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NOTE

Oceanobacillus gochujangensis sp. nov., Isolated from *gochujang* a Traditional Korean Fermented Food

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A Gram-stain-positive, polar flagella-containing, rod-shaped, obligate aerobic, endospore-forming bacterium, strain TK1655^T, was isolated from the traditional Korean food *gochujang*. The 16S rRNA sequence of strain TK1655^T was a member of the genus *Oceanobacillus* similar to that of the type strain of *Oceanobacillus oncorhynchi* subsp. *incaldanensis* DSM 16557^T (97.2%), *O. oncorhynchi* subsp. *oncorhynchi* JCM 12661^T (97.1%), *O. locisalsi* KCTC 13253^T (97.0%), and *O. sojae* JCM 15792^T (96.9%). Strain TK1655^T was oxidase and catalase positive. Colonies were circular, smooth, low convex, cream in colour, and measured about 0.5–1.0 mm in diameter. The range for growth was 20–40°C (optimal, 30°C), pH 6.0–10.0 (optimal, 7.0), and 2–16% (w/v) NaCl (optimal, 2%). Additionally, the cells contained *meso*-DAP, and the predominant isoprenoid quinone was MK-7. The complex polar lipids were consisted of diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylcholine (PC). The major cellular fatty acid components were *iso*-$C_{15:0}$, *anteiso*-$C_{15:0}$, *iso*-$C_{16:0}$, and *anteiso*-$C_{17:0}$, and the DNA G+C content was 40.5%. DNA-DNA relatedness of our novel strain and reference strain *O. locisalsi* KCTC 13253^T, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T, *O. oncorhynchi* subsp. *oncorhynchi* JCM 12661^T was 45.7, 43.8, and 41.9%. From the results of phenotypic, chemotaxonomic, and phylogenetic analyses of strain TK1655^T, we propose the novel species *Oceanobacillus gochujangensis* sp. nov. The type strain is TK1655^T (=KCCM 101304^T =KCTC 33014^T =CIP 110582^T =NBRC 109637^T).

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TK1655^T is JN808225.

Keywords: *Oceanobacillus gochujangensis*, *Oceanobacillus*, *gochujang*, Korean fermented food, 16S rRNA gene

Gochujang (hot pepper paste) is a traditional Korean fermented food prepared from *meju* (fermented soybeans), glutinous rice, and red pepper powder. Along with *kanjang* (fermented soy sauce) and *doenjang* (fermented soy paste), it is one of the most famous traditional Korean foods. Traditional *gochujang* has been shown to contain various bacterial strains, yeasts, and fungi. The most important bacteria found in *gochujang* are *Bacillus* species, *Corynebacterium xerosis*, *Enterococcus faecium*, *Pseudomonas paucimobilis* (Lee and Jang, 1996a). Additionally, the fungal and yeast strains, *Aspergillus oryzae*, *Zygosaccharomyces rouxii*, *Candida* species, *Cryptococcus uniguttulatus*, *Pichia farinosa*, *Rhodotorula glutinis*, *Saccharomyces cerevisiae*, and *S. rouzxii* (Jung and Choi, 1996; Jin and Kim, 2007) have been detected in *gochujang*. Consistent with the bacteria found in *gochujang*, *B. subtilis* is the most commonly detected microorganism in traditional Korean fermented foods (Shin, 2004).

The genus *Oceanobacillus* was first described by Lu *et al.* (2001), who isolated *O. iheyensis* from a deep-sea environment. Currently, the genus *Oceanobacillus* comprises 19 species and 2 subspecies. Only a few genus *Oceanobacillus* have been isolated from fermented food material (Namwong *et al.*, 2009; Tominaga *et al.*, 2009; Whon *et al.*, 2010). In this study, strain TK1655^T, isolated from the traditional Korean food *gochujang*, was identified and classified as a novel *Oceanobacillus* species, through phenotypic, chemotaxonomic, phylogenetic analyses based on 16S rRNA sequences and comparison of DNA-DNA relatedness.

