Weissella jogaejeotgali sp. nov., isolated from jogae jeotgal, a traditional Korean fermented seafood

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Strain FOL01^T was isolated from traditionally fermented Korean jogae jeotgal (fermented clams). Phylogenetic sequence analysis of the 16S rRNA gene from FOL01^T revealed that it is closely related to *Weissella thailandensis* FS61-1^T and *Weissella paramesenteroides* ATCC 33313^T with 99.39 % and 98.50 % 16S rRNA gene sequence similarities, respectively. API and VITEK analyses showed that strain FOL01^T could be separated from its nearest phylogenetic relatives with respect to carbohydrate fermentation and antibiotic resistance. Subsequent amplified rRNA gene restriction analysis of 16S rRNA genes and *Hae*III-restriction enzyme profiling of genomic DNAs revealed different band patterns. In addition, DNA–DNA hybridization of genomic DNAs showed 63.9 % relatedness. Analysis of the composition of cellular fatty acids confirmed that strain FOL01^T differs from its close relatives and supports the proposal to assign this organism to a novel species of the genus *Weissella*. Based on these results, strain FOL01^T could be classified as a novel species of the genus *Weissella*, for which the name *Weissella jogaejeotgali* sp. nov. is proposed. The type strain is FOL01^T (=KCCM 43128^T=JCM 30589^T).

Lactic acid bacteria (LAB) have been widely used for the fermentation of a variety of dairy products, vegetables, seafoods and meats over many years (Choi *et al.*, 2002; Guan *et al.*, 2011). They are generally recognized as safe microorganisms and play an important role in the fermentation of various foods for food preservation and the improvement of desired flavours via the production of organic

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A supplementary figure is available with the online Supplementary Material.

acids (mainly lactic and acetic acid), ethanol, aroma compounds, bacteriocins or exopolysaccharides (Kim *et al.*, 1999). Previous microbiological studies of various fermented foods have reported the presence of many different kinds of LAB for fermentation (Lee *et al.*, 2003). Species of the genera *Lactococcus* and *Lactobacillus* in fermented foods have been studied microbiologically over a long period; however, study of the genera *Leuconostoc* and *Weissella* has only begun recently. The genus *Weissella* is taxonomically located in the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* and family *Leuconostocaceae*, indicating that *Leuconostoc* and *Weissella* are closely related in their taxonomical classification (Collins *et al.*, 1993).

Members of the genus *Weissella* include facultatively anaerobic, heterofermentative, irregularly rod-shaped, Gram-positive bacteria (Collins *et al.*, 1993). Although the genus *Weissella* has been proposed as a probiotic bacterium due to frequent detection of strains in various

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Abbreviations: ARDRA, amplified rRNA gene restriction analysis; DDH, DNA–DNA hybridization; FE-SEM, field emission scanning electron microscopy; LAB, lactic acid bacteria; RE, restriction enzyme.

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene and *pheS* sequences of strain FOL01^T are KP027016 and KR822109, respectively.

fermented foods (Björkroth et al., 2002; De Bruyne et al., 2008, 2010; Lee et al., 2002; Padonou et al., 2010; Tohno et al., 2013; Vela et al., 2011), recent studies have suggested that this genus could be an opportunistic pathogen (Liu et al., 2009; Walter et al., 2001). At the time of writing there are 21 species with validly published names (Fusco et al., 2015; Parte, 2014). Recently, the composition of the microbiota in eight major Korean traditional fermented foods has been studied and it was found that species of the genus Weissella were present in six of these (75 %): kkangdugi (14 % of the strains of species of Weissella present), mugeungi (2.4 %), saeu-jeotgal (1.0 %), jogae jeotgal (55.2 %), myeolchi-jeotgal (4.4 %) and meju (2.7 %) (Lee et al., 2015). The composition of the microbiota and further molecular identification revealed a few strains that were candidates for novel species of the genus Weissella (data not shown).

