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Identification and Distribution of Predominant Lactic Acid Bacteria in Kimchi, a Korean Traditional Fermented Food

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Abstract To effectively investigate the identification and distribution of the lactic acid bacteria in Kimchi, polyphasic methods, including a PCR, SDS-PAGE of the whole-cell proteins, and 16S rRNA gene sequence analysis, were used. In various types of Kimchi fermented at 20°C, the isolate KHU-31 was found to be the predominant lactic acid bacteria. This isolate was identified as *Lactobacillus sake* KHU-31, based on SDS-PAGE of the whole-cell proteins and a 16S rRNA gene sequence analysis, which provided accurate and specific results. Accordingly, the approach used in the current study demonstrated that *Lactobacillus sake* KHU-31, together with *Leuconostoc mesenteroides*, were the most predominant lactic acid bacteria in all types of Kimchi in the middle stage of fermentation at 20°C.

Key words: Kimchi, *Lactobacillus sake*, fermented food

Kimchi, a Korean traditional fermented food, is made of Chinese cabbage along with various kinds of vegetables, condiments, and fermented fish juices. It is prepared through a series of processes, including the pretreatment of Chinese cabbage, brining, blending with various spices and other ingredients, and fermentation. In Korea, Kimchi is served as an indispensable side dish with all Korean meals and is considered as a nutritious and fundamental food [17]. Its fermentation is initiated by various microorganisms originally present in the raw materials. However, the fermentation becomes gradually dominated later on by lactic acid bacteria, such as *Leuconostoc* spp. and *Lactobacillus* spp. [13, 14, 16, 20, 21]. In previous microbial studies on the lactic acid bacteria present in Kimchi, it was found that *Leuconostoc mesenteroides* was the major microorganism during the optimum ripening period of Kimchi fermented at 5°C, 10°C, and 20°C [13, 14, 16, 21], and that *Lactobacillus plantarum* becomes the dominant microorganism during the later stage of fermentation. Consequently, *Lactobacillus plantarum* is generally regarded as the undesirable organism responsible for the acidic deterioration of Kimchi fermented at above 10°C [13, 14, 16, 20]. The quality of Kimchi depends on the composition of the lactic acid bacteria involved in the fermentation process. Therefore, in order to study the ecology of Kimchi, it is important to understand the components of the microbial community and identify the physiologically active organisms for Kimchi fermentation.

Since many lactic acid bacteria have similar nutritional and growth requirements, their identification is very difficult and also ambiguous, when conventional methods are used. Traditional identification methods based on physiological phenotypes are labor intensive and time consuming. Until recently, the identification of the lactic acid bacteria isolated from Kimchi has mostly depended on traditional phenotypic analyses [13, 14, 16, 20, 21]. However, this type of identification method using biochemical and morphological characteristics is limited in its discrimination and accuracy [21]. Accordingly, the effective study of Kimchi fermentation requires the development of a rapid and accurate lactic acid bacteria identification method, such as genotypic approach using modern molecular typing and identification tools. To this end, a variety of methods, including rRNA gene sequence analyses [15], rRNA-targeted probes [3, 12], DNA-DNA hybridization [5], randomly amplified polymorphic DNA (RAPD) [18], and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins [4, 6, 8, 19], have been used. Based on the above-mentioned methods, protein profiles can be stored in a database format and routinely used for the identification of unknown isolates. Although molecular biology-based methods have also become available, the

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Table 1. Kimchi samples used for isolation of lactic acid bacteria.

