Sphingobacterium jejuense sp. nov., with ginsenoside-converting activity, isolated from compost

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A Gram-stain-negative, strictly aerobic, non-motile, light yellow, short-rod bacterium (designated GJ30-7^T) isolated from compost, was characterized using a polyphasic approach, in order to clarify its taxonomic position. Strain GJ30-7^T was observed to grow optimally at 30 °C and at pH 7.0 on R2A agar medium. Strain Gsoil GJ30-7^T possessed β-glucosidase activity, which was responsible for its ability to transform ginsenosides Rb1 and Rc (the two main active components of ginseng) to ginsenoside F2. Phylogenetic analysis, based on 16S rRNA gene sequence similarities, indicated that GJ30-7^T belongs to the genus *Sphingobacterium* of the family *Sphingobacteriaceae* and was most closely related to *Sphingobacterium yanglingense* JCM 30166^T (92.6 %), *Sphingobacterium psychroaquaticum* KACC 18188^T (92.6 %), and *Sphingobacterium thermophilum* KCTC 23708^T (92.0 %). The DNA G+C content was 43 mol% and MK-7 was the major isoprenoid quinone. The main polar lipids were phosphatidylethanolamine, one unidentified phospolipid and one unknown polar lipid. In contrast to standard and reference strains, unidentified sphingolipid was also present. The predominant fatty acids of strain GJ30-7^T were iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ (summed feature 3) and iso-$C_{17:1}\omega9c$ and/or $C_{16:0}$ 10-methyl (summed feature 9), supporting the affiliation of strain GJ30-7^T to the genus *Sphingobacterium*. However, strain GJ30-7^T could be distinguished genotypically and phenotypically from species of the genus *Sphingobacterium* with validly published names. The novel isolate therefore represents a novel species, for which the name *Sphingobacterium jejuense* sp. nov. is proposed, with the type strain GJ30-7^T (=KACC 18625^T=JCM 30948^T).

The genus *Sphingobacterium*, which belongs to the family *Sphingobacteriaceae* (based on the presence of sphingolipids as a cellular lipid component), and the phylum *Bacteroidetes* was first described by Yabuuchi *et al.* (1983). Members of the genus *Sphingobacterium* are Gram-negative rods, non-spore forming and lack flagella, but may show a flagellum-independent sliding motility. Colonies usually become yellowish after several days at room temperature (Yabuuchi *et al.*, 1983). Species of the genus *Sphingobacterium* are positive for catalase and oxidase, negative for heparinase and gelatinase activities, and variable for indole production (Wauters *et al.*, 2012). They possess iso-$C_{15:0}$, iso-$C_{15:0}$ 2-OH, $C_{16:1}\omega7c$ and $C_{17:0}$ 3-OH as their major fatty acids (Takeuchi & Yokota, 1992; Steyn *et al.*, 1998) and menaquinone 7 (MK-7) as the predominant respiratory quinone. The range of G+C contents of the genomic DNA is 35–44 mol% (Lee *et al.*, 2013). At the time of writing, the genus *Sphingobacterium* comprises 33 species with validly published names (http://www.bacterio.net). Affiliates of the

Abbreviations: AL, aminolipid; L, lipid; PE, phosphatidylethanolamine; PL, phospholipid.

The GeneBank accession number for the 16S rRNA gene sequence of strain is KT950743.

Three supplementary figures are available with the online Supplementary Material.

genus *Sphingobacterium* are wide spread microorganisms, which have been commonly isolated from a variety of environments, including rhizosphere soil (Jiang *et al.*, 2014; Du *et al.*, 2015), lake water (Albert *et al.*, 2013), lichen [*Cladonia* sp (Lee *et al.*, 2013)], compost (Yabe *et al.*, 2013), activated sludge (Sun *et al.*, 2013), tobacco (Liu *et al.*, 2012), raw milk (Schmidt *et al.*, 2012) and clinical specimens, including blood, urine and the uterus of human patients with opportunistic infections (Holmes *et al.*, 1982; Yabuuchi *et al.*, 1983; Shivaji *et al.*, 1992). Morover, some species of this genus have been reported to have plant growth promoting activities (Marques *et al.*, 2010; Ahmad *et al.*, 2014; Ali *et al.*, 2015).