In this study, strain FOL01^T was isolated from jogae jeotgal and characterized. In addition, the strain was examined using several biochemical and molecular methods to ascertain whether it represents a novel species of the genus *Weissella*. The species *Weissella koreensis* (Lee *et al.*, 2002, 2010) was the first species of the genus *Weissella* isolated from Korean traditional fermented foods, and strain FOL01^T is identified here as a second species. The novel species described here also has the potential to be used in functional food applications as a novel probiotic strain.

Traditional fermented jogae jeotgal (clam-fermented seafood) was purchased at Sorae Port, Incheon, South Korea. A 25 g sample of the jogae jeotgal was mixed with 225 ml sterilized peptone water [0.1 % (w/v) final concentration]. The mixture was transferred into sterilized strainer bags (Seward) and homogenized with the stomacher (Seward). After stomaching, the supernatant was spread on agar plates of selected media and bacterial strains were isolated from the sample according to the following method: 1 ml supernatant from the jogae jeotgal sample was serially diluted 10-fold in sterilized 0.1 % peptone water (final concentration) and spread on 1.8 % agar plates made with four different types of media: Lactobacilli de Man-Rogosa-Sharpe (MRS; Difco) medium, Luria-Bertani (LB; Difco) medium, M17 (Difco) medium supplemented with 0.5 % D-glucose and Nutrient medium (Difco). Colonies were randomly selected and cultured in the same broth medium as the agar plate medium. Genomic DNA of each selected strain was prepared with Chelex 100 solution (Bio-Rad) using a protocol previously developed (Lu et al., 2006; Walsh et al., 2013). The 16S rRNA gene was PCR-amplified with the universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Choi et al., 2013) and the PCR product was purified with an AxyPrep DNA Gel Extraction kit (Axygen). The purified PCR product was sequenced in both directions, by Macrogen (Korea), using a capillary sequencing method. This 16S rRNA sequence was analysed using BLASTN (Altschul et al., 1990) and the Nucleotide Similarity Search program

(http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). Phylogenetic analysis was performed using MEGA 6.0 software (Tamura et al., 2013) with the neighbour-joining, maximum-parsimony and maximum-likelihood methods by using bootstrap values based on 1000 replications. Genetic distances were calculated by Kimura's twoparameter model (Kimura, 1980). Molecular identification of isolated bacterial strains from jogae jeotgal revealed that a few strains did not show 100 % 16S rRNA gene sequence similarities with other strains. Among them, strain FOL01^T showed a close relationship with Weissella thailandensis FS61-1^T, W. thailandensis FS45-1^T and Weissella paramesenteroides ATCC 33313^T, with 99.39 %, 98.43 % and 98.50 % 16S rRNA gene sequence similarities, respectively. Subsequent phylogenetic analysis of the 16S rRNA gene sequences of strain FOL01^T and strains of other closely related species of the genus Weissella supported this close phylogenetic relationship (Fig. 1a). While strain FOL01^T had the closest relationship with strains of the species W. thailandensis, phylogenetic analysis showed that FOL01^T differs from this species and represents a novel species of the genus Weissella. To verify this result, additional phylogenetic tree analyses were conducted using the maximum-parsimony and maximum-likelihood methods (Fig. S1, available in the online Supplementary Material). Furthermore, analysis of the pheS genes of strain FOL01^T and strains of other species of the genus Weissella was performed. The pheS gene of strain FOL01^T was PCR-amplified using the primers, pheS-21-F (5'-CAY-CCNGCHCGYGAYATGC-3') and pheS-23-R (5'-GGRT-GRACCATVCCNGCHCC-3') (Naser et al., 2005) and sequenced in both directions with these PCR primers using the capillary sequencing method (Macrogen). The sequences of pheS genes from other species of the genus Weissella were obtained from the GenBank database. The phylogenetic tree of pheS genes reconstructed using the neighbour-joining method indicated that FOL01^T was most closely related to W. thailandensis and W. paramesenteroides, but differed slightly to them (Fig. 1b). To further differentiate strain FOL01^T from closely related species of the genus Weissella (W. thailandensis, W. paramesenteroides and Weissella hellenica) in the phylogenetic tree, comparative sequence alignment analyses of their pheS genes were performed. The pheS gene sequence similarities between strain FOL01^T and the closely related species were 97.4 % (W. thailandensis), 85.1 % (W. paramesenteroides) and 84.4 % (W. hellenica), respectively, suggesting that strain FOL01^T is different from these species. Similar pheS gene sequence similarities have been previously reported as evidence to differentiate a novel species of the genus Weissella (Weissella uvarum) from other closely related species, supporting this (Nisiotou et al., 2014). Therefore, this comparative analyses of pheS genes supported strain FOL01^T representing a novel species of the genus Weissella.

