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Identifications of Predominant Bacterial Isolates from the Fermenting *Kimchi* Using ITS-PCR and Partial 16S rDNA Sequence Analyses

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Abstract Despites many attempts to explore the microbial diversity in *kimchi* fermentation, the predominant flora remains controversial to date. In the present study, major lactic acid bacteria (LAB) were investigated in Chinese cabbage *kimchi* in the early phase of fermention. For the samples over pH 4.0, viable cell counts of *Leuconostoc* and *Pediococcus* were 10^6 cfu/ml and below 10^2 cfu/ml, respectively, and 20 isolates out of 172 were subjected to a biochemical identification (API 50 CH kit) as well as molecular-typing methods including ITS-PCR with a RsaI digestion and 16s rRNA gene sequence analysis for species confirmation. Seven isolates were nicely assigned to *Lb. brevis*, 6 to *Leuconostoc* spp. (2 *mesenteroides*, 2 *citreum*, 1 *carnosum*, 1 *gasicomitatum*), 4 to *Weissella* (3 *kimchii/cibaria*, 1 *hanii*) and 2 to other *Lactobacillus* spp. (1 *farciminis*, 1 *plantarum*). On the other hand, the biochemical identification data revealed 9 strains of *Lb. brevis*, 6 strains of *Leuconostocs*, 2 strains of *Lb. plantarum* and 1 strain each of *Lb. coprophilus* and *Lactococcus lactis*. However, a single isolates, YSM 16, was not matched to the ITS-PCR database constructed in the present study. Two *Lb. brevis* strains by API 50 CH kit were reassigned to *W. kimchii/cibaria*, *Lb. coprophilus* or *W. hanii*, respectively, judging from the results by the above molecular typing approaches. As a whole, the identification data obtained by the biochemical test were different from those of ITS-PCR molecular method by about 63% at genus-level and 42% at species-level. The data by the ITS-PCR method conclusively suggest that predominant LAB species is probably heterolactic *Lb. brevis*, followed by *W. kimchii/cibaria*, *Leuc. mesenteroides*, and *Leuc. citreum*, in contrast to the previous reports [3] that *Leuc. mesenteroides* is the only a predominant species in the early phase *kimchi* fermentation.

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A variety of vegetables are fermented worldwide and consumed as foods, representing sauerkraut, fermented cucumber and olive, zukemono (Japan), and *kimchi* (Korea), where the lactic acid bacteria play a crucial role in the fermentation process [3]. For manufacturing *kimchi*, Chinese cabbage and/or radish is the most frequently brined among the raw materials from which various types of *kimchi* were made [16]. Since *kimchi* has been traditionally fermented by the natural microflora on the plant materials, fermentation failures are often experienced. Therefore, numerous efforts have been made to control the fermentation process especially in the industry. For the controlled fermentation, major bacterial species, which are actively involved in the fermentation, should be identified. In the previous studies, the major LAB isolated and identified from the *kimchi* include: *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuc. mesenteroides* subsp. *dextranicum*, *Leuc. citreum*, *Lactobacillus brevis*, *Lb. fermentum*, *Lb. plantarum*, *Pediococcus pentosaceus*, and *Streptococcus faecalis* [18]. However, these results about the predominant members in *kimchi* fermentation still remain controversial, since there are so many factors affecting the results, including isolation conditions, types of *kimchi*, variation in minor ingredients, fermenting temperatures, and so forth. Natural LAB populations on the plant materials brined are often very low and consist mostly of heterofermenters such as *Leuc. mesenteroides* which is predominant in the earlier stage and *Lb. plantarum*, a homofermenter, has been reported predominant in the later stage of fermentation [15, 21].

In recent years, molecular ecological studies have received increasing attentions for exploring the microbial

diversity in fermented foods. In prokaryotes, the rRNA genetic loci contain the genes for all three rRNA species: 16S, 23S, and 5S genes. These genes are separated by spacer regions which exhibit a large degree of sequence and length variation at the level of genus and species [12]. According to review papers [26], a variety of biochemical and molecular methods have identified lactic acid bacteria. As for molecular typing methods, PCR assays based on the genes or the intergenic spacer (ITS) regions of the rRNA locus have been very useful for the environmental detection of both prokaryotic and eukaryotic microorganisms [12, 25]. Because the rRNA genes are tandem repeated with high copy numbers and the small subunits are highly conserved [20], the genes have been the target site of choice for the development of molecular assays.

Despite the extensive works in the past 50 years, only a limited amount of information is available on the ecological changes in the fermenting *kimchi*. The present study was carried out to isolate and identify the predominant LAB strains from the *kimchi* samples in the early to mid-stage of the fermentation. The isolates were identified by a biochemical and ITS-PCR methods. In addition, 16S rRNA gene sequence was analyzed to supplement the ITS-PCR data, confirming the isolates at species-level.