Seven varieties of *gochujang* (TK1-7) manufactured by cottage industry manufacturers were purchased in the Sunchang province, Republic of Korea. All samples were stored at 4°C. Microbial communities from *gochujang* were enumerated and isolated. A total of 10 g of sample was homogenized for 2 h in 100 ml sterile water. Serial dilutions (10^1–10^{10}) of the sample were prepared, and 500 µl of each dilution was plated onto selective media. Halophilic bacteria were incubated for 2–3 days at 30°C on nutrient agar (Difco, USA) containing 10% NaCl. After counting the viable cells, the colnies were cultured. Colonies with distinct morphologies were isolated from the selection medium. The isolated colonies were purified by repeated streaking on the same medium. All cell cultures were freeze-dried and stored.

The isolated bacteria were characterized using a battery of

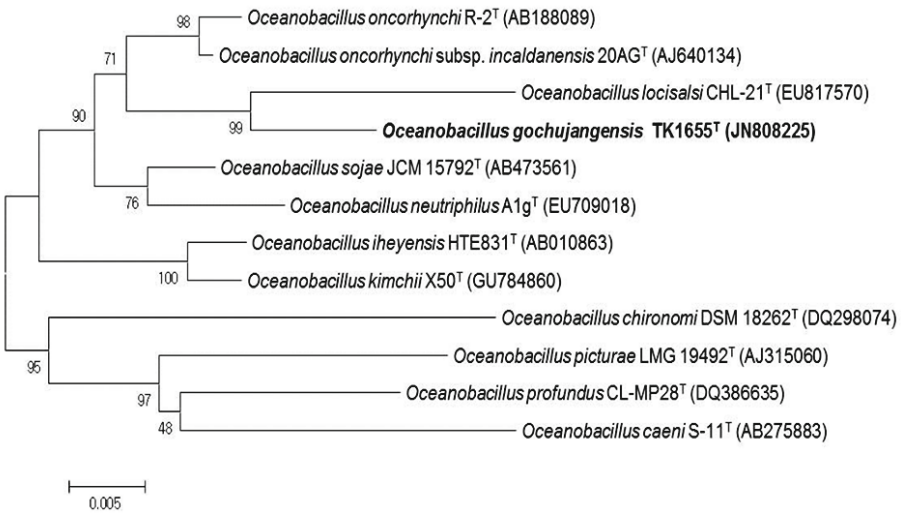

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences comparision of strain TK1655^T and the type strains of phylogenetically related representatives of the genus *Oceanobacillus* based on the neighbor-joining method. Scale bar represents 0.005 substitutions per nucleotide position. The numbers at each branch point are bootstrap value on 1,000 replicates.

common tests as follows: The Gram reaction was carried out using the Gram staining method (1884). The cell morphology of strain TK1655^T was examined by scanning electron microscope (SEM, Hitachi S-4700) and light microscope (Olympus BH-2) at 1000× magnification; by using cells grown for 2 days at 30°C in marine broth (Difco). For SEM analysis cells were examined on fixed material as described by Bozzola and Russell (1998) at 15.0 kV under standard conditions. Flagellation was determined by using a light microscope with cells from exponential phase of growth. To stain flagella of cell by using Leifson method (1930, 1951). The motility test was carried out as described by Tittsler and Sandholzer (1936) using semi-solid medium. Catalase activity was determined by observing bubble production in a 3% (v/v) hydrogen peroxide solution and oxidase activity was determined using an oxidase reagent (bioMérieux, France) according to the manufacturer's instructions. Growth at various pH values (4.0–12.0 at intervals of 1.0 pH unit) and optimal growth temperatures (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50°C) was examined for cells grown on marine broth. The pH of the medium was adjusted using 1 M HCl or 1 M NaOH. Various NaCl concentrations were evaluated using nutrient broth (Difco) supplemented with appropriate concentrations of NaCl (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20%, w/v). Anaerobe growth was investigated using an Anaeroack kit (Mitsubishi Gas Chemical, Japan) on marine agar (Difco). The antibiotic susceptibility test was performed using the disc diffusion method and antibiotics at the following concentrations: ampicillin (50 μg), carbenicillin (60 μg), chloramphenicol (35 μg), kanamycin (25 μg), spectinomycin (50 μg), streptomycin (50 μg), tetracycline (15 μg), and gentamycin (50 μg). Biochemical characteristics and enzyme activities were analyzed by API 20E, 20NE, 50CHB, and ZYM Kits (bioMérieux) according to the manufacturer's instructions. Hydrolysis of Tween 60 (Sigma, USA) was tested in marine agar containing 0.02% (w/v) $CaCl_2$ and 1% Tween 60 (Holding and Collee, 1971). Casein hydrolysis was tested in marine agar supplemented with 2% skim milk (Difco) (Cowan and Steel, 1974). Hydrolysis of gelatin was tested on medium containing 0.3% beef extract, 0.5% peptone, and 12% gelatin.