Jeju Island is the largest island in Korea and located to the southwest of the Korean Peninsula. Due to the envirement, Jeju island rarely produces rice. Instead, beans (paste or powder), barley, meat (pork, beef and chicken) and gogi-guksu (meat noodle dishes) are eaten by the population. During the course of a study to determine the types of aerobic bacteria in compost made from foodstuff waste, compost samples were collected from different locations on Jeju Island. Samples were plated on R2A (Difco) and incubated at 23–25 °C for 5 days. Numerous types of colonies were observed and one, designated GJ30-7, was chosen for taxonomic study. Strain GJ30-7^T was routinely cultured on R2A agar at 30 °C and was preserved at −80 °C in R2A broth (Difco) supplemented with 20 % (v/v) glycerol.

The genomic DNA of strain GJ30-7^T was extracted with a commercial genomic DNA extraction kit (Solgent); PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out according to Kim *et al.* (2015). Nearly full-length sequences of the 16S rRNA gene were compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database or [http://www.ezbiocloud.net/eztaxon; Kim *et al.* (2012)]. Multiple sequence alignments were performed using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were reconstructed by using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood methods, using the MEGA 6 program (Tamura *et al.*, 2013) with bootstrap values based on 1000 replications (Felsenstein, 1985).

An almost complete 16S rRNA gene sequence of strain GJ30-7^T (1452 nt) was determined and subjected to comparative analysis. Phylogenetic analysis using the neighbour-joining, maximum-likelihood and maximum-parsimony methods, based on 16S rRNA gene sequences, indicated that strain GJ30-7^T clustered within the genus *Sphingobacterium* and formed a clade with *Sphingobacterium arenae* KCTC 32294^T and *Sphingobacterium gobiense* ACCC 05757^T (Fig. 1). Strain GJ30-7^T showed 16S rRNA gene sequence similarities of less than 93 % with species of the genus *Sphingobacterium* with validly published names.

Based on 16S rRNA gene sequence and phylogenetic analysis, *Sphingobacterium yanglingense* JCM 30166^T, *Sphingobacterium psychroaquaticum* KACC 18188^T, *Sphingobacterium thermophilum* KCTC 23708^T, *Sphingobacterium arenae* KCTC 32294^T, *Sphingobacterium gobiense* ACCC 05757^T and type species of the genus *Sphingobacterium spiritivorum* KACC 10895^T were selected for further comparative studies. However, for the determination of the presence of sphingolipids *Sphingobacterium multivorum* KACC 12158^T, *Sphingobacterium canadense* LMG 23727^T and *Sphingobacterium cladoniae* KCTC 22613^T were used as reference strains, while *S. multivorum* KACC 12158^T was used for comparative polar lipid analysis.

The Gram reaction was determined using the non-staining method, as described by Buck (1982). Cell motility was determined using the hanging drop method. Cell morphology was examined by scanning electron microscopy (Hitachi; SEM-3500N), using cells grown for 2 days at 30 °C on R2A agar medium and prepared according to Schädler *et al.* (2008) and Wang *et al.* (2015). Biochemical tests were carried out using API 20NE, API ID 32GN and API ZYM kits, according to the manufacturer's instructions (bioMérieux). Tests for degradation of DNase (DNase agar medium, Sharlau), casein, starch (Atlas, 1993) and carboxyl methyl cellulose (Ten *et al.*, 2004) were performed and evaluated after 7 days incubation at 30 °C. Growth at different temperatures (5, 10, 15, 25, 30, 33, 37, 40, 42, 45 and 50 °C) and various pH values (pH 4–10, at intervals of 1 pH unit) was assessed on R2A agar medium after 5 days incubation at 30 °C. The following buffers (final concentration, 50 mM) were used to adjust the pH of R2A broth. Acetate buffer was used for pH 4.0–5.5, phosphate buffer was used for pH 6.0–8.0 and Tris buffer was used for pH 8.5–10.0. Salt tolerance was evaluated on R2A agar medium supplemented with 1–6 % NaCl (w/v, at intervals of 0.5 %) and was growth assessed after 7 days incubation at 30 °C. Anaerobic growth was observed in serum bottles by adding thioglycolate (1 g l^{-1}) to R2A broth and substituting the upper air layer with N_2 gas. Growth on nutrient agar (NA; Difco), trypticase soy agar (TSA; Difco), Luria-Bertani agar (LB; Difco) and MacConkey agar (Difco) was evaluated at 30 °C after 7 days of incubaition. Ginsenoside biotransformation was carried out as described by Kim *et al.* (2015).