MATERIAL AND METHODS

Isolation, Enumeration, and Morphological Tests

A total of thirty Chinese cabbage *kimchi* samples (~100 g each) were collected at local homes and groceries and pH was determined immediately after the sample was delivered to this laboratory in order to exclude the samples under pH 4.0, regardless of the manufacturing date and the minor ingredients used. Aliquots (10 g) of sample over pH 4.0 was blended with 90 ml of 0.85% sterile NaCl, and appropriate serial dilutions were made to isolate LAB on the modified MRS agar (MRS plus 10% Chinese cabbage juice: MMRS) with 0.004% bromocresol purple (Sigma, St. Louis, MO, U.S.A.) and incubated at 37°C for 72 h [10]. Approximately 5 distinct colonies from the highest dilution were randomly picked from individual plate and purified twice by streaking on MRS agar (Difco Laboratories, Detroit, MI, U.S.A.). Twenty of total 172 isolates were randomly chosen and given a serial YSM number to each of them. For long-term storage, 5 ml of pure culture was prepared in the modified MRS broth at 30°C for 24 h, washed briefly by centrifugation and the pellet was suspended in MRS broth, followed by mixing with sterile glycerol to 25% (v/v) and stored at −70°C until use. For *Leuconostoc* enumeration, viable cells were counted directly from the samples by plating appropriate dilutions on the NLS agar [5] containing vancomycin (5 μg/ml) and novobiocin (0.5 mg/ml) using Spiral Plater 4000 (Spiral Biotech, Norwood, MA, U.S.A.). Pediococci were selectively counted on GYP agar [10.0 g of glucose, 5.0 g of yeast extract, 5.0 g of peptone, 2.0 g of sodium acetate, 0.25 g of Tween 80, 200 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot 4H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 5.0 g of NaCl, 5 g of $CaCO_3$, and 1,000 ml of distilled water, pH 6.8] after incubation in an anaerobic jar (Anaerocult®, Merck, Darmstadt, Germany) at 35°C for 2 to 3 days, and were selectively counted based on morphological observations [10]. Bacterial strains used as reference LAB cultures and are listed in Table 1.

Table 1. List of reference strains used for the molecular-typing methods in this study.

Bacterial Strains		Source
Lactobacillus plantarum	KCCM[a] 11322	Lab. Stock
Lactobacillus plantarum	ATCC[b] 14917	NCSU[c]
Lactobacillus brevis	KCCM 11904	Lab. Stock
Lactobacillus brevis	ATCC 14869	NCSU
Pediococcus pentosaceus	ATCC 33314	NCSU
Weissella kimchii/cibaria	KCCM 41287	Lab. Stock
Pediococcus pentosaceus	ATCC 33316	NCSU
Leuconostoc mesenteroides	KCCM 11324	Lab. Stock
Leuconostoc mesenteroides	ATCC 23386	NCSU
Lactococcus lactis subsp. *lactis*	KCCM 32041	Lab. Stock
Lactococcus lactis subsp. *lactis*	ATCC 7962	NCSU

[a]KCCM: Korean Culture Center of Microorganisms (Seoul, Korea)
[b]ATCC: American Type Culture Collection (Rockville, MD, U.S.A.)
[c]NCSU: USDA-ARS Food Fermentation Laboratory Culture Collection, North Carolina State University (Raleigh, NC, U.S.A.).

Biochemical Tests

All the *kimchi* isolates were identified using by API 50 CH strips and API 50 CHL medium (BioMérieux, Marcy, l'Etoile, France) at 30°C for 48 h according to the manufacturer's instructions. The results were obtained using the APIWEB version 5.0 (BioMérieux). Other biochemical or microbiological experiments were performed by the standard methods [27], unless described otherwise.

ITS-PCR Procedure

The internal transcribed spacer region (ITS) between the 16S and 23S rRNA gene cluster was carried out by using the PCR method described by Breidt and Fleming [2], and the amplification was performed with the *Taq* DNA polymerase (Promega, Madison, WI, U.S.A.) in a Gradient 96 RoboCycler (Stratagene La Jolla, CA, U.S.A.); reaction mixtures consisted of 70 μl of deionized water, 10 μl of PCR buffer (500 mM KCl, 100 mM Tris, pH 8.3), 1% Triton X-100 (Sigma), 10 μl of 25 mM $MgCl_2$, 4 μl each of four 50 mM deoxynucleoside triphosphates (dNTP), 0.8 μl of *Taq* polymerase (5 U/ml, Promega), 2 μl of primer (Genosys Biotechnologies, Woodland, TX, U.S.A.), and 4 μl of appropriately diluted template DNA in a final volume of 100 μl. These regions of various isolates of LAB were

amplified using two primer pairs of G1-16S 5'-GAAGTC-GTAACAAGC-3' and L2-23S 5'-GGGTTTCCCCATTCGGA-3'; G2-16S 5'-TGCGGCTGGATCACC-3' and L1-23S 5'-CAAGGCATCCACCGT-3'. After initial denaturation at 94°C for 5 min, PCR was repeated for 24 cycles at 94°C for 1 min, at 55°C for 5 min, and at 72°C for 2 min, and the reaction mixture was cooled immediately to 4°C and kept at -20°C for electrophoresis. Each amplicon was separated on 5% polyacrylamide gel, and DNA was extracted from the gel slice with a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. ITS-PCR products (100 μg) were digested with 16 U of RsaI (Stratagene) at 37°C for 30 min, and 50 μl aliquots were stored at -70°C.