Cellular fatty acid composition of strain TK1655^T was analysed by the method described by Miller *et al.* (1982). Briefly, bacterial cultures were grown on marine agar at 30°C for 2 days. After harvesting the bacteria, fatty acid methyl esters were extracted and analyzed using the Microbial Identification System (MIDI, Inc., USA) (Lee and Jung, 1996b). Isoprenoid quinone was extracted with chloroform:methanol (2:1, v/v) and was analyzed using high-performance liquid chromatography (HPLC; Younglin, Korea) with a Spherisorb 5 μm ODS2 4.6 mm × 250 mm column (Waters, USA)

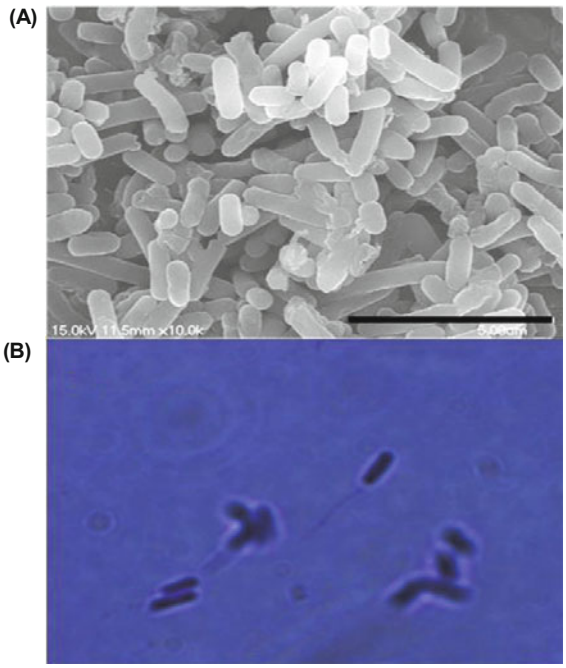

Fig. 2. Scanning Electron Microscope and light microscope at ×1000 of strain TK1655^T. The cells grown for 2 days at 30°C on marine broth. (A) Scanning Electron Microscope. Bar, 5 μm; cells are 0.5–0.6 × 1.1–2.6 μm. (B) Flagellation was determined by using light microscope at ×1000. To stain flagella of cell by using Leifson method with tannic acid.

(Hiraishi *et al.*, 1996). Polar lipids were extracted, analyzed using two dimensional TLC as previously described (Minnikin *et al.*, 1984; Komagata and Suzuki, 1987). The amino acids of cell wall peptidoglycan were analyzed using one-dimensional TLC, with alanine, glycine, glutamine, lysine, diaminopimelic acid (DAP, Sigma) were determined using the method of Kim (1993). The G+C content of DNA was determined as described by Shin *et al.* (1996), with the modification that DNA was hydrolyzed using P1 nuclease (Sigma) and then analyzed by HPLC with a Symmetry C18 column (Waters).

The genomic DNA of the relevant strain TK1655^T was extracted and purified according to the methods described by Yoon *et al.* (1996). Isolated single colonies were cultured in each selection medium. Lysozyme (50 mg/ml; Sigma) was added to single colonies resuspended in 50 mM EDTA buffer, and samples were incubated in a water bath for 12 h at 37°C. Genomic DNA was isolated using a Wizard genomic DNA purification kit (Promega, USA) according to the manufacturer's instructions. Genomic DNA was stored at -20°C until DNA amplification. DNA-DNA hybridization was carried out at 30°C for 24 h and measured fluorometrically by the method of Ezaki *et al.* (1989), using photobiotin-labeled DNA probes in microplate wells. The 16S rRNA sequence of bacteria was PCR amplified using the universal primer pairs 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-GGATACCTTGTTACGACTT-3′) (Gurtler and Stanisich, 1996) as well as 530F (5′-GTGCCAGCMGCG-3′) and 1100R (5′-GGGTTGCGCTCGTTG-3′) (Yoon *et al.*, 1996). The PCR products were purified using the Wizard SV Gel and PCR Clean-up system (Promega). The purified PCR product was direct sequenced using an ABI PRISM BigDye Terminator

Table 1. Differential characteristics of strain TK1655^T and the phylogenetically related to *Oceanobacillus* species

Strains: 1, Strain TK1655^T; 2, *O. iheyensis* JCM 11309^T; 3, *O. oncorhynchi* subsp. *oncorhynchi* JCM 12661^T; 4, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T; 5, *O.sojae* JCM 15792^T; 6, *O. locisalsi* KCTC 13253^T. All strains are positive for acid production from glucose, maltose, mannose. Data were obtained in the present study unless indicated.