Cells of strain GJ30-7^T were strictly aerobic, non-motile, Gram-stain-negative short rods (Fig S1, available in the online Supplementary Material). Colonies of strain GJ30-7^T grown on R2A agar were circular, convex, opaque and light yellow after 48 h incubation at 30 °C. The isolate did not grow on TSA (Difco), LB agar (Difco) and MacConkey agar (Difco), whereas weak growth was observed on nutrient and DNase agar at 30 °C after 3 days of incubation. Physiological characteristics of strain GJ30-7^T are summarized in the species description whilst a comparison of some selective characteristics of the isolated strain and related type strains are given in Table 1. A time-course study of the biotransformation of the ginsenosides, Rb1 and Rc, by strain

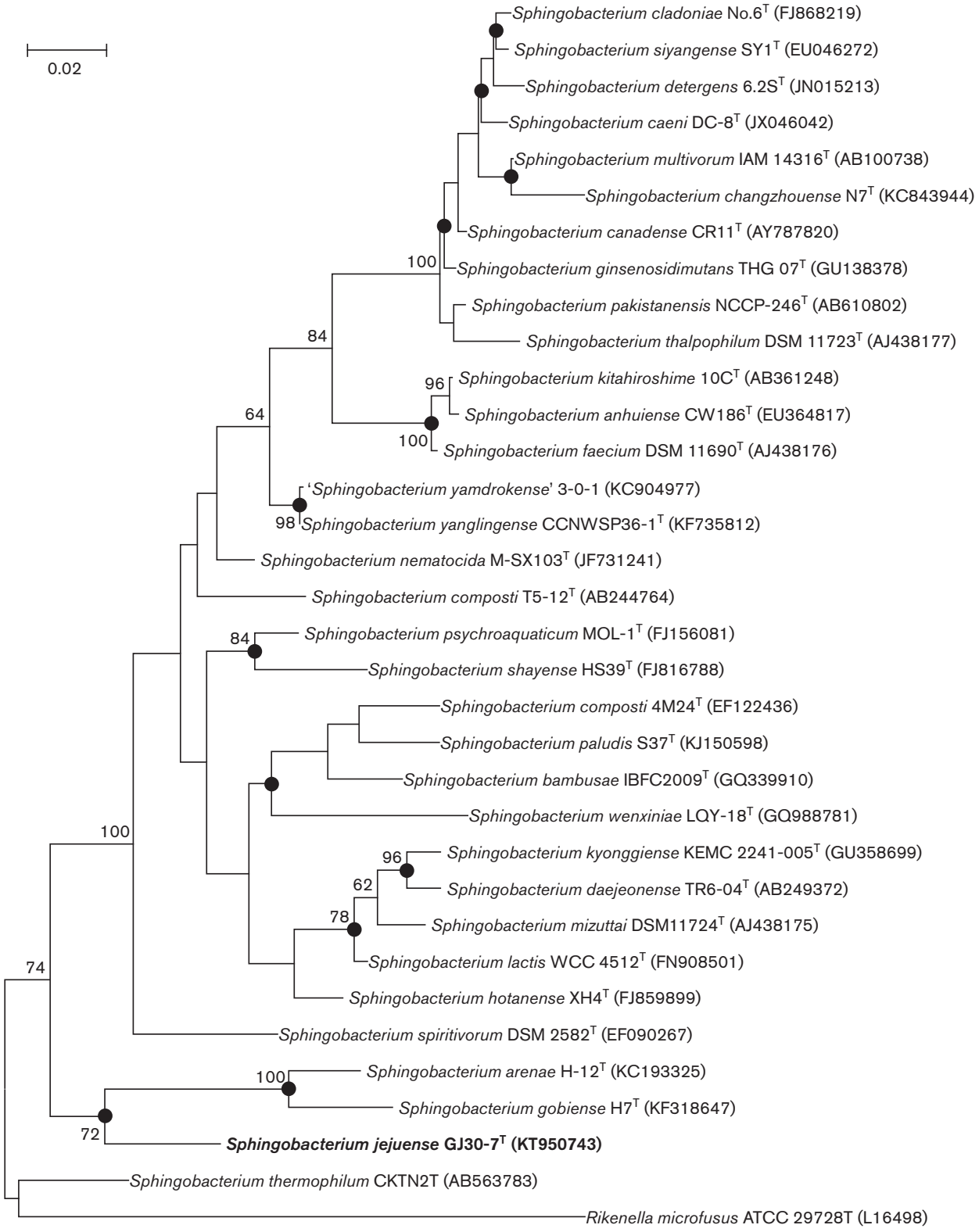

Fig. 1. Phylogenetic tree reconstructed from comparative analysis of 16S rRNA gene sequences, showing the relationships of strain GJ307^T with related species. The tree was reconstructed using the maximum-likelihood method with a two-parameter distance matrix and complete deletion. Dots indicate generic branches also recovered by using maximum-parsimony and neighbour-joining algorithms. Bootstrap values (expressed as percentages of 1000 replications) greater than 60 % are shown at the branching points. *Rikenella microfusus* ATCC 29728^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Table 1. Differential characteristics of strain GJ30-7^T and the type strains of related species of the genus *Sphingobacterium*