Randomly Amplified Polymorphic DNA Analyses

RAPD-PCR amplification was performed according to the procedure of Johansson *et al.* [13] with a pair of random primers described below. The reaction mixtures consisted of 70 μl of deionized water, 10 of μl PCR buffer (500 mM KCl, 100 mM Tris, pH 8.3), 1% Triton X-100 (Sigma), 10 μl of 25 mM $MgCl_2$, 4 μl each of four 50 mM deoxynucleoside triphosphates (dNTP), 0.8 μl of *Taq* polymerase (5 U/ml, Promega), 2 μl of primer (Genosys Biotechnologies, Woodland, TX, U.S.A.), and 2 μl of appropriately diluted template DNA in a final volume of 100 μl. The genomic DNA of various LAB isolates was amplified using primers of ED-01 5'-ACGCGCCCT-3' and ED-02 5'-CCGAGTCCA-3'. After denatured at 94°C for 10 min without *Taq* polymerase, PCR was repeated for 4 cycles at 94°C for 45 s, at 30°C for 2 min, and at 72°C for 45 s, and then additional 26 cycles at 94°C for 15 s, at 36°C for 30 s, at 72°C for 45 s and final reaction of 72°C for 10 min. the PCR products were cooled immediately to 4°C and kept at -20°C until use.

Sequencing of the 16S Ribosomal DNA

A partial 16S rRNA gene was sequenced according to Kullen *et al.* [17]. Cells grown in MMRS broth at 30°C for 8 h were used for genomic DNA extraction with Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's protocol, using 10 mg/ml of final lysozyme concentration. Sequencing was performed on both strands by the dideoxy method using a PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, U.S.A.) in combination with an Applied Biosystems Automated Sequencing System (Model 310A).

Sequence Alignments and Phylogenetic Inference

Sequence similarity search was performed using BLAST program in GenBank data library. The sequences were aligned using CLUSTAL W (default setting), and the sequence information was then imported into the CLUSTAL W software program (Hitachi Software Eng. Japan) for assembly and alignment. The 16S rDNA sequences of the isolates were compared with sequences from type LAB strains held in GenBank, nucleotide substitution rates were calculated, and phylogenetic trees were constructed by the neighbor-joining method [1]. *Bacillus subtilis* NCDO 1769 was used as an out-group organism. The reliability of clustering pattern in trees was evaluated by bootstrap analysis [9] of the sequence data with CLUSTAL W software.

RESULTS

Enumerations and Biochemical Identifications

To uncover the predominant LAB species occurring in the early phase of the fermentation, the properly-diluted each *kimchi* sample was plated on the modified MRS agar, and the colonies grown on the surface were picked, based on its distinct morphology as described in the Materials and Methods. Twenty out of total 172 isolates were selected because of their frequent occurrence in the samples. Fifteen strains among them were shown to produce gas from glucose (heterolactic fermenters). API 50 CHL kit (BioMérieux, Lyon, France) was used to identify the common 20 isolates (Table 2), all of which were able to utilize glucose and fructose as a carbon source. Among the others, YSM 19 and 20 distinctly grew in sorbitol and melezitose, YSM 8 in 5-ketogluconate, and YSM 17 did not in maltose. Twelve isolates were able to grow at between 10°C and 45°C. Only two isolates grew at 45°C (data not shown) and six were able to grow at between 10 and 45°C up to 5-days after inoculation. Nine isolates (YSM 1, 5, 6, 9, 10, 11, 12, 13, 15) were tentatively identified as *Lb. brevis,* based on the API database, even though YSM 1 didn't produce gas from D-xylose. Six isolates (2, 8, 14, 16, 17, 18) were assigned to *Leuc. mesenteroides*, two isolates to *Lb. plantarum*, and a single isolate each to *Lb. coprophilus* and *Lactococcus(Lc.) lactis* (Table 3).

ITS-PCR Profiles

As reported earlier, conventional identification methods among the LAB based on the phenotypic differences may lead to identification error or misrepresent the phylogenetic relationships among this group of microorganisms [2]. Therefore, ITS-PCR was chosen as a method of choice for identifying the isolates. Some reference strains listed in Table 1 were *Lb. brevis*, *Lb. plantarum*, *P. pentosaceus*, *Leuc. mesenteroides*, and *Lc. lactis* subsp. *lactis*, because they were previously reported as the dominant microflora found in *kimchi* fermentation. As shown in Fig. 1, the PCR products for the reference strains showed similar banding patterns among strains of a given LAB species and resulted in formation of 450 and 700 bp for *Lb. plantarum*, 430 bp and 650 bp for *Lb. brevis* (Group 1), 500 bp and 600 bp (Group 3) for *P. pentosaceus* and *W. kimchii/cibaria. Leuc. mesenteroides* produced one single band (Group 2) of

Table 2. Carbohydrate utilization test of the *kimchi* isolates using a API 50 CH kit.

Carbohydrate	*Kimchi* Isolates (YSM No.)																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
L-Arabinose	+	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
Ribose	+	+	+	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	+	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–
Galactose	+	–	–	+	+	+	+		+	+	+	+	+	+	+	+	–	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	–	+	+	–	+	–	–	–	+	–	–	+	+	+	+
Mannitol	–	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	+	+
Sorbitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
Methyl-D-glucoside	–	+	+	–	–	–	–	+	+	–	+	+	+	+	–	+	+	+	+	+
Acetyl-glucosamine	–	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	+	+	+	+
Amygdalin	–	–	–	–	+	–	+	–	+	–	+	+	+	+	–	–	+	+	+	+
Arbutin	–	–	–	–	+	–	+	–	+	–	–	–	–	+	–	–	+	+	+	+
Esculin	–	+	+	–	+	+	+	+	+	–	–	–	–	+	–	–	+	+	+	+
Salicin	–	–	–	–	+	–	+		+	–	–	–	–	+	–	–	+	+	+	+
Cellobiose	–	+	+	–	+	–	+	+	+	–	–	–	–	+	–	–	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
Lactose	–	–	–	–	–	+	–	–	+	–	+	+	+	+	–	–	–	+	+	+
Melibiose	+	–	–	+	–	+	–	+	–	+	+	+	+	+	–	–	–	+	+	+
Sucrose	+	+	+	+	+	–	+	+	+	+	+	+	+	+	–	–	+	+	+	+
Trehalose	–	+	+	–	–	–	–	+	+	–	–	–	–	+	–	–	+	+		+
Melezitose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
Raffinose	+	–	–	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–	+	–
Gentiobiose	–	+	+	–	+	–	+	–	+	–	–	–	–	+	–	–	+	+	+	+
D-Turanose	–	+	+	–	–	–	–	+	–	–	–	–	–	+	–	–	+	+	+	–
D-Arabitol	+	–	–	–	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–
Gluconate	+	–	+	–	+	–	+	–	+	+	+	+	+	–	+	+	–	–	+	+
5-Ketogluconate	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–

