J. Microbiol. Biotechnol. (2004), **14**(1), 105–109

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Isolation and Identification of *Weissella kimchii* from Green Onion by Cell Protein Pattern Analysis

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Received: May 28, 2003
Accepted: September 16, 2003

Abstract This study was conducted to investigate the potential origin of *Weissella* species, which were found in ingredients of kimchi, such as salted Chinese cabbage, radish, green onion, red pepper powder, pickled shrimps, garlic, and ginger. Ten strains of *Weissella* species (*Weissella thailandensis*, *W. kimchii*, *W. koreensis*, *W. minor*, *W. halotolerans*, *W. hellenica*, *W. kandleri*, *W. confusa*, *W. viridescens*, and *W. paramesenteroides*) and lactic acid bacteria isolated from ingredients of kimchi were analyzed by SDS-PAGE of whole-cell proteins. Several strains with patterns identical to those of *Weissella kimchii* were isolated from green onion. On the basis of biochemical characteristics and 16S rDNA sequence comparisons, these strains were identified as *Weissella kimchii*, suggesting green onion as a major origin of *Weissella kimchii* found in kimchi.

Key words: *Weissella*, kimchi, ingredients, SDS-PAGE

Kimchi, a Korean traditional fermented vegetable food, is prepared by fermentation of salted Chinese cabbage with various spices and other ingredients. Chinese cabbage and radish are the main ingredients, and red pepper powder, garlic, green onion, and ginger are generally used as seasoning. Kimchi fermentation is markedly affected by environmental factors, such as temperature [19], salt concentration [15, 18], and various ingredients [5, 9]. Different microorganisms originally present in the raw materials initiate kimchi fermentation, and lactic acid bacteria then gradually dominate fermentation. Thus, studies might be needed to detect and understand the characteristics of lactic acid bacteria in ingredients for controlling and improving the quality of kimchi.

The *Weissella* species have been increasingly isolated from a variety of sources [2, 3, 14]. The phylogeny of the bacteria currently classified in the genus *Weissella* was clarified in 1993 on the basis of 16S rRNA gene sequences [6]. Before reclassification, the *Weissella* species had been classified as *Leuconostoc* and *Lactobacillus* species. Several *Weissella* species have been found in kimchi, and *Leuconostoc paramesenteroides* is one of the predominant lactic acid bacteria of kimchi fermentation [16, 19, 21] and is now named as *Weissella paramesenteroides* as per the change in the classification. *Weissella paramesenteroides* was reported to play an important role in the sensory acceptability of kimchi, since they produce organic acids, CO_2, and ethanol, in addition to lactic acid from a hexose as the hetero-fermentative lactic acid bacteria [16, 11]. Recently, two novel species, *Weissella kimchii* [4] and *Weissella koreensis* [13], were isolated from kimchi and have been suggested to belong to this genus. Thus, it is important to elucidate the origin and physiological characteristics of *Weissella* species found in kimchi. Lactic acid bacteria are one of the most diverse groups of bacteria known, and these organisms have been extensively characterized by various techniques [10, 12, 22, 23]. Since SDS-PAGE of whole-cell proteins reveals homogeneity of patterns between strains of the same species under highly standardized conditions, it has been shown to be useful to quickly identify large numbers of strains at the species or subspecies levels without performing any pre-identification experiments [24]. In this paper, *Weissella* species from ingredients of kimchi were screened using SDS-PAGE of whole-cell proteins, and the phenotypic characteristics and 16S rRNA gene sequence analysis of *Weissella kimchii* GO-1 and GO-2 strains isolated from green onion are described.

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MATERIALS AND METHODS

Bacterial Strains and Isolation of Strains

The reference *Weissella* strains were obtained from the KCTC (Korean Collection for Type Cultures) (Daejon, Korea) and cultured in an MRS broth (Difco, Detroit, U.S.A.) at 30°C. *Weissella* species used in this study are as follows: *Weissella thailandensis* KCTC 3751, *W. kimchii* KCTC 3746, *W. koreensis* KCTC 3621, *W. minor* KCTC 3604, *W. halotolerans* KCTC 3595, *W. hellenica* KCTC 3668, *W. kandleri* KCTC 3610, *W. confusa* KCTC 3499, *W. viridescens* KCTC 3504, and *W. paramesenteroides* KCTC 3531. Ingredients of kimchi including Chinese cabbage, radish, red pepper powder, green onion, garlic, pickled shrimps, and ginger were used for isolation of *Weissella* species strains. Several grams of samples were suspended in sterilized water. After stirring, dilution solution was spread onto the surface of an MRS agar plate containing 0.2% $CaCO_3$ and 0.01% bromocresol purple, and incubated anaerobically (AnaeroPack, Mitsubishi Gas Chemical Co., Japan) for 48 h at 30°C. 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.

