Alishewanella jeotgali sp. nov., isolated from traditional fermented food, and emended description of the genus *Alishewanella*

Min-Soo Kim,[1,2] Seong Woon Roh,[1,2] Young-Do Nam,[1,2] Ho-Won Chang,[1] Kyoung-Ho Kim,[1] Mi-Ja Jung,[1] Jung-Hye Choi,[1] Eun-Jin Park[1] and Jin-Woo Bae[1,2]

Correspondence
Jin-Woo Bae
baejw@khu.ac.kr

[1]Department of Life and Nanopharmaceutical Sciences and Department of Biology, Kyung Hee University, Seoul 130-701, Republic of Korea

[2]University of Science and Technology, Biological Resources Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Republic of Korea

A novel Gram-negative and facultative anaerobic strain, designated MS1^T, was isolated from gajami sikhae, a traditional fermented food in Korea made from flatfish. Strain MS1^T was motile, rod-shaped and oxidase- and catalase-positive, and required 1–2 % (w/v) NaCl for growth. Growth occurred at temperatures ranging from 4 to 40 °C and the pH range for optimal growth was pH 6.5–9.0. Strain MS1^T was capable of reducing trimethylamine oxide, nitrate and thiosulfate. Phylogenetic analysis placed strain MS1^T within the genus *Alishewanella*. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain MS1^T was related closely to *Alishewanella aestuarii* B11^T (98.67 % similarity) and *Alishewanella fetalis* CCUG 30811^T (98.04 % similarity). However, DNA–DNA reassociation experiments between strain MS1^T and reference strains showed relatedness values <70 % (42.6 and 14.8 % with *A. aestuarii* B11^T and *A. fetalis* CCUG 30811^T, respectively). Genotypic, physiological and biochemical analyses allowed the differentiation of strain MS1^T from type strains of species belonging to the genus *Alishewanella*. Therefore, we propose that strain MS1^T (=KCTC 22429^T =JCM 15561^T) is assigned to a novel species, *Alishewanella jeotgali* sp. nov.

The genus *Alishewanella* is one of the major branches of the family *Alteromonadaceae* and was first proposed by Fonnesbech Vogel *et al.* (2000) to accommodate *Alishewanella fetalis*, isolated from an autopsy of a human fetus in 1992. Recently, another species, *Alishewanella aestuarii*, from a marine environment, has been reported (Roh *et al.*, 2009). Here, we describe a third novel species that belongs to the genus *Alishewanella*, isolated from a traditional fermented food in Korea.

Strain MS1^T was isolated from gajami sikhae (jeotgal), which is a traditional fermented food in Korea. A sample of gajami sikhae was diluted 10^{-6}-fold with PBS and cultured on R2A agar (Difco). The 16S rRNA gene sequence was amplified by the colony PCR method with PCR Pre-Mix (Intron Biotechnology) and two universal primers for bacteria (Baker *et al.*, 2003). The PCR products were sequenced with a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) after purification according to the manufacturer's instructions. The reaction mixtures were analysed with an automated system (ABI Prism 3730XL DNA Analyzer; Applied Biosystems) and the 16S rRNA gene sequences were assembled with SEQMAN (DNASTAR). Comparison of the 16S rRNA gene sequence of strain MS1^T with those deposited in GenBank revealed that strain MS1^T belongs to the genus *Alishewanella* in the phylum *Proteobacteria* and that its 16S rRNA gene sequence shares 98.67 and 98.04 % similarity with the sequences of *A. aestuarii* B11^T and *A. fetalis* CCUG 30811^T, respectively. The 16S rRNA gene sequence of the isolate was aligned with those of reference strains by using the multiple-sequence alignment program CLUSTAL X (1.83) (Thompson *et al.*, 1997). The phylogenetic relationships between strain MS1^T and the representative type strains of *Alishewanella* species were defined by MEGA4 (Tamura *et al.*, 2007). In the randomly generated neighbour-joining, maximum-parsimony and maximum-likelihood consensus trees constructed from 1000, 1000 and 300 bootstrap replicates, respectively (Felsenstein, 1981; Kluge & Farris, 1969; Saitou & Nei, 1987), strain MS1^T formed a monophyletic clade that was separate

Abbreviation: TMAO, trimethylamine oxide.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MS1^T is EU817498.

from *A. aestuarii* B11^T. The phylogenetic relationships between strain MS1^T and its relatives are shown in Fig. 1.