Positive (+) and negative (–) symbols are whether or not each carbon source was utilized by the test organism.

550–600 bp in size, and 500 bp for *Lc. lactis* subsp. *lactis*. For the 20 isolates, ITS-PCR experiment was performed and the results are shown in Fig. 2: YSM 1, 5, 7, 8, 10, 11, 12, 13, and 20 were nicely assigned to *Lb. plantarum* or *Lb. brevis* (Group 1); YSM 2, 4, 6, 14, 17, and 18 were *Leuc. mesenteroides* or *Lc. lactis* subsp. *lactis* (Group 2); The banding pattern of YSM 9 was very close to a member of *Pediococcus* or *Weissella kimchii/cibaria* (Group 3). Some of the isolates (YSM 3, 15, 16, 19: Group 4) produced one to four bands within the same migrating range on the gel, while PCR profiles of the reference strains as a whole showed one or two bands between 430 to 700 bp. Thus, they were unidentified since their fingerprints were hardly matched to those of the reference strains, suggesting that they are possibly new species which has not earlier been published. There were also some isolates with similar ITS-PCR profiles, for which additional experiment was required to unequivocally differentiate the LAB isolates. Toward this end, PCR products were digested with RsaI, a restriction endonuclease, because this enzyme is active in PCR reaction buffer, thus eliminating the need to purify the PCR products prior to digestion [2]. The banding profiles presented in Fig. 3 and 4 showed that YSM 1 of Group 1 was assigned to *Lb. plantarum* and 4, 14, and 18 of the group 2 presumably belong to *Leuc. mesenteroides*, based on the data from the digested amplicons. YSM 5, 9, and 15 among the *Lb. brevis* by the API 50 CH kit were identified as *W. kimchii/cibaria*.

16s rRNA Sequence Analyses

To the best of our knowledge, 16s rRNA sequence analysis is, in spite of the limitations such as its expenses and laborious procedure, the best reliable identification method at species-level [25]. To confirm the results on species obtained by the above ITS-PCR method, the 300 to 350 bp products from 16s rDNA PCR were sequenced, followed by comparing its homology with those of GenBank database. As shown in Fig. 5, 18 with exception of two isolates had more than 95% homology to the database, and were assigned 7 strains to *Lb. brevis* (1, 3, 6, 8, 11, 12, 13), 3 strains to *W. kimchii/cibaria* (5, 9, 15), 2 strains to *Leuc. medenteroides* (14, 18), 2 strains to *Leuc. citreum* (4, 17), a

Table 3. Identification of the 20 *kimchi* isolates by API 50 CH kit and ITS-PCR with 16S rDNA sequence analyses.

Strains	Biochemical data (API 50 CH kit)		ITS-PCR+16S rDNA sequence	
	species	% identity	species	% homology
YSM-1	*Lb. brevis*	98.7	*Lb. brevis*	100(330/330)[a]
YSM-2	*Leuc. mesenteroides* subsp. *mesen/dextr*[b]	74.3	*Leuc. carnosum*	100(298/298)
YSM-3	*Lc. lactis* subsp. *lactis*	–[c]	*Lb. brevis*	91(291/319)
YSM-4	*Leuc. lactis*	95.7	*Leuc. citreum*	99(301/304)
YSM-5	*Lb. brevis*	70.2	*W. kimchii/cibaria*[c]	99(330/332)
YSM-6	*Lb. brevis*	99.6	*Lb. brevis*	98(320/326)
YSM-7	*Lb. corprophilus*	74.3	*W. hanii*	99(326/327)
YSM-8	*Leuc. mesenteroides* subsp. *mesen/dextr*	99.9	*Lb. brevis*	97(315/322)
YSM-9	*Lb. brevis*	99.7	*W. kimchii/cibaria*	99(335/337)
YSM-10	*Lb. brevis*	98.7	*Leuc. gasicomitatum*	100(303/303)
YSM-11	*Lb. brevis*	75.3	*Lb. brevis*	96(313/326)
YSM-12	*Lb. brevis*	75.3	*Lb. brevis*	96(313/325)
YSM-13	*Lb. brevis*	75.3	*Lb. brevis*	98(320/326)
YSM-14	*Leuc. mesenteroides* subsp. *mesen/dextr*	99.9	*Leuc. mesenteroides*	100(309/309)
YSM-15	*Lb. brevis*	95.0	*W. kimchii/cibaria*	99(330/332)
YSM-16	*Leuc. mesenteroides* subsp. *mesen/dextr*	75.5	(–)[d]	
YSM-17	*Leuc. mesenteroides* subsp. *mesen/dextr*	97.2	*Leuc. citreum*	100(309/309)
YSM-18	*Leuc. mesenteroides* subsp. *mesen/dextr*	99.9	*Leuc. mesenteroides*	100(309/309)
YSM-19	*Lb. plantarum*	99.9	*Lb. farciminis*	95(244/255)
YSM-20	*Lb. plantarum*	98.1	*Lb. plantarum*	99(323/326)