Samples	Characteristics		Preparation	Region
	pH	Total acidity (%)		
Chinese-cabbage Kimchi A	4.5	0.61	Store-bought	Suwon
Chinese-cabbage Kimchi B	4.3	0.70	Laboratory-made	Yongin
Chinese-cabbage Kimchi C	4.7	0.52	Commercial manufacture	H Company
Chinese-cabbage Kimchi D	4.2	0.68	Commercial manufacture	D Company
Cucumber Kimchi	5.0	0.40	Homemade	Bundang
Yeolmoo Kimchi	4.8	0.50	Homemade	Anyang
Welsh onion Kimchi	4.7	0.63	Homemade	Anyang
Kakdugi	4.2	0.76	Homemade	Bundang

identification of lactic acid bacteria in Kimchi using these methods have rarely been reported [10, 11, 24] and have not sufficiently been examined to identify a large variety of lactic acid bacteria from various types of Kimchi. The aim of the current study is to investigate a reliable identification method and the distribution of the predominant lactic acid bacteria in various types of Kimchi using polyphasic methods. It is hoped that information regarding the identification and distribution of the predominant lactic acid bacteria involved in Kimchi fermentation will help in the understanding of the Kimchi fermentation process.

MATERIALS AND METHODS

Kimchi Samples

Various types of Kimchi, homemade, laboratory-made, and commercially-sold (store-bought), were collected. Chinese cabbage Kimchi, cucumber Kimchi, Yeolmoo Kimchi, Welsh onion Kimchi, and kakdugi were used for isolation of lactic acid bacteria. Characteristics of the various types of Kimchi are shown in Table 1. The Chinese cabbage Kimchi A was purchased from a store in Suwon and fermented at 20°C. The other Kimchi were prepared either at the home, laboratory, or factory and fermented at 20°C.

Bacterial Strains and Isolation of Strains

The reference strains used in the current study were obtained from the Korean Collection for Type Cultures (Daejon, Korea) and Korean Culture Center of Microorganisms (Seoul, Korea), and cultured in an MRS broth (Difco, Detroit, U.S.A.) at 30°C. Lactic acid bacteria were isolated from various types of Kimchi. The juice of the Kimchi was diluted to 10^5–10^6 with distilled water, spread onto the surface of an MRS agar plate, and incubated for 2–3 days at 30°C to allow colonies to develop. The colonies were collected for testing with PCR and SDS-PAGE. The selected colonies were recultured in a liquid MRS broth for 18 h at 30°C, and then respread on an MRS agar plate for purification and identification.

PCR for Detection of *Leuc. mesenteroides*

The PCR primers were selected from specific 16S/23S rRNA spacer gene sequences and the 23S rRNA gene of *Leuc. mesenteroides*. The GenBank (National Center for Biotechnology Information, Bethesda, U.S.A.) BLAST was used to ensure that the designed primers were only complementary with the target species and not other species. The primers were synthesized by Bionix (Seoul, Korea), and the sequences of the primers (LMf and LMr) used to detect *Leuc. mesenteroides* are shown in Table 2. The colonies on the MRS plates were selected using sterile toothpicks, diluted with 30 µl of distilled water in an Eppendorf tube, and mixed by shaking the tube for 30 sec. The bacterial suspensions were boiled for 6 min, immediately cooled on ice, and tested directly using a PCR without isolation of the DNA. The PCR amplification was performed as described by Kim *et al.* [12], except for the annealing temperature (64°C).

SDS-PAGE of Whole-Cell Proteins and Identification of Isolates

The whole-cell proteins were analyzed by the modified method of Pot *et al.* [19]. The strains were incubated

Table 2. Sequence of the oligonucleotide primers used for PCR amplification and sequencing.

Primer	Location	Sequence (5'→3')	Reference
LMf*	16S/23S spacer region of *Leuc. mesenteroides*	GGACTTAAGTGTCAATTTGT	Present study
LMr*	23S rRNA gene of *Leuc. mesenteroides*	AGATACAGACAACTCTTACG	Present study
16f	16S rRNA gene position 9-27 of *E. coli*	GAGTTTGATCCTGGCTCAG	[7]
16r	16S rRNA gene position 1525-1544 of *E. coli*	AGAAAGGAGGTGATCCAGCC	[7]

*f indicates forward and r reverse.

overnight at 30°C in 5 ml of an MRS broth and centrifuged at 12,000 ×g for 3 min at 4°C. The cell pellet was washed twice with deionized water and suspended in 50 µl of a Tris-HCl buffer (50 mM, pH 8.0). Approximately 50 mg of glass beads (diameter, 425 to 600 microns; Sigma, St. Louis, U.S.A.) was added to the tubes and vortexed for 5 min.