The morphology of the cells was determined using field emission scanning electron microscopy (FE-SEM). For

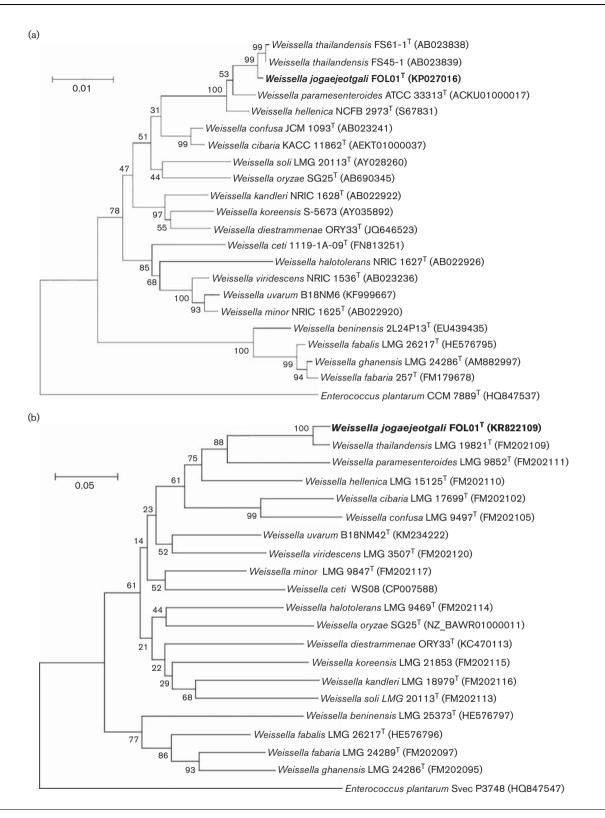


Fig. 1. Phylogenetic relationship of strain FOL01^T among other species of the genus *Weissella* using the neighbour-joining method based on 16S rRNA gene sequences (a) and on *pheS* gene sequences (b). *Enterococcus plantarum* was included as an outgroup. Bootstrap values (%) derived from 1000 replicates are given at branching points. Bars, 0.01 (a) and 0.05 (b) substitutions per nucleotide position.

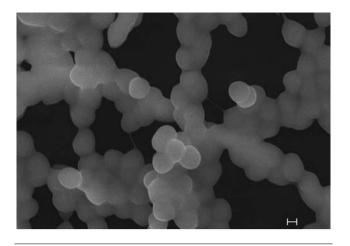


Fig. 2. Scanning electron micrographs of strain FOL01^T. The cells were freeze-dried and the cell powder was used for FE-SEM observations. Bar, 300 nm.

preparation of the samples, FOL01^T cells were incubated for 24 h in MRS broth at 37 °C and freeze-drying was performed using a miniVac (LaboGene). Powdered cells were spread onto a carbon disc and coated with platinum. Samples were observed using a LEO SUPRA 55 (Carl Zeiss) at an acceleration voltage of 10 kV. Motility was observed using a phase-contrast microscope (Stereoscan 440, Leica Cambridge). Bacterial cells showed the typical characteristics of species of the genus *Weissella*, which included being irregularly shaped rods with no flagella and a size of $0.4-0.6 \times 0.6-0.8 \ \mu m$ (Fig. 2).