[a]Numbers in the parenthesis represent the homology sequence in number
[b]*Leuc. mesenteroides* subsp. *mesenteroides* or *dextranicum*
[c]*Weissella kimchii* was annexed to *W. cibaria*: *Int. J. Syst. Evol. Microbiol.* (2004)
[d]not able to type based on the identity percentage, but closely related to a species by API 50 CH kit (BioMérieux).

single strain each to *Leuc. carnosum* (2), *Leuc. gasicomitatum* (10), *Lb. plantarum* (20), and *Lb. farciminis* (19) (Table 3). *W. hanii* (7) with a ITS-PCR method was identified from a

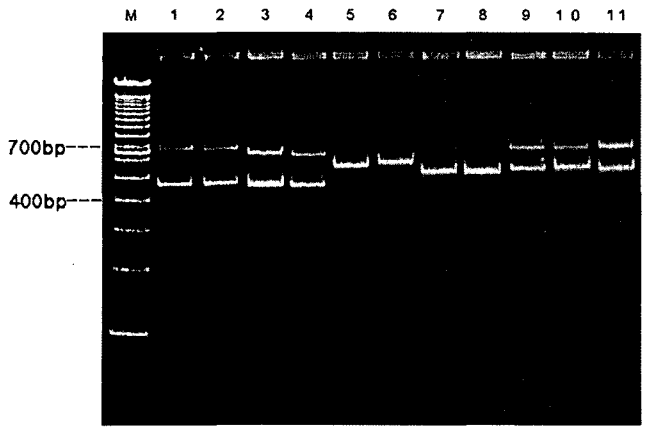

Fig. 1. ITS-PCR profiles of the reference LAB strains used in this study.
Lane 1, *Lb. plantarum* KCCM 11322; lane 2, *Lb. plantarum* ATCC 14917; lane 3, *Lb. brevis* KCCM 11904; lane 4, *Lb. brevis* ATCC 14869; lane 5, *Leu. mesenteroides* KCCM 11324; lane 6, *Leu. mesenteroides* ATCC 23386; lane 7, *Lc. lactis* subsp. *lactis* KCCM 32041; lane 8, *Lc. lactis* subsp. *lactis* ATCC 7962; lane 9, *P. acidilactici* KCCM 11746; lane 10, *P. pentosaceus* ATCC 23386; lane 11, *Weissella kimchii* KCCM 41287; M, molecular weight marker, 100 bp DNA ladder.

Lb. coprophilus with API 50 CH kit. Among the isolates unidentified (Group 4: 3, 15, 16, 19), YSM 3 was confirmed to be *Lb. brevis*, 15 to *W. kimchii/cibaria*, and 19 to *Lb. farciminis* with 16S rRNA gene sequencing approach, but 16 was still unidentified with an ITS-PCR experiment. The biochemical data were different from those of ITS-PCR molecular method by about 63% at genus-level (12/19 isolates) and 42% at species-level (8/19 isolates). These data have conclusively shown that *Lb. brevis* was the most prevalent, followed by *W. kimchii/cibaria* and *Leuc. citreum* in this particular experiment. These data are to some extent compatible with recent publications [6, 19].

DISCUSSION

Along with the recent urbanization of Korean society, the factory-made *kimchi* products are gradually preferred to home-made one which relies on the natural fermentation [4, 16]. Therefore, many food microbiologists have been intrigued by the main bacterial flora involved in the fermentation and affecting the overall flavors. In recent years, much effort has been made to introduce the starter-added technology in the *kimchi* industry. *Leuc. citreum* IH22 [6], which was claimed as a possible dominant strain during the early to mid-stage of *kimchi* fermentation, was