The cells were then resuspended in an equal volume of the sample buffer [2×SDS sample buffer; 25 ml of 4×Tris-HCl/SDS (pH 6.8), 20 ml of glycerol, 4 g of sodium dodecyl sulfate, 2 ml of 2-mercaptoethanol, 1 mg of bromophenol blue and H_2O were added to 100 ml].

For protein denaturation, the tubes including the samples were heated for 5 min at 95°C. The cell debris was removed by centrifugation and the supernatants were analyzed by gradient (8–16%) SDS-PAGE. After the electrophoresis, the gels were stained for 2 h with 0.05% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, U.S.A.), and destained with 10% acetic acid and 30% methanol solution. The destained gels were scanned for further analysis. The identification of the isolates was based on a comparison of the protein patterns with those of protein fingerprints which were derived from almost all known reference strains of lactic acid bacteria previously isolated from Kimchi. The pattern storage and comparison were performed using 1D main software (Bioneer, Korea).

DNA Isolation for PCR Amplification of 16S rRNA Gene

The chromosomal DNA was isolated using a modification of the method of Ausubel *et al.* [1]. Five ml of the culture was harvested by centrifugation. The cells were then washed in a TE buffer (50 mM Tris-HCl, 50 mM EDTA, pH 8.0) and resuspended in 0.5 ml of another TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Lysis was initiated by the addition of 50 µl of 10 mg/ml lysozyme. After incubation, 30 µl of 10% (w/v) SDS and 3 µl of 20 mg/ml proteinase K (Sigma) were added, gently mixed, and further incubated for 1 h at 37°C. Next, the samples were treated with 100 µl of 5 M NaCl and 80 µl of a CTAB/NaCl solution, and incubated for 10 min at 65 µl. The protein was removed by treatment with an equal volume of phenol-chloroform. After centrifugation at 12,000 ×g for 5 min, the supernatant was added to a 0.6 volume of isopropanol, gently mixed, and spun down at 12,000 ×g for 10 min. The pellet was then washed twice in ice-cold ethanol and resuspended in ultra pure water.

16S rRNA Gene Sequence Analysis

The 16S rRNA gene was amplified using a universal primer pair [7] (Table 2). The PCR products were then purified using a QIAquick gel extraction kit (Qiagen, Valencia, U.S.A.), ligated into a pEZ-T vector (RNA Inc., Korea), and transformed into *Escherichia coli* DH5α competent cells. The recombinant plasmids were purified using a QIAprep spin miniprep kit and digested with *Eco*RI to confirm the insert. The nucleotide sequences of the plasmids were determined using an ABI PRISM Dye Terminator sequencing kit and ABI PRISM 377 sequencer (Perkin-Elmer, Norwalk, U.S.A.), according to the manufacturer's instructions. The T7 (forward) and M13 (reverse) primers were used as the sequencing primers.

Phenotypical Characterization of Isolates

Before testing, the strains were subcultured twice overnight in an MRS broth at 30°C. The capacity of the strains to grow at pH 3 or pH 8.5, at 10°C or 45°C, and in the presence of 6.5% or 8% NaCl (w/v) was tested in the MRS broth. The gas production was tested using Durham tubes. The strains were also tested for the production of acids from carbohydrate and related compounds by the use of API 50 CH strips and an API CHL medium (Montalieu Inc., Vercieu, France). The tests were performed according to the manufacturer's instructions and the results read after incubating the strains at 30°C for 2 days.

Scanning Electron Microscopy

The cell pellets were fixed with glutaraldehyde and dehydrated with ethanol, as described by Yamauchi and Johannes [23]. The ethanol was then removed by adding CO_2 at the critical point. Thereafter, the dried specimens were coated with platinum and examined using a Spectroscan Leica Model 440 microscope at Kyung Hee University in Korea.