In addition, biological characterization of strain FOL01^T was performed, including gas production with inverted Durham tubes from glucose, ammonia production and dextran production. Strain FOL01^T produced gas from glucose, ammonia and dextran. Gas was produced from glucose in MRS medium with a Durham tube (Holzapfel & Gerber, 1983). However, ammonia was not produced from arginine (Table 1). Strain FOL01^T was incubated on an modified MRS agar plate containing sucrose, but not glucose [5 % (w/v) final concentration] and no extracellular polysaccharide was produced, indicating that strain FOL01^T does not produce dextran from sucrose. The lactic acid configuration was determined using a DL-lactate test kit (Boehringer Mannheim/R-Biopharm) (Table 1).

For ribotyping and confirmation of differences among selected and reference strains, amplified rRNA gene restriction analysis (ARDRA) of 16S rRNA genes was performed (Choi *et al.*, 2013). For this, type strains of three closely related species (*W. paramesenteroides* ATCC 33313^T, *W. thailandensis* FS61-1^T and *W. hellenica* ATCC 51523^T) and *Weissella cibaria* LMG 17699^T for comparative analysis, were purchased from the Korean Collection for Type Cultures (KCTC). Amplification of 16S rRNA genes was performed with the 27F/1492R 16S rRNA universal primer set as previously described for PCR of the 16S rRNA

Table 1. Characteristics that differentiate strain FOL01^T from three closely related species of the genus *Weissella*

Strains: 1, FOL01^T (data from this study); 2, *W. thailandensis* FS61-1^T; 3, *W. paramesenteroides* ATCC 33313^T; 4, *W. hellenica* ATCC 51523^T. Data partially adapted from Collins *et al.* (1993), Tanasupawat *et al.* (2000) and Snauwaert *et al.* (2013). ND, No data available.

Characteristic	1	2	3	4
Acid production from:				
Arabinose	+	+	+	+
Cellobiose	+	_	_	_
Fructose	+	+	+	+
Galactose	_	+	+	+
Lactose	_	_	_	—
Maltose	+	+	+	+
Melibiose	_	+	+	_
Raffinose	_	+	_	—
Ribose	_	+	+	—
Salicin	_	_	_	_
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Xylose	+	_	_	_
Hydrolysis of aesculin	+	_	+	ND
NH ₃ from arginine	_	_	_	—
Lactic acid configuration	D	D	D	D
DNA G+C content (mol%)	39.6	41.2	40.4	39.4
16S rRNA gene sequence similarity with strain FOL01 ^T	100	99.39	98.50	92.74

genes and 16S rRNA gene sequence analysis. After purification of the PCR products from strain FOL01^T and the selected reference strains, the products were doubledigested with a mixture of *Hae*III (New England Biolabs) and HhaI (New England Biolabs) at 37 °C for 24 h. Their restriction fragment patterns were compared and analysed using 2 % (w/v) agarose gel electrophoresis. While strain FOL01^T showed different ARDRA band patterns from those of type strains of two of the closely related species as well as from a strain of W. cibaria, strain FOL01^T had the same ARDRA band pattern as W. thailandensis FS61-1^T (Fig. 3). To confirm the differences in 16S rRNA gene sequences between these strains, a 1417 bp sequence of the 16S rRNA gene of W. thailandensis FS61-1^T (Gen-Bank accession no. AB023838) and 1336 bp of strain FOL01^T (GenBank accession no. KP027016) were compared using the Blast2seq program. Comparative 16S rRNA gene sequence analysis showed that 25 nt differed among 1335 nt (data not shown), suggesting that strain FOL01^T is different to the four closely related species and may belong to a novel species of the genus Weissella.