In order to determine the extent of genetic relatedness, DNA–DNA hybridization experiments (Ezaki *et al.*, 1989) were performed with modifications (Hirayama *et al.*, 1996), with probe DNA labelled with photobiotin under a 400 W mercury-vapour lamp and boiling (100 °C) for 30 min. The genomic DNA reassociation values of strain MS1^T with *A. fetalis* CCUG 30811^T and *A. aestuarii* B11^T were 14.8 and 42.6 %, respectively. Bacterial strains with 16S rRNA gene sequence similarities of 98 % or lower are considered to be different species unless the extent of DNA–DNA relatedness is >70 % (Stackebrandt & Goebel, 1994).

Media supporting the growth of strain MS1^T included tryptic soy agar, marine agar, nutrient agar, R2A agar, Luria agar (LA) (all from BBL/Difco) and 5 % sheep blood agar. Optimal growth was seen with LA. To determine the optimal ranges of temperature, NaCl concentration and pH for growth, the strain was incubated on LA for 48 h at temperatures of 0, 4, 10, 15, 25, 30, 37, 40 and 45 °C, with NaCl concentrations ranging from 0 to 14 % (w/v) and with pH ranging from 4.0 to 13.0 (with increments of 0.5 pH units). Gram staining was performed according to the method described by Gram (1884). Cell shape, motility and size were determined by light microscopy (ECLIPSE 80*i*; Nikon) and transmission electron microscopy (JEM-1010; JEOL). In addition, a flagellum-staining method (Heimbrook *et al.*, 1989) clarified the presence of a single, polar flagellum. The motility of the isolate was observed directly by examination of the flagellum after growth on motility test medium (BBL) supplemented with 1 % (w/v) NaCl. Catalase activity was examined by determining bubble production in 3 % (v/v) hydrogen peroxide solution. Oxidase activity was investigated by using 1 % (w/v) *p*-tetramethyl phenylenediamine (bioMérieux). Enzyme activities, eight biochemical characters and utilization of sole carbon sources were determined with the API ZYM, API 20NE and API 50CH systems (bioMérieux), according to the manufacturer's instructions, with incubation at 30 °C for 24 h for the biochemical tests and for 72 h for the utilization tests. Reduction of trimethylamine oxide (TMAO), nitrite, ferric iron, thiosulfate and sulfite was defined by observing visible growth at 30 °C for 7 days under anaerobic conditions in modified LA medium [l^{-1}: 5.0 g yeast extract, 10.0 g tryptone, 20.0 g NaCl, 0.5 g $MgCl_2.6H_2O$, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.015 g $CaCl_2.2H_2O$, 1 ml trace element solution A (Loffler *et al.*, 1996), 0.137 g sodium thioglycolate, 2.52 g $NaHCO_3$, 15.0 g agar, 1 ml filter-sterilized vitamin solution (Wolin *et al.*, 1963); final pH adjusted to pH 7.0–7.5, autoclaved and cooled to below 60 °C]. The medium was poured and stored in an oxygen-free N_2/CO_2 (80 : 20) anaerobic chamber for at least 1 day. The final concentrations of various electron acceptors were 20 mM each. The G+C content was estimated by a fluorimetric method employing SYBR green and a real-time PCR thermocycler (Gonzalez & Saiz-Jimenez, 2002). The method of phenol/chloroform extraction followed by ethanol precipitation was used to extract template chromosomal DNA (Sambrook *et al.*, 1989). The genomic DNA of *Escherichia coli* KCTC 2441^T was tested at the same time as the calibration reference. The cultural, physiological and biochemical characteristics of strain MS1^T are shown in Table 1 and Fig. 1.

To determine the cellular fatty acid composition of strain MS1^T, *A. fetalis* CCUG 30811^T and *A. aestuarii* B11^T, cultures were prepared on 5 % sheep blood agar at 30 °C for 48 h before harvesting. The cellular fatty acids were extracted as described by MIDI (1999), analysed by gas chromatography (HP 6890; Hewlett Packard) and identified by using the Microbial Identification software package (Sasser, 1990). The cellular fatty acid profiles of the three isolates are presented in Table 2.