RESULTS AND DISCUSSION

PCR Specificity for Detection of *Leuc. mesenteroides*

In previous studies on the lactic acid bacteria isolated from Kimchi, *Leuc. mesenteroides* was identified as the major bacterial population in Kimchi during the initial and the middle stages of Kimchi fermentation, while *Lb. plantarum*

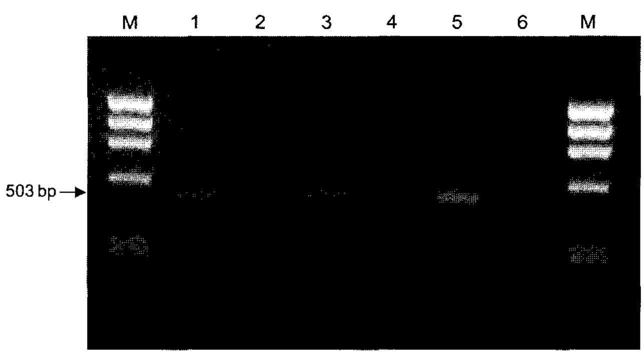

Fig. 1. PCR products obtained by amplification of type strains and lactic acid bacteria isolated from Kimchi using LMf/LMr primers.

Lanes: M, *Hae*III-X174 DNA marker (Gibco BRL, U.S.A.); 1, *Leuc. mesenteroides* KCTC 3100; 2, *Lb. plantarum* KCTC 3104; 3-6, isolates from Kimchi.

Table 3. Distribution of lactic acid bacteria isolated from various types of Kimchi.

Samples	Number of isolates (%)			Unknown	Total
	KHU-31	KHU-32 (*Lb. sake*)	KHU-38 (*Leuc. mesenteroides*)		
Chinese-cabbage Kimchi A	11 (55%)	1 (5%)	4 (20%)	4 (20%)	20 (100%)
Chinese-cabbage Kimchi B	23 (57%)	14 (35%)	-	3 (8%)	40 (100%)
Chinese-cabbage Kimchi C	6 (33%)	3 (17%)	5 (28%)	4 (22%)	18 (100%)
Chinese-cabbage Kimchi D	6 (33%)	-	7 (39%)	5 (28%)	18 (100%)
Cucumber Kimchi	19 (67%)	1 (3.5%)	6 (21%)	2 (7.1%)	28 (100%)
Yeolmoo Kimchi	5 (20%)	-	10 (40%)	10 (40%)	25 (100%)
Welsh onion Kimchi	25 (96%)	-	-	1 (4%)	26 (100%)
Kakdugi	5 (20%)	3 (12%)	6 (24%)	11 (44%)	25 (100%)

was found to continuously increase quantitatively during the last stage of fermentation [13, 14, 16, 20]. In the current study, the PCR method was used for the rapid identification and detection of *Leuc. mesenteroides* in Kimchi. The specificity of the primers was tested by performing a PCR with various reference strains of lactic acid bacteria (*Lb. plantarum* KCTC 3104, *Lb. brevis* KCTC 3498, *Lb. casei* KCTC 3109, *Lb. sake* 3603, *Lb. sake* 3598, *Lb. acidophilus* KCTC 3142, *Leuc. mesenteroides* subsp. *mesenteroides* KCTC 3505, *Leuc. mesenteroides* subsp. *dextranicum* KCCM 35046, *Leuc. argentinum* KCCM 40710, *Leuc. citreum* KCTC 3524, *Leuc. lactis* KCTC 3528, and *Ped. pentosaceus* KCTC 3507). It was found that PCR amplification using the LMf/LMr primer set consistently produced a unique DNA fragment of the expected size without producing PCR products from nontarget species, thus suggesting that the primer set was species-specific. As expected, the LMf/LMr primer set yielded a 503 bp PCR product for *Leuc. mesenteroides*. The DNA templates for the primers were obtained from the colonies using a simple lysis procedure, as described in Materials and Methods. This procedure produced the same result as the experiments where DNA isolated from the strains was used. To investigate the distribution of *Leuc. mesenteroides*, a PCR was carried out using 200 strains isolated from various types of Kimchi fermented at 20°C. The use of species-specific primers designed in the current study allowed successful identification of *Leuc. mesenteroides* in the mixed microbial populations in Kimchi (Fig. 1). Unlike previous reports, *Leuc. mesenteroides* was not detected as the predominant population in various types of Kimchi fermented at 20°C (data not shown). Therefore, it would appear that another type of lactic acid bacteria rather than *Leuc. mesenteroides* is the predominant species in Kimchi, when fermented at 20°C. To test this hypothesis, all 200 isolates were examined using SDS-PAGE of the whole-cell proteins.