Restriction enzyme (RE) profiling and DNA–DNA hybridization (DDH) using Southern blot hybridization were carried out to evaluate the DNA–DNA relatedness values between strain FOL01^T and *W. thailandensis* FS61-1^T, as this strain had the highest similarity score (99.39 %) in

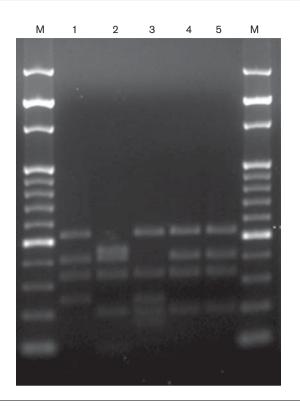
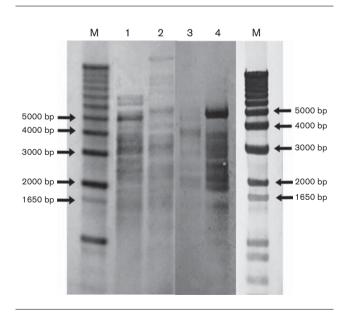


Fig. 3. ARDRA analysis of strain FOL01^T and closely related species using *Hae*III and *Hha*I. 1, *W. cibaria* LMG 17699^T; 2, *W. hellenica* ATCC 51523^T; 3, *W. paramesenteroides* ATCC 33313^T; 4, *W. thailandensis* FS61-1^T; 5, Strain FOL01^T. M, 100 bp DNA ladder marker (MGmed)

16S rRNA gene sequence analysis. For these analyses, genomic DNA isolation was performed with the G-spin Genomic DNA Extraction kit for Bacteria (iNtRON) according to the manufacturer's protocol. The extracted genomic DNA of strain FOL01^T was digested using HaeIII at 37 °C for 12 h. The probe for RE profiling and DDH was then prepared using the HaeIII-digested genomic DNA and labelled using the DIG High Prime DNA labelling kit (Roche) according to the manufacturer's instructions. For preparation of the probe, 15 µl solution containing 1 µg HaeIII-digested genomic DNA was heated in boiled water for 10 min and quickly chilled in ice for denaturation; then, 2 μ l hexanucleotide mix (10 ×), 2 μ l dNTP labelling mix and 1 µl Klenow fragment enzyme (labelling grade) included in the kit (Roche) were added to the denatured genomic DNA. After the mixture was incubated at 37 °C overnight, the reaction was stopped by the addition of 2 µl 0.2 M EDTA (pH 8.0) with subsequent heating to 65 °C for 10 min. The prepared probe was stored at -20 °C. For RE profiling analysis, the genomic DNAs from both strains were digested with HaeIII at 37 °C for 12 h and the DNA fragments were separated by electrophoresis on a 1 % (w/v) agarose gel. After gel separation of the DNA bands, they were transferred onto Zeta-Probe blotting membranes (Bio-Rad) and fixed by UV

radiation. For detection of homologous DNA bands between two strains, Southern blot analysis was conducted using the probe of strain FOL01^T and DIG High Prime DNA labelling kit according to the manufacturer's standard protocol (Roche). DDH was carried out by the slot-blot hybridization method (Lee et al., 2011). The purified genomic DNA was diluted to 0, 8, 16, 24, 32, 40, 60, 80, 120 and 160 ng and then each diluted genomic DNA sample was slot-blotted onto Zeta-Probe blotting membranes (Bio-Rad) using a Bio-Dot SF microfiltration system in triplicate. Similarly to RE profiling, Southern blotting was conducted using the same kit. Additionally, hybridization signals were captured and analysed using a Bio-Rad Quantity One (v. 4.62). Signals produced by hybridization of the probe to homologous target DNA were regarded as 100 %, and signal intensities obtained from selfhybridization with the series of dilutions were used to calculate the DNA-DNA relatedness values between strains FOL01^T and FS61-1^T. RE profiling results showed that W. thailandensis FS61-1^T had larger DNA fragments than strain FOL01^T, suggesting that they differ at the genomic level (Fig. 4). In addition, DNA-DNA hybridization analysis revealed that the DNA-DNA relatedness value between strain FOL01^T and W. thailandensis FS61-1^T was 63.9 % + 4.2 %, suggesting that strain FOL01^T can be accepted as a novel species (Wayne et al., 1987).