Strains; 1, GJ30-7^T; 2, *S. psychroaquaticum* KACC 18188^T (this study); 3, *S. thermophilum* KCTC 23708^T (this study); 4, *S. spiritivorum* KACC 10895^T (this study); 5, *S. arenae* KCTC 32294^T (data from Jiang *et al.*, 2014); 6, *S. gobiense* ACCC 05757^T (data from Zhao *et al.*, 2014); 7, *S. yanglingense* JCM 30166^T (data from Peng *et al.*, 2014). All strains are short rods, Gram-stain-negative, positive for aerobic growth and catalase activity, but negative for nitrate reduction and indole production. +, Positive; W; weakly positive; –, negative; ND, no data available/not determined.

Characteristics	1	2	3	4	5	6	7
Catalase	+	W*	+†	ND	+	+	+
Oxidase	+	–*	+†	ND	+	+	+
Temperature range (°C)	20–40	5–37	20–50	15–37	20–37	8–40	10–40
Optimum temperature (°C)	30	30	50	30	30	30	28–30
Enzyme activity							
Urease	–	+	–	–	+	+	+
Valine arylamidase	–	+	+	+	–	ND	+
α-Chymotrypsin	–	–	+	+	–	ND	+
α-Galactosidase	+	+	–	+	+	ND	+
β-Galactosidase	+	–	–	+	+	ND	–
β-Glucuronidase	+	–	+	–	–	ND	–
α-Mannosidase	+	+	+	–	+	+	–
α-Fucosidase	–	–	+	+		ND	+
Assimilation of:							
D-Glucose	+	+	–	+	+	+	+
L-Arabinose	–	+	–	–	+	+	+
L-Rhamnose	–	+	–	+	+	+	–
N-Acetyl-D-glucosamine	–	+	+	+	+	–	–
D-Ribose	–	+	–	–	ND	ND	+
D-Saccharose	–	+	+	+	–	–	–
Sodium acetate	–	–	–	+	ND	ND	ND
Lactic acid	–	–	–	+	ND	ND	+
D-Mannitol	–	–	–	+	–	–	–
L-Fucose	–	–	–	+	+	–	+
L-Histidine	–	+	+	+	–	–	–
3-Hydroxybutyric acid	–	–	+	–	–	–	+
L-Proline	–	+	+	+	–	–	+
DNA G+C content (mol%)	43.0	ND	40.3†	39.0–42.0‡	44.1	44.3	41.1

*Data taken from Albert *et al.* (2013).
†Data taken from Yabe *et al.* (2013).
‡Data taken from Yabuuch *et al.* (1983) & Takeuchi & Yokota (1992).