+, Positive; -, negative; W, weekly positive reaction; ND, not data. Growth conditions for analysis; 1, 2 days at 30°C in marine agar; 2, 2 days at 30°C in marine agar; 3, 2 days at 26°C in marine agar; 4, 2 days at 30°C in marine agar; 5, 2 days at 30°C in BHI agar; 6, 2 days at 37°C in marine agar.

Characteristics	1	2	3	4	5	6
Colony colour*	Cream	Cream-white	White	Cream-beige	Cream	Cream-beige
Spore formation*	+	+	+	-	+	+
Anaerobic growth	-	-	+	-	-	+
Hydrolysis of						
Gelatin	-	+	-	-	-	-
Tween 60	-	+	-	-	+	+
Casein	-	+	-	-	-	-
Esculin	+	+	+	+	+	+
Reduction of nitrate to nitrite	-	-	+	+	-	+
Reduction of nitrite to N2	-	-	-	+	-	+
Growth temperature range (°C)*	20–40	15–42	15–40	10–40	15–45	10–45
Optimum growth temp. (°C)*	30	30	30–36	37	30–35	30–37
pH range for growth*	6.0–10.0	6.5–10.0	9.0–10.0	6.5–9.5	6.0–10.0	6.0–9.0
Optimum PH*	7.0	7.0–9.5	9.0–10.0	9.0	8.5	7.0–7.5
Nacl range for growth (%)*	2–16	0–21	0–22	5–20	0–15	0–25
NaCl Optimum (%)*	2	3	3	10	ND	5-10
Fermentation of:						
Arabinose	+	-	-	+	-	+
Arabitol	+	-	-	-	W	-
Cellobiose	+	-	+	+	+	W
Fructose	+	-	+	+	+	W
Galactose	+	-	W	+	-	-
Lactose	-	-	-	-	-	-
Melezitol	+	-	-	+	+	W
Melibiose	+	-	W	-	-	-
Raffinose	-	-	-	+	-	-
Ribose	+	-	-	+	+	-
Sorbitol	-	-	-	-	+	-
Sorbose	-	-	+	W	-	-
Sucrose	+	-	+	+	+	-
Trehalose	+	-	+	+	+	-
Xylose	+	-	-	+	-	-
DNA G+C content (mol%)	40.5	37.5	40.8	40.8	38.2	40.3
Isoprenoid quinone	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7

* Data from; 2, Lu *et al.* (2001); 3, Yumoto *et al.* (2005); 4, Romano *et al.* (2006); 5, Tominaga *et al.* (2009); 6, Lee *et al.* (2010).

Table 2. Cellular fatty acid composition (%) of strain TK1655^T and the type strains of related species of the genus *Oceanobacillus*
Strain : 1, Strain TK1655^T ; 2, *O. iheyensis* JCM 11309^T; 3, *O. oncorhynchi* subsp. *oncorhynchi* JCM 12661^T; 4, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T; 5, *O. sojae* JCM 15792^T; 6,*O.locisalsi* KCTC 13253^T. All data are from this study. Only those fatty acids amounting to >1.0% in all strains are shown. -, Not detected. tr, trace amounts (<1%).