To further differentiate strain FOL01^T from other closely related species of the genus *Weissella*, the VITEK system [bioMérieux; (Putnam *et al.*, 1997)] with a GPI card for Gram-positive bacteria and API50 CHL strips (bioMérieux) were used, according to the manufacturer's directions. VITEK analysis of strain FOL01^T, *W. thailandensis*



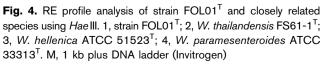


Table 2. Comparison of the cellular fatty acids compositions of					
strain FOL01 ^T , W. thailandensis FS61-1 ^T and W. parames-					
enteroides ATCC 33313 ^T					

Strains: 1, FOL01^T; 2, *W. thailandensis* FS61-1^T; 3, *W. paramesenteroides* ATCC 33313^T. –, Not detected.

Fatty acid	1	2	3
Straight-chain saturated	l		
C _{14:0}	4.92	4.11	4.66
C _{16:0}	30.91	32.52	29.25
C _{18:0}	0.84	1.87	1.91
Branched saturated			
iso-C _{19:0}	0.81	0.61	1.40
Unsaturated			
cyclo-C _{17:0}	3.95	0.48	_
$C_{18:1}\omega 9c$	8.03	16.9	32.61
$cyclo-C_{19:0}\omega 8c$	20.6	12.26	_
Summed features*			
3	5.93	4.37	2.45
7	22.02	23.42	24.35
8	2.61	3.40	3.38

*Summed features are two or three fatty acids that could not be separated using the Microbial Identification System. Summed feature 3 comprises $C_{16:1}\omega7c/C_{16:1}\omega6c$; summed feature 7 comprises $C_{19:0}$ cyclo $\omega10c/19\omega6$; summed feature 8 comprises $C_{18:1}\omega7c/C_{18:1}\omega6c$. Bold numbers indicate that the composition of cellular fatty acids is >10 %.

FS61-1^T and W. paramesenteroides ATCC 33313^T revealed that only strain FOL01^T produced β -galactosidase, α -galactosidase and a-glucosidase. However, only W. thailandensis FS61-1^T was resistant to antibiotics, including polymyxin B and bacitracin. In addition, the results for W. paramesenteroides ATCC 33313^T were similar to those of strain FOL01^T except for its sensitivity to novobiocin (while W. paramesenteroides is susceptible, strain FOL01^T is resistant to novobiocin), further suggesting that strain FOL01^T belongs to a novel species of the genus Weissella. Furthermore, API analysis of strain FOL01^T and type strains of three closely related species (W. thailandensis FS61-1^T, W. paramesenteroides ATCC 33313^T and W. hellenica ATCC 51523^T) showed that only strain FOL01^T could ferment cellobiose and xylose, but it could not ferment galactose. In addition, the fermentation activities of strain FOL01^T and the other strains were different in their fermentation of melibiose, ribose, raffinose and aesculin, suggesting that strain FOL01^T is markedly different from the other three closely related species (Table 1). On the basis of the comparative analyses of the biological characteristics of FOL01^T and three other closely related species FOL01^T belongs to a novel species of the genus Weissella.

Recently, composition analysis of cellular fatty acids has been widely used for bacterial identification. For the analysis of cellular fatty acids, cells of strain FOL01^T and the reference strains were harvested at the exponential growth stage on MRS agar for 2 days at 37 °C. The cellular

fatty acids were converted to the corresponding methyl esters, extracted according to the protocol of the Sherlock Microbial Identification System (MIDI) with TSBA database version 6.1, analysed by gas chromatography (Hewlett Packard 6890) and identified using Microbial Identification software package (Sasser, 1990). For this analysis, three strains of species of the genus Weissella were selected, including W. thailandensis FS61-1^T, W. paramesenteroides ATCC 33313^T and strain FOL01^T. The results revealed that their major cellular fatty acids were C_{16+0} and C_{19+0} cyclow8c. However, strain FOL01^T had a markedly different composition of $C_{17:0}$ cyclo, $C_{18:1}\omega 9c$ and $C_{19:0}$ cyclo $\omega 8c$ (Table 2), suggesting that strain FOL01^T is different from the two closely related species, W. thailandensis FS61-1^T and W. paramesenteroides ATCC 33313^T. Strain FOL01^T may, therefore, belong to a novel species of the genus Weissella.