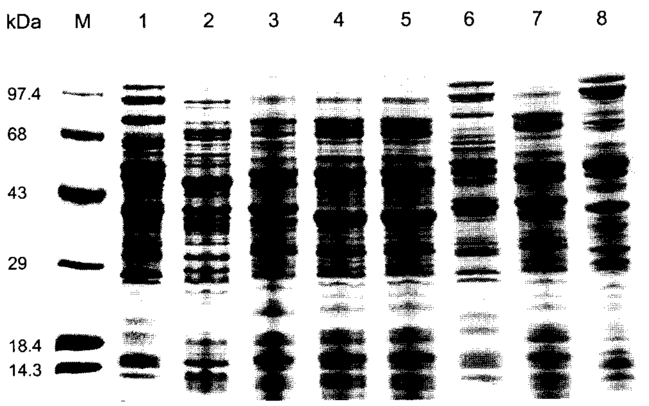

Fig. 2. SDS-PAGE profiles of whole cell proteins of reference strains and isolates from Kimchi.
Lanes: M, Protein molecular weight markers (kDa); 1, *Leuc. mesenteroides* KCTC 3100; 2, *Lb. plantarum* KCTC 3104; 3, *Lb. sake* KCTC 3598; 4, 5, Isolate KHU-31; 6, Isolate identified as *Leuc. mesenteroides*; 7, Isolate KHU-32 identified as *Lb. sake*; 8, Unidentified isolate.

Identification of Isolates Based on Whole Protein Patterns Using SDS-PAGE

The reproducibility of the SDS-PAGE technique was investigated by comparing duplicate extract preparations

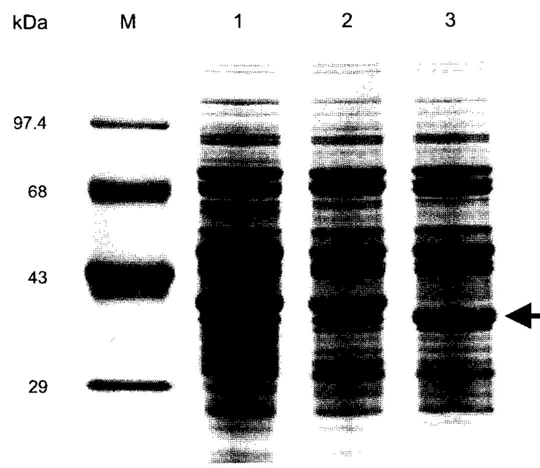

Fig. 3. SDS-PAGE profiles of whole-cell proteins of *Lb. sake*, KHU-31, and KHU-32 isolated from Kimchi.
Lanes: M, Protein molecular weight markers (kDa); 1, *Lb. sake* KCTC 3598; 2, KHU-32 strain isolated from Kimchi; 3, KHU-31 strain isolated from Kimchi. Arrow indicates distinct 38 kDa band of KHU-31.