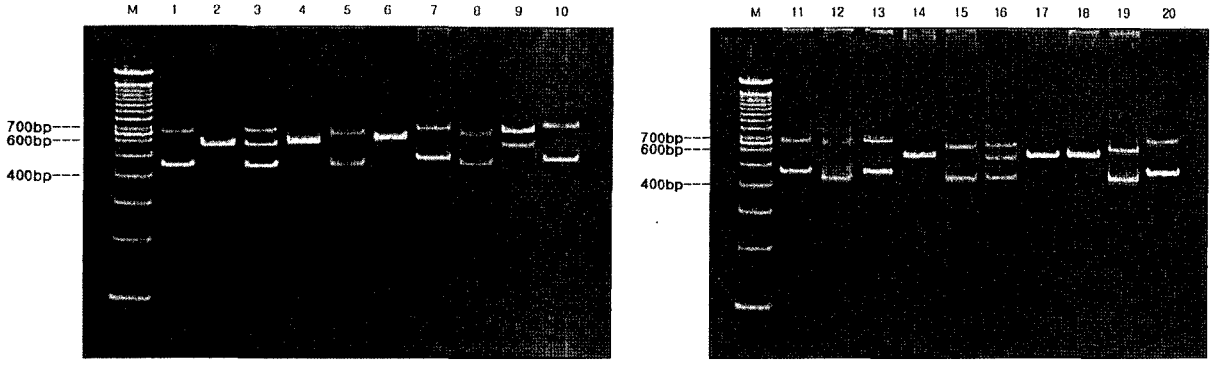

Fig. 2. ITS-PCR profiles of the 20 LAB isolates from the fermenting *kimchi*.
Lane 1, YSM 1; lane 2, YSM 2; lane 3, YSM 3; lane 4, YSM 4; lane 5, YSM 5; lane 6, YSM 6; lane 7, YSM 7; lane 8, YSM 8; lane 9, YSM 9; lane 10, YSM 10; lane 11, YSM 11; lane 12, YSM 12; lane 13, YSM 13; lane 14, YSM 14; lane 15, YSM 15; lane 16, YSM 16; lane 17, YSM 17; lane 18, YSM 18; lane 19, YSM 19; lane 20, YSM 20; M, molecular weight marker, 100 bp DNA ladder.

tentatively used as a starter in the fermentation. The strain consistently constituted over 95% of the population in the IH22-inoculating *kimchi* for 5-day fermentation, while heterogeneous LAB were observed in the non-starter *kimchi*. In the present study, leuconostocs were easily differentiated on the MMRS agar plates. Furthermore they showed typical physiological characteristics: small, grey or white colonies, Gram-positive, non-motile, catalase negative, and non-sporogenous (data not shown). Since *Leuconostoc* and *Pediococcus* strains have been reported to frequently occur in the fermented vegetables, especially in the early phase of *kimchi* fermentation, their levels throughout the fermentation period are of interests [14, 18]. The genus *Leuconostoc*, non-sporeforming and obligate heterofermenter, consists of eight species [29, 31]. Thus, the leuconostocs were selectively counted using its vancomycin and novobiocin resistance as described above, because there are no reference

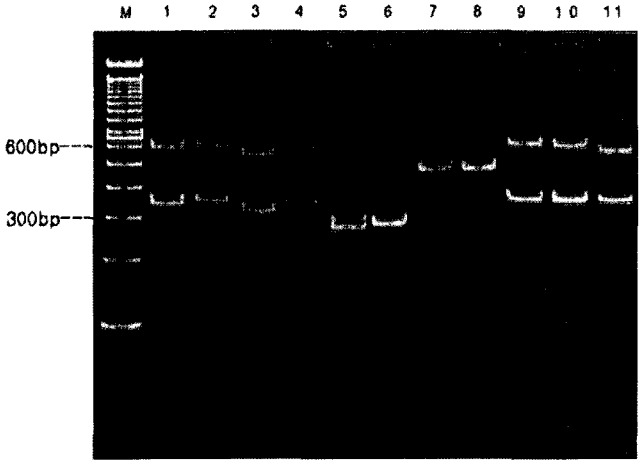

Fig. 3. *Rsa*I digestion profiles of the ITS-PCR products of reference LAB strains.
Lane 1, *Lb. plantarum* KCCM 11322; lane 2, *Lb. plantarum* ATCC 14917; lane 3, *Lb. brevis* KCCM 11904; lane 4, *Lb. brevis* ATCC 14869; lane 5, *Leuc. mesenteroides* KCCM 11324; lane 6, *Leuc. mesenteroides* ATCC 23386; lane 7, *Lc. lactis* subsp. *lactis* KCCM 32041; lane 8, *Lc. lactis* subsp. *lactis* ATCC 7962; lane 9, *P. acidilactici* KCCM 11746; lane 10, *P. pentosaceus* ATCC 23386; lane 11, *Wessella kimchii* KCCM 41287; M, molecular weight marker, 100 bp DNA ladder.

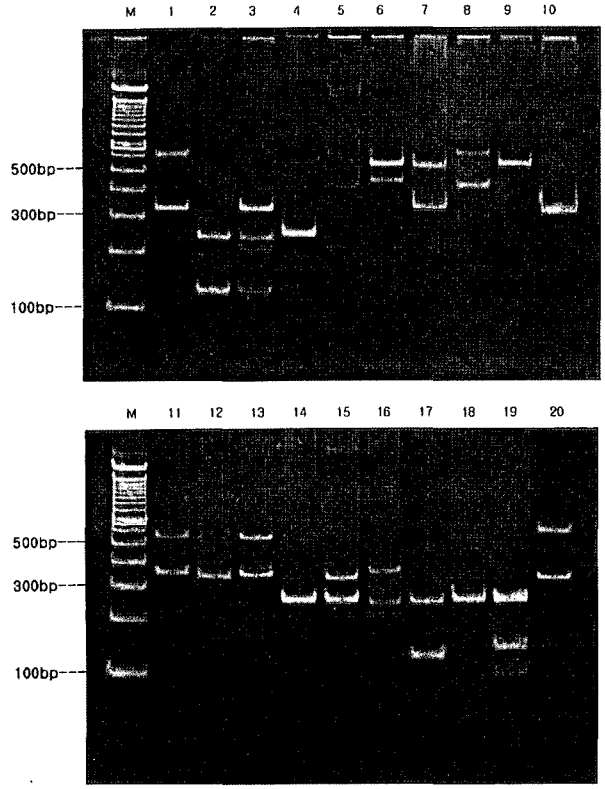

Fig. 4. *Rsa*I digestion profiles of the ITS-PCR amplicons of 20 LAB isolates from the fermenting *kimchi*.
Lane 1, YSM 1; lane 2, YSM 2; lane 3, YSM 3; lane 4, YSM 4; lane 5, YSM 5; lane 6, YSM 6; lane 7, YSM 7; lane 8, YSM 8; lane 9, YSM 9; lane 10, YSM 10; lane 11, YSM 11; lane 12, YSM 12; lane 13, YSM 13; lane 14, YSM 14; lane 15, YSM 15; lane 16, YSM 16; lane 17, YSM 17; lane 18, YSM 18; lane 19, YSM 19; lane 20, YSM 20; M, molecular weight marker, 100 bp DNA ladder.