SDS-PAGE of Whole-Cell Proteins

SDS-PAGE of whole-cell proteins was performed as described by Kim *et al.* [10]. Strains were cultured overnight at 30°C in 5 ml of MRS broth and centrifuged at 12,000 ×g for 3 min at 4°C. The pellet was washed twice with deionized water and suspended in 50 µl of 50 mM Tris-HCl buffer (pH 8.0). Fifty mg of glass beads (diameter, 425 to 600 microns; Sigma, St. Louis, U.S.A.) were added to the tubes, and the bacteria were vortexed for 5 min. The pellet was resuspended in an equal volume of 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 was added to 100 ml]. For protein denaturation, samples were heated for 5 min at 95°C. The cell debris was settled by centrifugation and the supernatants were collected for analysis by gradient (8–15%) SDS-PAGE. SDS-PAGE was performed on vertical slab gels. After electrophoresis, the gel was 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 for 2 h. The destained gels were scanned for further analysis. Grouping of the patterns for whole-cell proteins among reference strains was performed with the program NTSYS-pc (Numerical taxonomy system by using multivariate statistical programs, version 2.02j) by using UPGMA (unweighted pair group method using average linkage) cluster analysis [20].

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, and the sedimented 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 a TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The cells were lysed 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°C. An equal volume of a phenol-chloroform treatment removed the protein. 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]. The sequences of the 16f primer (forward) and 16r primer (reverse) were 5'-GAGTTTGATCCTGG-CTCAG-3' (16S rRNA gene position 9–27 of *E. coli*) and 5'-AGAAAGGAGGTGATCCAGCC-3' (16S rRNA gene position 1525–1544 of *E. coli*), respectively. The PCR products were purified using a QIAquick gel extraction kit (Qiagen, Valencia, U.S.A.), ligated into a pGEM-T easy vector (Promega, Madison, U.S.A.), 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 T3 (reverse) primers were used as the sequencing primers.

Physiological Characterization of Isolates

Sugar fermentation profiles and hydrolysis of esculin were determined using the API 50 CH strips and an API CHL medium (Bio-Merieux, Inc., France) in duplicate. Before testing, the strains were subcultured twice overnight in an MRS broth at 30°C. The tests were performed according to the manufacturer's instructions, and the results were read after incubating the strains for 2 days at 30°C.

RESULTS AND DISCUSSION

Isolation of Lactic Acid Bacteria from Ingredients of Kimchi

Various ingredients of kimchi, such as salted Chinese cabbage, radish, red pepper powder, green onion, garlic, pickled shrimps, and ginger, were used for isolation of

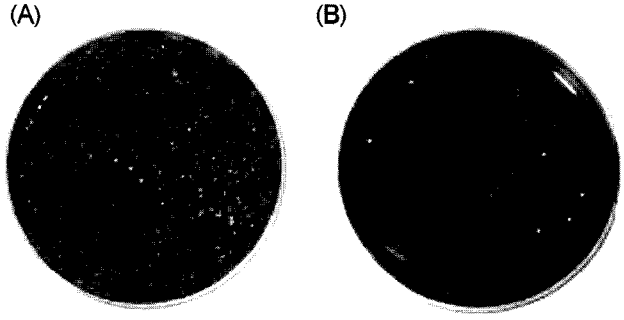

Fig. 1. Isolation of lactic acid bacteria from ginger under different culture conditions.
(A) Aerobic culture condition. (B) Anaerobic culture condition.

Weissella species strains. Figure 1 shows lactic acid bacteria isolated from ginger on an MRS agar plate containing 0.2% $CaCO_3$ and 0.01% bromocresol purple incubated aerobically (A) and anaerobically (B). Anaerobic culture condition could isolate lactic acid bacteria effectively from ingredients of kimchi. Numbers of lactic acid bacteria in ingredients varied between 3.0 and 7.0 log CFU/g, and the developed colonies were picked from higher dilution plate for analysis. Numbers of isolates selected in each ingredient ranged from about 20 to 30.