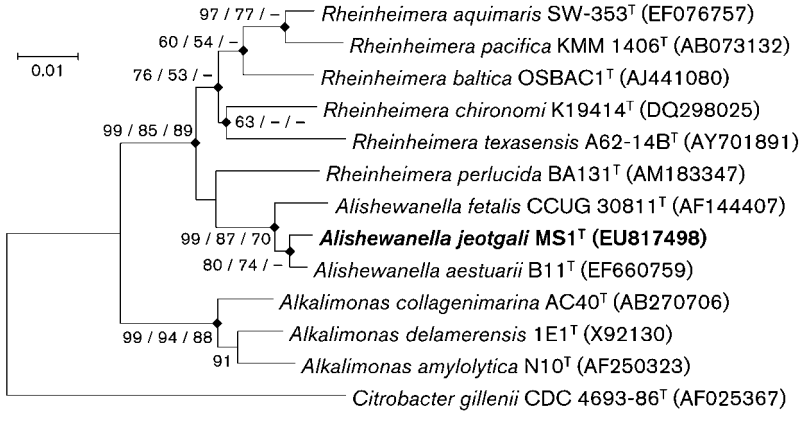

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, constructed with the neighbour-joining, maximum-parsimony and maximum-likelihood methods and based on the neighbour-joining consensus tree. Percentages at nodes (>50 %) are levels of bootstrap support based on the neighbour-joining/maximum-parsimony/maximum-likelihood methods as percentages of 1000/1000/300 replicates, respectively. Filled diamonds indicate generic branches that were present in phylogenetic trees generated by the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms. *Citrobacter gillenii* CDC 4693-86^T was used as the outgroup. Bar, 0.01 substitutions per nucleotide position.

Table 1. Comparison of the characteristics of strain MS1^T and its closest phylogenetic neighbours

Strains: 1, *Alishewanella jeotgali* sp. nov. MS1^T; 2, *A. fetalis* CCUG 30811^T (data from Fonnesbech Vogel *et al.*, 2000); 3, *A. aestuarii* B11^T (Roh *et al.*, 2009). All of the biochemical data are from this study. All strains were positive for hydrolysis of gelatin, assimilation of maltose, sucrose and starch and reduction of TMAO, thiosulphate and nitrate. All strains were negative for production of indole, fermentation of D-glucose, hydrolysis of L-arginine, urea and *p*-nitrophenyl *β*-D-galactopyranoside and reduction of sulfite, nitrite and ferric iron. +, Positive; −, negative; W, weak.

Characteristic	1	2	3
Isolation source	Gajami sikhae (jeotgal)	Human fetus	Tidal flat sediment
Motility	+	−	+
Growth at/in:			
4 °C	W	−	−
10 °C	+	−	−
40 °C	−	+	+
0 % NaCl	−	−	+
6 % NaCl	−	+	−
8 % NaCl	−	+	−
Hydrolysis of aesculin	+	−	−
Assimilation of:			
Aesculin	+	−	−
D-Fructose	−	+	+
D-Glucose	+	−	−
Glycerol	−	+	−
Glycogen	+	−	−
Inositol	−	+	−
Malate	−	+	−
D-Mannitol	−	+	+
Raffinose	−	−	+
Trehalose	+	−	−
G+C content (mol%)	53.6	51.0	49.5

Phylogenetic analysis and the cultural, physiological and biochemical characteristics of strain MS1^T show that it is phylogenetically and phenotypically distinct from *A. fetalis* CCUG 30811^T and *A. aestuarii* B11^T. We propose that strain MS1^T represents a novel species that belongs to the genus *Alishewanella*, one of the major branches of the family *Alteromonadaceae*, and that this species should be named *Alishewanella jeotgali* sp. nov.

Emended description of the genus *Alishewanella*

The characteristics of the genus *Alishewanella*, described previously by Fonnesbech Vogel *et al.* (2000) and Roh *et al.* (2009), are as follows: Gram-negative, motile or non-motile rods, oxidase- and catalase-positive, and the species vary in terms of the concentration of NaCl needed for growth. In addition, growth of species in the genus *Alishewanella* occurs at 4 °C or above. Utilization of glucose and reduction of nitrite are species-dependent.