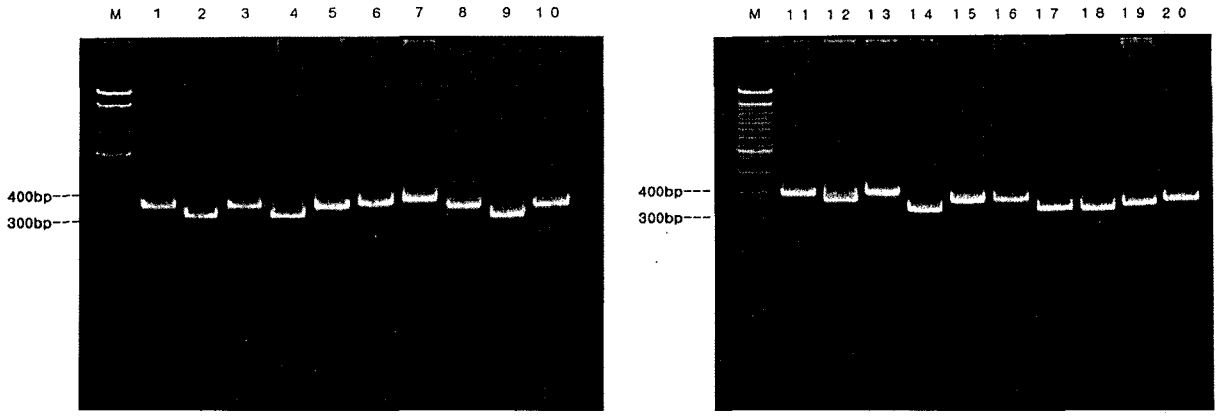

Fig. 5. 16s rDNA PCR profiles of the 20 LAB isolates from the fermenting *kimchi*.
Lane 1, YSM 1; lane 2, YSM 2; lane 3, YSM 3; lane 4, YSM 4; lane 5, YSM 5; lane 6, YSM 6; lane 7, YSM 7; lane 8, YSM 8; lane 9, YSM 9; lane 10, YSM 10; lane 11, YSM 11; lane 12, YSM 12; lane 13, YSM 13; lane 14, YSM 14; lane 15, YSM 15; lane 16, YSM 16; lane 17, YSM 17; lane 18, YSM 18; lane 19, YSM 19; lane 20, YSM 20; M, molecular weight marker, 100 bp DNA ladder.

methods available to enumerate the genus *Leuconostoc* in food products. The viable count was about 10^6 cfu/ml, which were fewer than the expected level, probably due to plating conditions including the selective pressure used. The genus *Pediococcus* is also commonly found in association with plant materials, dairy products, and foods produced by LAB, representing *P. acidilactici* (facultative heterofermenter) and *P. pentosaceus* (obligate heterofermenter). Few papers have reported pediococci as one of the dominant bacterial populations in the early phase of *kimchi* fermentation [18]. Available phenotypic characteristics to assign the natural isolates to this group were limited, and it is not easy to clearly differentiate between species of Pediococci [29]. Pediococci was recovered at a lower level (below 10^2 cfu/ml) in this study (data not shown). In other words, they are unlikely the major type in the early to mid-stage of the fermentation. The genus *Weissella* were previously classified as *Lactobacillus* or *Leuconostoc* species [8] and comprises seven members, namely *W. confusa*, *W. minor*, *W. cibaria*, *W. halotolerans*, *W. viridescens*, *W. kandleri*, *W.*

Table 4. 16S rRNA gene sequence information of the 20 *kimchi* isolates.

strains	accession No.	longest common fragment			variable region[b]		
		length	position	Ns[a]	length	position	Ns
YSM-1	AB024299	1475	1-1475	0	330	1-330	0
YSM-2	AB022925	1450	2-1451	0	298	12-309	0
YSM-3	AB024299	1467	1-1467	2	319	5-323	2
YSM-4	AF111949	1505	1-1505	0	304	1-304	0
YSM-5	AJ295989	1515	25-1518	0	332	1-332	0
YSM-6	M58810	1569	29-1597	0	326	1-326	0
YSM-7	AF295984	1507	1-1507	0	327	11-337	0
YSM-8	M58810	1569	33-1601	0	322	1-322	1
YSM-9	AF295989	1515	20-1523	0	337	1-337	0
YSM-10	AF231131	1500	8-1507	0	303	7-309	0
YSM-11	M58810	1569	29-1597	0	326	1-326	0
YSM-12	M58810	1569	30-1598	0	325	1-325	0
YSM-13	M58810	1569	29-1597	0	326	1-326	0
YSM-14	M23035	506	11-516	0	309	1-309	0
YSM-15	WCI295989	1515	21-1535	0	332	1-332	0
YSM-17	AF195784	1407	15-1421	0	309	1-309	0
YSM-18	M23035	506	11-516	0	309	1-309	0
YSM-19	M58817	1563	24-1586	0	255	1-255	1
YSM-20	AF108384	506	11-516	0	326	1-326	0

[a]Ns: undefined nucleotide sequence.
[b]Sequenced fragments in this study.