Screening of *Weissella* Species Isolates Based on Whole-Protein Patterns

The SDS-PAGE method of whole-cell proteins has proven to be extremely reliable for comparing and grouping large numbers of strains related at or below the species level [17]. Kim *et al.* [10] have earlier demonstrated the usefulness of SDS-PAGE of whole-cell proteins in identification of some lactic acid bacteria isolated from kimchi. Reference strains of *Weissella* species and the isolates could be characterized through their whole-cell protein patterns

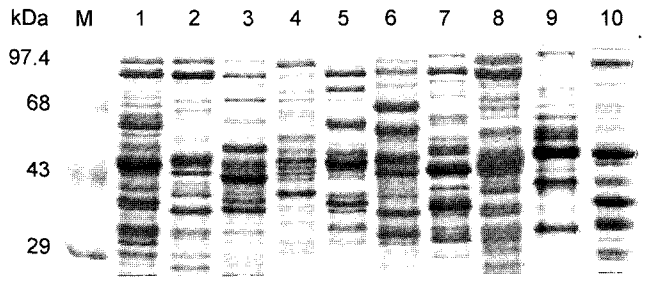

Fig. 2. SDS-PAGE profiles of whole-cell proteins of *Weissella* species.
Lanes: M, Protein molecular weight markers (kDa); 1, *Weissella thailandensis* KCTC 3751; 2, *W. kimchii* KCTC 3746; 3, *W. koreensis* KCTC 3621; 4, *W. minor* KCTC3604; 5, *W. halotolerans* KCTC3595; 6, *W. hellenica* KCTC 3668; 7, *W. kandleri* KCTC 3610; 8, *W. confusa* KCTC 3499; 9, *W. viridescens* KCTC 3504; 10, *W. paramesenteroides* KCTC 3531.

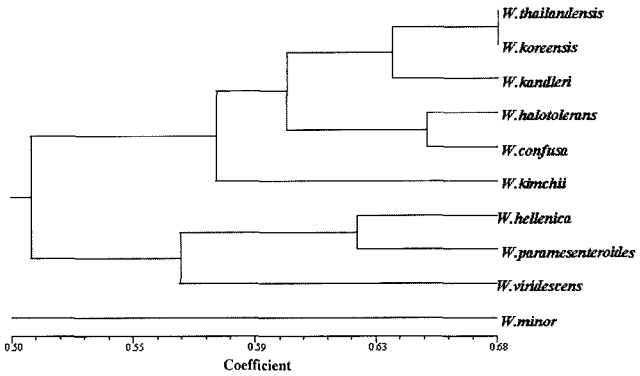

Fig. 3. Dendrogram of similarity of *Weissella* species based on SDS-PAGE of whole-cell protein pattern analysis.
Genetic similarity is analyzed by UPGMA clustering. *W. koreensis* KCTC 3621; *W. kandleri* KCTC 3610; *W. halotolerans* KCTC3595; *W. confusa* KCTC 3499; *W. kimchii* KCTC 3746; *W. hellenica* KCTC 3668; *W. paramesenteroides* KCTC 3531; *W. viridescens* KCTC 3504; *W. minor* KCTC3604.

obtained by SDS-PAGE. The protein patterns of 10 *Weissella* strains (*Weissella thailandensis* KCTC 3751, *W. kimchii* KCTC 3746, *W. koreensis* KCTC 3621, *W. minor* KCTC3604, *W. halotolerans* KCTC3595, *W. hellenica* KCTC 3668, *W. kandleri* KCTC 3610, *W. confusa* KCTC 3499, *W. viridescens* KCTC 3504, and *W. paramesenteroides* KCTC 3531) are shown in Fig. 2, and clearly and effectively discriminated other species of *Weissella*. It is difficult to identify these *Weissella* species by classical phenotypic methods [8]. The dendrogram is derived from the UPGMA clustering of correlation coefficients of the SDS-PAGE protein patterns (Fig. 3). Based on these results, *Weissella* species were screened from ingredients of kimchi mentioned above and several strains were isolated from green onion with protein patterns identical to those of *Weissella kimchii* (KCTC 3746) found in kimchi. *Weissella kimchii* was not detected from the other ingredients used in this study. A total of 90 strains isolated from four green onion samples harvested at spring and one welsh onion sample were characterized using their whole-cell protein patterns obtained by SDS-PAGE. Distribution of *Weissella kimchii* in green onion samples purchased in different regions is shown in Table 1. *Weissella kimchii*

Table 1. Distribution of *Weissella kimchii* isolated from green onion samples.