Table 2. Comparison of the fatty acid contents of strain MS1^T and its closest phylogenetic neighbours

Strains: 1, *Alishewanella jeotgali* sp. nov. MS1^T; 2, *A. aestuarii* B11^T; 3, *A. fetalis* CCUG 30811^T. All data were taken from this study. All strains were grown on 5 % sheep blood agar at 30 °C for 48 h. Values are percentages of the total fatty acids. tr, Trace (<1.0 %); −, not detected.

Fatty acid (%)	1	2	3
$C_{11:0}$ 3-OH	2.3	2.3	1.7
$C_{12:0}$	−	tr	1.0
$C_{12:0}$ 3-OH	3.3	4.0	3.7
$C_{14:0}$ 3-OH/$C_{16:1}$ iso I	1.9	3.2	3.2
$C_{15:0}$	1.4	1.2	1.9
$C_{15:1}\omega 8c$	1.6	1.1	tr
$C_{16:0}$	13.4	11.4	14.3
$C_{16:0}$ iso	2.4	1.5	tr
$C_{17:0}$	8.9	8.9	8.7
$C_{17:1}\omega 6c$	1.9	1.6	tr
$C_{17:1}\omega 8c$	19.8	18.4	16.0
$C_{18:0}$	−	1.6	1.3
$C_{18:0}$ iso	2.0	1.8	tr
$C_{18:1}\omega 7c$	21.7	23.4	18.0
$C_{18:1}\omega 9c$	1.3	1.4	2.0
Summed features*			
1	1.8	2.4	1.9
2	1.9	3.2	3.2
3	16.5	13.4	15.0

*Summed features are groups of two or three fatty acids that cannot be separated by GC with the MIDI system. Summed feature 1 contained $C_{13:0}$ 3-OH and/or iso-$C_{15:1}$ H. Summed feature 2 contained $C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I. Summed feature 3 contained $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH.

Description of *Alishewanella jeotgali* sp. nov.

Alishewanella jeotgali (je.ot.ga′li. N.L. gen. n. *jeotgali* of jeotgal, a traditional Korean fermented seafood).

Motile, rod-shaped, Gram-negative, facultative anaerobe with a single, polar flagellum. Cells are 2–6 μm long and about 1 μm wide and are generally observed as single cells, although they can occur in pairs. Colonies are 3–4 mm in diameter, circular and raised, initially light ivory in colour and dark ivory later. Growth occurs at 4–40 °C, in 1–2 % (w/v) NaCl and at pH 6.5–9.5. Optimal growth occurs at 37 °C, in 1 % (w/v) NaCl and at pH 6.5–9.0. Growth at 4 °C takes >72 h. Generally grows well for >24 h on LA. MA, NA and TSA are also suitable for growth. Oxidase- and catalase-positive. Does not produce indole or L-arginine dihydrolase. Positive for alkaline phosphatase, esterase (C_4), esterase lipase (C_8), leucine arylamidase, trypsin, α-chymotrypsin, *β*-glucosidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase, but negative for lipase (C_4), valine arylamidase, cystine arylamidase, α- and *β*-galactosidase, *β*-glucuronidase, α-glucosidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase and α-fucosidase (API

ZYM). Positive for reduction of nitrate to nitrite, β-glucosidase (aesculin hydrolysis) and protease (gelatin hydrolysis), but negative for production of indole, fermentation of D-glucose, L-arginine dihydrolase, urease and β-galactosidase. Utilizes TMAO and thiosulfate as electron acceptors, but not nitrite, sulfite or ferric iron. Assimilates D-glucose and maltose, but not L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, potassium gluconate, capric acid, adipic acid, malate, trisodium citrate or phenylacetic acid (API 20NE). Assimilates aesculin, maltose, sucrose, trehalose, starch and glycogen, but not glycerol, erythritol, D- and L-arabinose, D-ribose, D- and L-xylose, D-adonitol, methyl β-D-xyloside, D-galactose, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, lactose, melibiose, insulin, melezitose, raffinose, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, gluconate or 2- or 5-ketogluconate (API 50CH). Major cellular fatty acids are $C_{16:0}$, $C_{17:0}\omega 8c$, $C_{18:0}\omega 7c$ and summed feature 3 ($C_{16:1}\omega 7c$/iso-$C_{15:0}$ 2-OH). The DNA G+C content of the type strain is 53.6 mol%.

The type strain, MS1^T (=KCTC 22429^T =JCM 15561^T), was isolated from gajami sikhae (jeotgal), a traditional Korean fermented seafood.

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