Fatty acid	1	2	3	4	5	6
Saturated						
$C_{16:0}$	1.7	tr	tr	tr	1.9	2.9
Unsaturated						
$C_{16:1}$ *ω7c* alcohol	-	6.4	-	tr	-	-
Branched chain						
iso-$C_{14:0}$	4.0	21.7	10.6	21.6	5.1	1.8
iso-$C_{15:0}$	17.8	30.0	20.2	14.8	20.0	9.4
anteiso-$C_{15:0}$	48.6	24.2	45.3	30.8	46.1	52.1
iso-$C_{16:0}$	8.2	10.8	12.1	21.8	10.9	5.2
iso-$C_{17:0}$	4.7	1.2	2.1	2.6	3.1	3.8
anteiso-$C_{17:0}$	14.5	2.5	8.1	6.3	11.9	23.8

Cycle Sequencing Kit (Applied Biosystems, USA) and ABI PRISM 3730XL Analyzer (96 capillary type) according to the manufacturer's instructions. Sequences similarity was calculated using the EzTaxon-e server (Kim *et al*., 2012) to identify the nearest taxa. Multiple alignments were conducted using CLUSTAL X program (Thompson *et al*., 1997). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) based on distance matrix data. The MEGA 6 program (Tamura *et al*., 2013) was used for all analyses. The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 replications.

The 16S rRNA sequence of strain TK1655^T was compared with those of already published strains. A phylogenetic tree constructed based on the 16S rRNA sequence showed that strain TK1655^T was included in the genus *Oceanobacillus* (Fig. 1). Similarities in the 16S rRNA sequence indicated that the closest relatives of strain TK1655^T were *Oceanobacillus oncorhynchi* subsp. *incaldanensis* DSM 16557^T (97.2% similarity), *O. oncorhynchi* subsp. *oncorhynchi* JCM 12661^T (97.1% similarity), *O. locisalsi* KCTC 13253^T (97.0% similarity), *O. sojae* JCM 15792^T (96.9% similarity), and *O. iheyensis* JCM 11309^T (95.7% similarity). These results demonstrated that strain TK1655^T represented a novel species in the genus *Oceanobacillus* (Stackebrandt and Goebel, 1994; Rossello Mora and Amann, 2001).

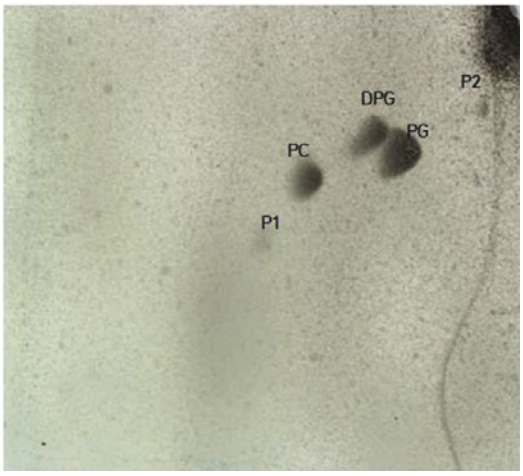

Fig. 3. Total polar lipids profile of strain TK1655^T after by two-dimensional TLC. Spraying one plate with 5% ethanolic molybdophosphoric acid reagent. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; P1-2, unidentified polar lipids.

DNA-DNA hybridization was performed to compare strain TK1655^T and reference strain. The levels of DNA-DNA relatedness between strain TK1655^T and *O. locisalsi* KCTC 13253^T, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T, *O. oncorhynchi* subsp. *oncorhynchi* JCM 12661^T, *O. sojae* JCM 15792^T and *O. iheyensis* JCM 11309^T were 45.7%, 43.8%, 41.9%, 22.7%, and 13.7%, respectively. These DNA-DNA reassociation values were below the 70% limit recommended for species classification (Wayne *et al*., 1987).

Strain TK1655^T was characterized as Gram-stain-positive aerobic rods (0.5–0.6 × 1.1–2.6 μm) (Fig. 2A) that produced centrally positioned ellipsoidal spores. For cells grown for 2 days at 30°C on marine agar, colonies were circular, smooth, low convex, cream in colour and measured about 0.5–1.0 mm in diameter. Cells were motile by means of polar flagella (Fig. 2B).

The results of physiological and biochemical analyses are shown in Table 1. Strain TK1655^T was positive for catalase and oxidase activity and could hydrolyze esculin, but not Tween 60 and casein, gelatin. Acid production from sugar or alcohol is also shown in Table 1. Strain TK1655^T could grow in 2–16% (w/v) NaCl and at pH 6.0–10.0 and 20–40°C. Optimal growth occurred at 2% (w/v) NaCl, pH 7.0, and 30°C. Additionally, strain TK1655^T was susceptible to ampicillin (50 μg/L), carbenicillin (60 μg/L), chloramphenicol (35 μg/L), kanamycin (25 μg/L), spectinomycin (50 μg/L), streptomycin (50 μg/L), tetracycline (15 μg/L), and gentamycin (50 μg/L). Therefore, strain TK1655^T could be distinguished from other closely related species on the basis of phenotypic characteristics.