To determine the G + C content of the chromosomal DNA, genomic DNA was extracted from strain FOL01^T using the G-spin Genomic DNA Extraction kit for Bacteria (iNtRON) and measured by the fluorometric method (Gonzalez & Saiz-Jimenez, 2002) using SYBR Green I and a real-time PCR thermocycler (Bio-Rad). The DNA G + C content of strain FOL01^T was 39.6 mol%, indicating that it fits with the standard DNA G + C content range of the genus *Weissella* (37–40 mol%; Padonou *et al.*, 2010).

Strain FOL01^T was tested using biochemical and molecular experiments to confirm whether this strain was a novel species. API and VITEK analyses revealed that strain FOL01^T had unique characteristics related to carbohydrate fermentation and antibiotic resistance. In addition, ARDRA and DNA G+C content analyses showed that strain FOL01^T had unique ribotype patterns and its DNA G+C content was located in the range of the general G+C content for the genus Weissella. RE profiling and DDH experiments also showed that strain FOL01^T had different band patterns from the other closely related species of the genus Weissella. Furthermore, analysis of the cellular composition of fatty acids revealed that the cellular fatty acid composition of strain FOL01^T was distinct from that of the closely related species, W. thailandensis FS61-1^T and W. paramesenteroides ATCC 33313^T, also supporting that this strain may belong to a novel species of the genus Weissella. These results provide evidence that strain FOL01^T represents a novel species of the genus Weissella, for which the name Weissella jogaejeotgali sp. nov. is proposed.

Description of Weissella jogaejeotgali sp. nov.

Weissella jogaejeotgali (jo.gae.je.ot.ga'li. N.L. gen. n. jogaejeotgali of jogae jeotgal, from which the type strain was isolated).

Cells are Gram-stain-positive, non-motile, irregularly rodshaped or coccoid (0.4–0.6 × 0.6–0.8 µm) occurring singly or in pairs or chains. After 24 h of facultative aerobic growth on MRS plates at 37 °C, colonies were 1 mm in diameter, creamy white, smooth, circular and swollen. Grows at 15-37 °C, but not at 4-10 or 42 °C. Grows with 0-6.5% (w/v) NaCl, but not with 8% (w/v) NaCl. No growth is observed with 8 % (w/v) NaCl. Gas is produced from glucose, but ammonia is not produced from arginine. Dextran is not formed from sucrose. D-Lactic acid is produced from glucose. Acid is produced from Larabinose, D-xylose, D-glucose, D-fructose, D-mannose, mannitol, methyl a-D-glucoside, arbutin, aesculin, cellobiose, maltose, sucrose and trehalose, but not from glycerol, erythritol, D-arabinose, ribose, L-xylose, adonitol, methyl β -xyloside, galactose, L-sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α -D-mannoside, N-acetylglucosamine, amygdalin, salicin, lactose, melezitose, inulin, melezitose, raffinose, starch, glycogen, xylitol, β -gentiobiose, turanose, D-lyxose, D-tagatose, DL-fucose, DL-arabitol, gluconate, 2ketogluconate or 5-ketogluconate. Positive for α-galactosidase, β -galactosidase and α -glucosidase activities. Resistant to novobiocin and optochin. The major components of cellular fatty acids include $C_{16.0}$, cyclo- $C_{19.0}\omega 8c$ and summed feature 7 containing cyclo- $C_{19.0}\omega 10c/19\omega 6$.

The type strain FOL01^T (=KCCM 43128^{T} =JCM 30580^{T}) was isolated from jogae jeotgal, a traditional fermented shrimp in Incheon, South Korea. The genomic DNA G+C content of the type strain is 39.6 mol%.

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