Samples	Number of isolates (%)			Purchasing region
	W. kimchii	Unknown	Total	
Green onion 1	15 (94%)	1	16	Anyang
Green onion 2	11 (85%)	13	24	Osan
Green onion 3	14 (78%)	4	18	Suwon
Green onion 4	11 (92%)	1	12	Yongin
Welsh onion	0 (0%)	20	20	Anyang

was found to be the predominant species in most of the green onion samples used. Among these isolates, GO-1 and GO-2 isolates were selected from green onion Sample 1 and Sample 3, respectively, for further examination by 16S rRNA gene sequence analysis and physiological characteristics.

Analysis of 16S rRNA Gene Sequence and Physiological Characteristics of Isolates

Two isolates, GO-1 and GO-2, were subjected to 16S rRNA gene sequence analysis and physiological characterization using sugar fermentation patterns. Thus, the 16S rRNA genes of two isolates were amplified by universal primers and sequenced, and the sequences achieved from the two isolates were used for searches in the public databases of GenBank (National Center for Biotechnology Information, Bethesda, U.S.A.). The 16S rRNA gene sequences from strains GO-1 and GO-2 showed 99% identity with that of *Weissella kimchii* (GenBank AF183558). Also, the sugar fermentation profiles of the above two isolates and *Weissella kimchii* were investigated using API 50 CH strips and API CHL medium (Bio-Merieux, Inc., France) according to the manufacturer's instructions.

Table 2. Characteristics of sugar fermentation for *Weissella kimchii*, GO-1, and GO-2 isolates.

Carbohydrates	Fermentation		
	W. kimchii KCTC 3746	GO-1	GO-2
Acid from:			
N-Acetyl-glucosamine	+	+	+
Amygdaline	+	+	+
L-Arabinose	$+^{w}$	+	+
Arbutine	+	+	+
Cellobiose	+	+	+
D-Fructose	+	+	+
Galactose	+	$+^{w}$	-
D-Glucose	+	+	+
Gluconate	$+^{w}$	$+^{w}$	$+^{w}$
Lactose	-	-	-
Maltose	+	+	+
Mannitol	-	-	-
D-Mannose	+	+	+
Melezitose	-	-	-
Melibiose	-	-	-
D-Raffinose	-	-	-
Rhamnose	-	-	-
Ribose	-	$+^{w}$	-
Salicin	+	+	+
Sorbitol	-	-	-
Sucrose	+	+	+
Trehalose	-	-	-
D-Xylose	+	+	+
Hydrolysis of esculin	+	+	+
Dextran formation	+	+	+

Symbols; +, positive; $+^{w}$, weak positive; -, negative.

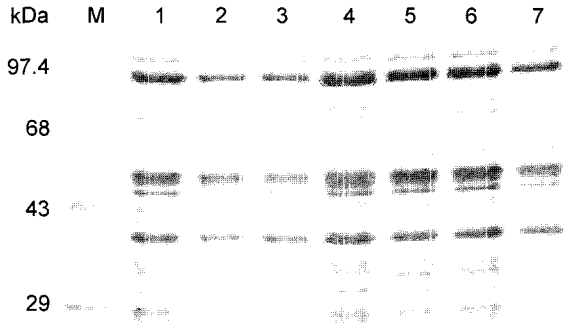

Fig. 4. SDS-PAGE profiles of whole-cell proteins of *Weissella kimchii* and isolates from green onion.
Lanes: M, Protein molecular weight markers (kDa); 1, *W. kimchii* KCTC 3746; 2-6, GO-1, GO-2, GO-3, GO-4, Go-5, and GO-6 strain isolated from green onion.

The patterns of sugar fermentation for GO-1 and GO-2 were almost similar to that for *Weissella kimchii* KCTC 3746, except for utilization of ribose and galactose (Table 2): *Weissella kimchii* and GO-1 isolates utilized galactose, whereas GO-2 isolate did not, and GO-1 isolate utilized ribose, whereas *Weissella kimchii* and GO-2 isolate did not. In general, even though both strains belonged to the same species, the sugar fermentation patterns were somewhat different [8]. On the basis of SDS-PAGE of whole-cell proteins, 16S rDNA sequence comparisons, and physiological characteristics, these two isolates were identified as *Weissella kimchii*. There has been no report that *Weissella kimchii* has previously been isolated in ingredients of kimchi. Taken together, these results suggest that green onion might be the major source of *Weissella kimchii* found in kimchi.

Acknowledgment

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (02-PJ1-PG1-CH08-0002).

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