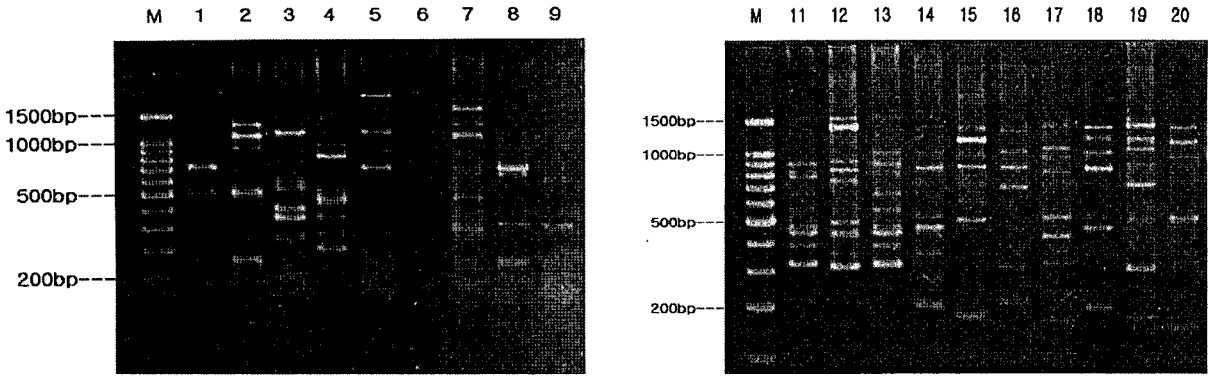

Fig. 6. RAPD-PCR profiles of the 20 LAB isolates from the fermenting *kimchi*.
Lane 1, YSM 1; lane 2, YSM 2; lane 3, YSM 3; lane 4, YSM 4; lane 5, YSM 5; lane 6, YSM 6; lane 7, YSM 7; lane 8, YSM 8; lane 9, YSM 9; lane 10, YSM 10; lane 11, YSM 11; lane 12, YSM 12; lane 13, YSM 13; lane 14, YSM 14; lane 15, YSM 15; lane 16, YSM 16; lane 17, YSM 17; lane 18, YSM 18; lane 19, YSM 19; lane 20, YSM 20; M, molecular weight marker, 100 bp DNA ladder.

paramesenteroides, and *W. hellenica*. Most recently, *W. thailandensis*, *W. soli*, *W. kimchii* (annexed to *W. cibaria*, 2004), and *W. koreensis* have additionally been published and listed in NCBI Taxonomy as a new species [11]. If bacterial successions take place during the *kimchi* fermentation [22], it is noteworthy that instead of *Leuc. mesenteroides* *Lb. brevis* and *W. kimchii/cibaria* more frequently occur at 20 in the initial to mid-stage of the fermentation. This result is in line with the previous studies in which *Lb. plantarum*, *Lb. brevis*, *Leuc. mesenteroides* subsp. *mesenteroides*, and *Lb. citreum* [6] play a critical role, particularly in the some acid-fermented vegetables such as sauerkraut, cucumber, and *kimchi*. Therefore it is highly possible that *Lb. brevis* and *W. kimchii/cibaria* are probably the predominating species in the initial to mid-stage (*kimchi* over pH 4.0) of fermenting *kimchi*. In contrast, Lee *et al.* [19] performed microbial fingerprints by a denaturing gradient gel electrophoresis (DGGE) to investigate the distribution of microorganisms in *kimchi* fermented at 10 to 20°C, and concluded that *W. confusa*, *Lb. sakei*, *Leuc. citreum*, and *Lb. curvatus* were dominant. Interestingly, *Lb. plantarum*, which has been known as a main strain in the later stage of fermented vegetables by culture-dependent approaches, were not detected by the culture-independent DGGE analysis. According to a publication [15], *Lb. sake* and *Leuc. mesenteroides* are the most predominant LAB in all types of *kimchi* in the mid-stage of fermentation at 20°C.

On the other hand, identification methods based on biochemical traits are not always reproducible and often give ambiguous results, which we experienced much difficulties in identifying the *kimchi* isolates. In the present study, data obtained by ITS-PCR method coincided merely 63% with those of API 50 CH kit at genus-level as well as not more than 42% at species-level. Given the discrepancy existing in the identification results by biochemical and molecular-typing methods such as ITS-PCR approach, rRNA gene sequencing analysis was thought to be the best alternative to confirm the respective data for precise identification. For species- and strain-specific identification, RAPD-PCR method is frequently conducted. According to Moschetti *et al.* [23], 66 strains of *Leuc. mesenteroides* subsp. *mesenteroides* were specifically differentiated using the primer 239 by RAPD analysis, and they insist that the method could be used for monitoring this microorganism in mixed microbial populations. In the present study, RAPD experiments were also carried out with a pair of RAPD primers, ED-01 and ED-02 [7], for the isolates, resulting in a variety of banding patterns ranging from 150 to 1500 bp (Fig. 6). Moreover, the banding patterns were not only reproducible, but also not in accord with the band profiles in number and size between the isolates. Bacterial diversity observed by RAPD can partially be explained by the presence or absence of extra-chromosomal plasmid DNA of *Bacillus cereus* [28, 30]. To understand the phylogenic relatedness between the isolates, a dendrogram was constructed (data not shown). We are now in a process to LAB diversity or successions further during *kimchi* fermentation by using the culture-independent approaches.

Acknowledgments

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