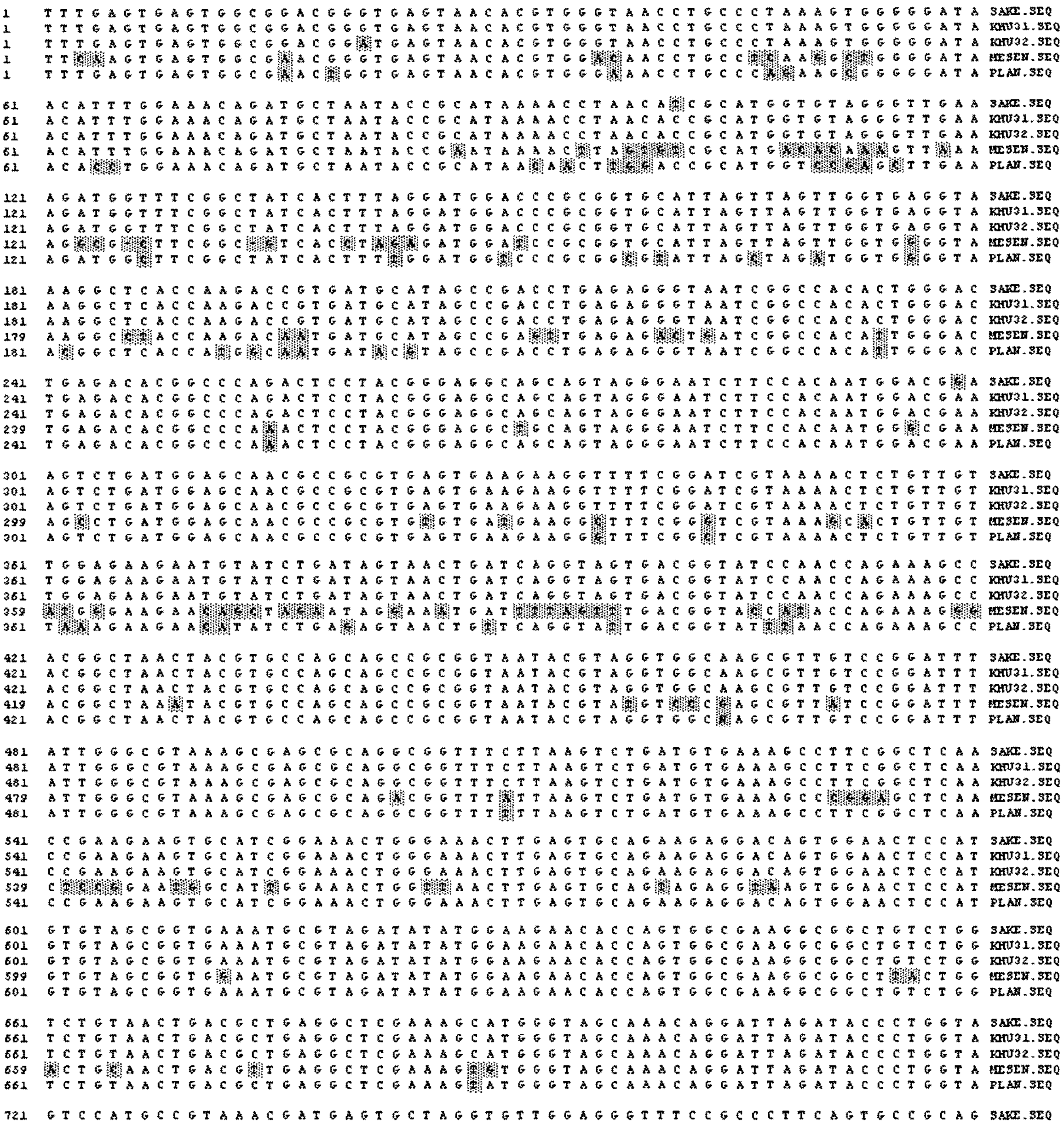

Fig. 4. Comparison of 16S rRNA gene partial sequence of strains aligned with that of *Lb. sake*.

SAKE; *Lb. sake* (GenBank AF401673); KHU31, Isolate KHU-31; KHU32, Isolate KHU-32; MESEN, *Leuc. mesenteroides* (GenBank AB023242); PLAN, *Lb. plantarum* (GenBank M58827). Sequences were aligned by using MegAlign software (Windows version 3.03c; DNASTAR, Madison, WI, U.S.A.). Differences between sequences are indicated by a gray box.

on different gels. The whole patterns were very similar (96±1%), therefore, the 200 isolates mentioned above could be characterized by their whole-cell protein patterns obtained by SDS-PAGE. SDS-PAGE of whole-cell proteins of the reference strains and lactic acid bacteria isolated from Kimchi yielded different banding patterns which provided results specific for identification of lactic acid bacteria (Fig. 2).

