reconfirmed that 16S rDNA

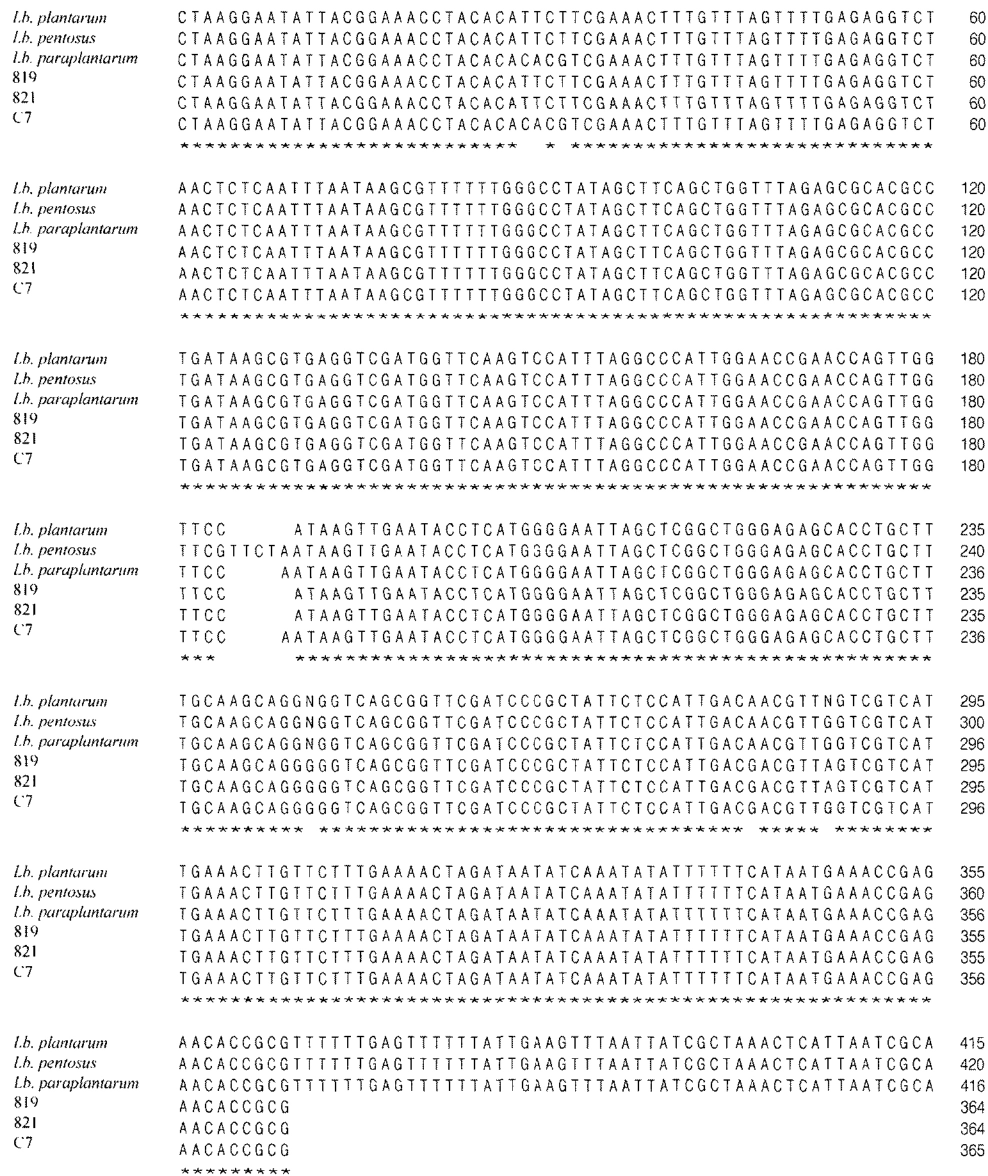

Fig. 3. Alignment of the large SR sequences of *Lb. plantarum* ATCC 8014 (accession No. U97139), *Lb. pentosus* CNRZ 1544 (accession No. U97141), *Lb. paraplantarum* CNRZ 1885 (accession No. U97138), strain KFRI 819, strain KFRI 821, and the isolate C7.

Gaps introduced to maintain alignment are indicated by '-'. Identical residues in the sequences are indicated by '*'.

sequencing is not enough for the identification of closely related lactobacilli, and that additional DNA-based information needs to be checked.

Identification of the Isolate Based on Whole-Cell Protein SDS-PAGE Pattern

Recently, Kim *et al.* [17] reported that SDS-PAGE fingerprinting of whole-cell proteins can be used for the identification of LAB in kimchi. Therefore, for further confirmation of our identification results, SDS-PAGE of the whole-cell protein of the isolate was performed (Fig. 4). SDS-PAGE of whole-cell proteins from the reference strains and the isolate cultured at 30°C in MRS broth was conducted according to the method of Kim *et al.* [17].

As shown in Fig. 4, the whole-cell protein pattern of the isolate was almost identical with that of reference *Lb. paraplantarum.* However, some bands distinguishable from that of reference *Lb. plantarum* were detected. This result further confirmed our identification result for the isolate, and we therefore concluded that the isolate is *Lb. paraplantarum.*

Two strains of LAB isolated from beer and one strain from human feces were proposed by Curk *et al.* [10] in 1996 as new species of *Lb. paraplantarum* because of its

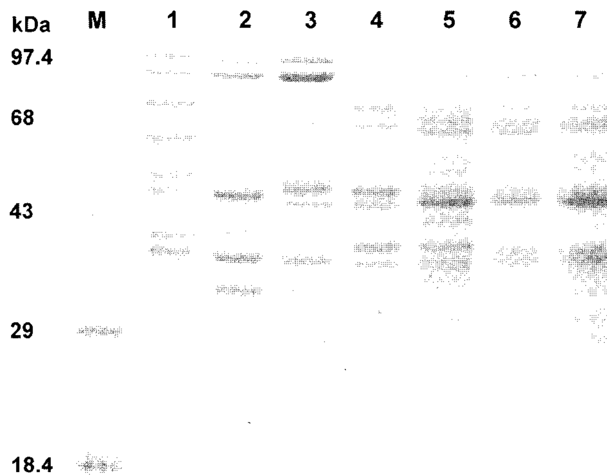

Fig. 4. SDS-PAGE profiles of whole-cell proteins from the isolate and the reference strains.
Lanes: M, protein molecular weight marker (kDa); 1, *Leu. mesenteroides* KCTC 3505; 2, *W. paramesenteroides* KCTC 3531; 3, *W. kimchii* KCTC 3746; 4, *Lb. sake* KCTC 3598; 5, *Lb. plantarum* KCTC 3104; 6, *Lb. paraplantarum* ATCC 700211; 7, *Lb. paraplantarum* C7.

phenotypic similarity to *Lb. plantarum*. Since the description of *Lb. paraplantarum*, the existence of the species in cheese has also been reported [2, 32]. To the best of our knowledge, strain C7 is the first *Lb. paraplantarum* isolated and identified from kimchi.

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