The predominant cellular fatty acid of strain TK1655^T was *anteiso*-$C_{15:0}$, and additional measurable components, i.e., *iso*-$C_{15:0}$, *iso*-$C_{16:0}$, and *anteiso*-$C_{17:0}$ were also detected. The major cellular fatty acid of strain TK1655^T (*anteiso*-$C_{15:0}$) was the same as that of other species belonging to the genus

Oceanobacillus. In particular, the fatty acid composition of this novel strain was comparatively similar to that of *O. locisalsi* KCTC 13253^T but differences were found in the relative amounts of each fatty acid (Table 2). The main isoprenoid quinone in strain TK1655^T was MK-7. The polar lipids of strain TK1655^T were consisted of diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylcholine (PC) and small amounts of unidentified polar lipid(P1-2) (Fig. 3). The major DAP isomer in the cell wall peptidoglycan of strain TK1655^T were *meso*-DAP. The DNA G+C content of strain TK1655^T was 40.5%, which falls within the 37.5–40.8% range reported for other species within *Oceanobacillus* and related organisms, as shown in Table 1.

Thus, the results of phenotypic, chemotaxonomic, and phylogenetic analyses indicated that strain TK1655^T represented a novel species, for which the name *Oceanobacillus gochujangensis* sp. nov. is proposed.

Description of *Oceanobacillus gochujangensis* sp. nov.

Oceanobacillus gochujangensis (go.chu′jang.en.sis. N.L. gen. n. *gochujangensis* of *gochujang*, a traditional Korean fermented food).

Cells are Gram-stain-positive, oxidase and catalase reaction-positive, rod-shaped (0.5–0.6 × 1.1–2.6 μm), and obligate aerobic, contain polar flagella, and produce centrally positioned ellipsoidal spores. Colonies grown on marine agar are circular, smooth, low convex, cream in colour, and usually measure 0.5–1.0 mm in diameter after 2 days of growth at 30°C. The temperature range for growth is 20–40°C (optimal, 30°C). The pH values required for growth are 6.0–10.0 (optimal, 7.0). The NaCl concentration required for growth is 2–16% (w/v) (optimal, 2%). Cells can hydrolyze esculin but not Tween 60 and casein, gelatin. Nitrates were not reduced to nitrite. Cells were positive for alkaline phosphatase, esterase (C4), acid phosphatase, and naphtol-AS-Bl-phosphohydrolase, but negative for esterase (C8), lipase, leucine arylamidase, arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, α-glactosidase, β-glucurosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Cells are susceptible to ampicillin, carbenicillin, chloramphenicol, kanamycin, spectinomycin, streptomycin, tetracycline, and gentamycin. Acid is produced from D-arabitol, cellobiose, D-fructose, galactose, D-glucose, maltose, D-mannose, melezitol, melibiose, ribose, sucrose, trehalose, D-xylose, glycerol, mannitol, glycogene, D-fucose, and L-arabinose. No acid is produced from ertythritol, lactose, D-raffinose, sorbitol, L-sorbose, L-xylose, D-arabinose, adonitol, β-methyl-xyloside, rhamnose, dulcitol, inositol, α-methyl-D-mannoside, α-methyl-D-glucoside, *N*-acetyl glucosamine, amygdaline, arbutine, salicine, inuline, amidon, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, L-fucose, L-arabitol, gluconate, 2-ceto-gluconate, and 5-ceto-gluconate. The cell contains *meso*-DAP in the cell wall peptidoglycan. Principal cellular fatty acids (>5%) components are *iso*-$C_{15:0}$ (17.8%), *anteiso*-$C_{15:0}$ (48.6%), *iso*-$C_{16:0}$ (8.2%), and *anteiso*-$C_{17:0}$ (14.5%). The predominant isoprenoid quinone is MK-7. The major polar lipids were consisted of diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylcholine (PC). The DNA G+C content is 40.5%.

The type strain, TK1655^T (=KCCM 101304^T =KCTC 33014^T =CIP 110582^T =NBRC 109637^T) was isolated from the traditional Korean food *gochujang*.

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