The whole-cell protein patterns of the KHU-32 and the KHU-38 strains were found to be identical with those of the reference strains of *Lb. sake* KCTC 3598 and *Leuc. mesenteroides* KCTC 3100, respectively (Fig. 2), thus identifying the KHU-32 and KHU-38 strains to be *Lb. sake* and *Leuc. mesenteroides*, respectively. In various types of Kimchi, KHU-31 and KHU-32 (*Lb. sake*) and KHU-38 (*Leuc. mesenteroides*) were found to be present as the predominant species (Table 3). In particular, the KHU-31 isolate was more predominant than *Leuc. mesenteroides* in various types of Kimchi. The protein pattern of the KHU-31 strain was almost identical to the reference strains of *Lb. sake*, yet distinctly different in one band (indicated by the arrow) with 38 kDa molecular weight (Fig. 3). This strain was further examined using a 16S rRNA gene sequence analysis.

16S rRNA Gene Sequence Analysis of Isolates

Two isolates, KHU-32 identified as *Lb. sake* and KHU-31 identified as a *Lb. sake*-like strain by SDS-PAGE, were further examined by a 16S rRNA gene sequence analysis. The 16S rRNA genes from the two isolates were amplified. As expected, fragments with an approximate 1,500-bp size, corresponding to the 16S rRNA gene, were obtained. The sequences of the two isolates were subjected to a similarity search using the GenBank databases. The 16S rRNA gene sequences of two isolates were aligned with those of *Leuc. mesenteroides* (GenBank AB023242), *Lb. plantarum* (GenBank M58827), and *Lb. sake* (GenBank AF401673) (Fig. 4). The 16S rRNA gene sequence of two isolates matched perfectly with that of *Lb. sake* (99.6%). However, the similarity among the two isolates, *Leuc. mesenteroides* and *Lb. plantarum*, were low, therefore, KHU-31 strain was named *Lb. sake* KHU-31.

Morphological and Physiological Characteristics of Isolates

The phenotypic characteristics of the two isolates, KHU-31 and KHU-32, were found to be Gram-positive, non-spore forming, rod-shaped (Fig. 5), facultatively anaerobic and catalase-negative. The isolates did not produce any gas from glucose and grew at both 10°C and 40°C. The optimal temperature for growth was approximately 30°C. The isolates grew optimally at pH 6.0–7.0, yet were not inhibited at pH 3 and pH 8.5. The strains also exhibited salt tolerance in the presence of 6.5% (w/v) NaCl, yet not in 8% (w/v) NaCl. The patterns of carbohydrate fermentation for KHU-31 and KHU-32 were slightly similar to that for *Lb. sake* KCTC 3598 (Table 4). The two isolates utilized arabinose, rhamnose, and xylose, whereas *Lb. sake* KCTC 3598 did not. Therefore, even though the strains belong to the same species, the carbohydrate fermentation patterns were different [9]. It would appear that the major microorganisms in Kimchi were affected by special environments, such as a low pH and salts. The adaptation of microorganisms to special environmental conditions has been previously reported in other fermented foods [2, 22]. Hence, it would seem that

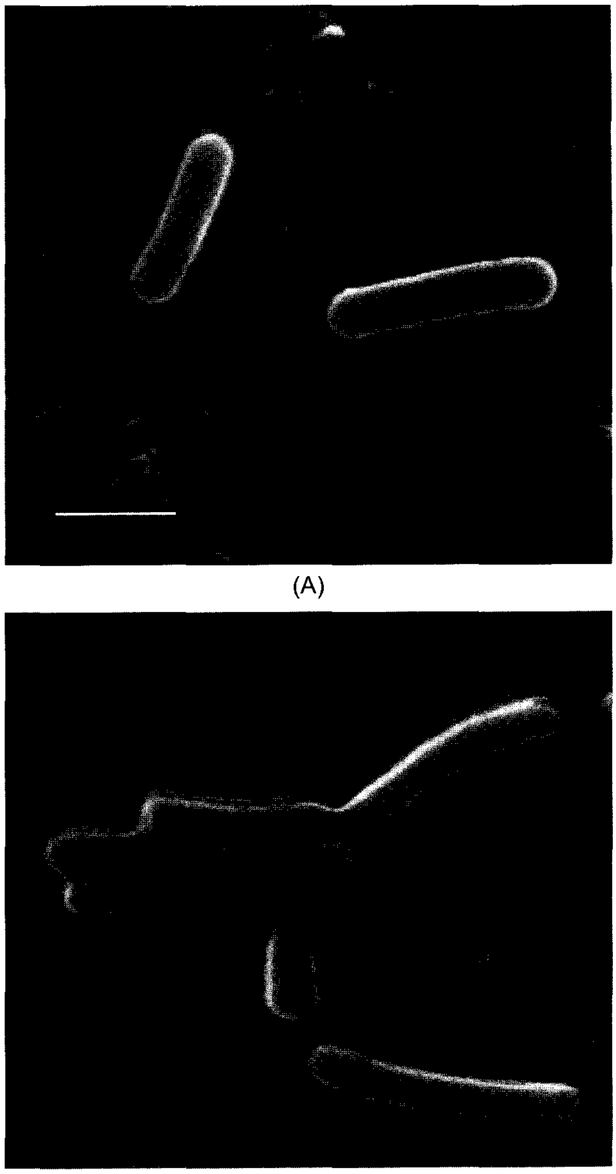

Fig. 5. SEM micrographs of strains isolated from Kimchi.
(A) KHU-31 strain and (B) KHU-32 strain, isolated from Kimchi. Bar represents 1 μm.

Table 4. Characteristics of carbohydrate fermentation for *Lb. sake* KCTC 3598, and KHU-31 and KHU-32 isolates.

Carbohydrates	Fermentation		
	Lb. sake KCTC3598	KHU-31	KHU-32
Acid from			
Arabinose	–	+	+
Cellobiose	+	+	+
Esculin	–	–	–
Fructose	+	+	+
Galactose	+	+	+
Glucose	+	+	+
Gluconate	$+^w$	$+^w$	$+^w$
Lactose	+	+	+
Maltose	–	–	–
Mannitol	–	–	–
Mannose	+	+	+
Melezitose	–	–	–
Melibiose	+	+	+
Raffinose	–	–	–
Rhamnose	–	$+^w$	$+^w$
Ribose	+	+	+
Salicin	$+^w$	+	+
Sorbitol	–	–	–
Sucrose	+	+	+
Trehalose	+	+	+
Xylose	–	+	+

Symbols; +, positive; $+^w$, weak positive; –, negative.

a genotypic approach could offer more reliable and specific results than conventional identification methods for the detection and identification of the lactic acid bacteria in Kimchi.

CONCLUSION

The use of a PCR, SDS-PAGE of whole cell proteins, and 16S rRNA gene sequence analysis was able to offer rapid and accurate results in the identification and detection of the lactic acid bacteria in Kimchi and could be employed for the analysis and monitoring of the lactic acid bacteria during Kimchi fermentation. This approach would be particularly useful when the species are phenotypically closely related and nondistinguishable by conventional microbiological approaches, as in the case of the lactic acid bacteria in Kimchi. The current results showed that *Lb. sake* KHU-31 together with *Leuc. mesenteroides* were the predominant lactic acid bacteria in various types of Kimchi in the middle stage of fermentation at 20°C. Accordingly, it is proposed that *Lb. sake* may play an important role in various types of Kimchi fermented at a high temperature. In particular, *Lb. sake* seems to be the main microorganism responsible for the acidification of Kimchi, because it exhibited tolerance at low pH and characteristics of homofermentative lactic acid bacteria. For a better understanding of Kimchi fermentation, further studies on the lactic acid bacteria present in Kimchi fermented at low temperatures (i.e. 4°C or 10°C) and with various sub-ingredients using polyphasic methods are necessary.

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