GJ30-7^T showed that they were completely transformed into ginsenoside F2 after 3 days of incubation at 30 °C.

For the measurement of DNA G+C, the genomic DNA of strain GJ30-7^T was extracted and purified, as described by Moore & Dowhan (1995), and degraded enzymatically into nucleosides, then the DNA G+C content was determined, as described by Mesbah *et al.* (1989) using reversed-phase HPLC. Isoprenoid quinone was extracted with chloroform/methanol (2 : 1, v/v), evaporated under vacuum conditions, and re-extracted in n-hexane/water (1 : 1, v/v). The crude n-hexane-quinone solution was purified using Sep-Pak Vac cartridges silica (Waters) and subsequently analyzed by HPLC, as previously described (Hiraishi *et al.*, 1996) with a reversed-phase Nova-pak C18 column (3.9×300 mm). The menaquinone of strain GJ30-7^T was compared with the (MK-7) quinone standard. Cellular fatty acid profiles were determined after 48 h of growth at 30 °C on R2A agar medium. The cellular fatty acids were saponified, methylated and extracted, according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids, analysed with a gas chromatograph (6890; Hewlett Packard), were identified by the Microbial Identification software package, based on the Sherlock Aerobic Bacterial Database (TSBA60) (Sasser, 1990). The polar lipid profile of strain GJ30-7^T was determined by using the method of Minnikin *et al.* (1984).

For the analysis of sphingolipids, cells of strain GJ30-7^T, *S. multivorum* KACC 12158^T, *S. canadense* LMG 23727^T and *S. cladoniae* KCTC 22613^T were harvested from R2A broth after incubation for 2 days at 30 °C. The sphingolipids were

extracted and analysed by TLC using the method of Dees *et al.* (1985).

The DNA G+C contents of strain GJ30-7^T was 43, which was similar to that of *S. thermophilum* KCTC 23708^T, *S. psychroaquaticum* KACC 18188^T and *S. spiritivorum* KACC 10895^T (Table 1). The major fatty acids of strain GJ30-7^T were iso-$C_{15:0}$ (38.2 %), iso-$C_{17:0}$ 3-OH (14.6 %), $C_{16:1}$ ω7*c* and/or $C_{16:1}\omega$6*c* [(summed feature 3) 20.9 %] and iso-$C_{17:1}$ ω9*c* and/or $C_{16:0}$ 10-methyl [(summed feature 9) 10.4 %]. The large amount of summed feature 9 (10.4 %) along with qualitative and quantitative differences distinguishes strain GJ30-7^T from other species of the genus *Sphingobacterium* with validly published names (Table 2). The major polar lipids of strain GJ30-7^T were phosphatidylethanolamine (PE), one unknown phospholipid (PL1) and one unknown polar lipid (L4), while the minor polar lipids were two unknown phospholipids (PL2 and PL3), one unknown aminolipid (AL1) and five unknown polar lipids (L1, L2, L3, L5 and L6) (Fig. S2). Strain GJ30-7^T shared the similar major polar lipid, PE, with a recently described species of the genus *Sphingobacterium* (Jiang *et al.*, 2014; Du *et al.*, 2015). Comparative analysis of strain GJ30-7^T with *S. multivorum* KACC 12158^T indicates that both strains share PE as a major polar lipid, and L2 (unidentified polar lipid) and AL1 (unidentified aminolipid) as minor lipids. Similarly, the differences in the polar lipids between strain GJ30-7^T and *S. multivorum* KACC 12158^T were indicated by labelling the spots in Fig. S2 (A, B). The only predominant isoprenoid quinone of strain GJ30-7^T was MK-7. As illustrated in Fig S3, sphingolipids were detected in strain GJ30-7^T and in three reference strains (*S. multivorum* KACC 12158^T, *S. canadense* LMG 23727^T and *S. cladoniae* KCTC 22613^T).

Based on phylogenetic and phenotypic comparisons, strain GJ30-7^T shares several common features with members of the genus *Sphingobacterium*, e.g. MK-7 as the major respiratory quinone, PE as the major polar lipid and iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and summed feature 3 ($C_{16:1}\omega$7*c* and /or $C_{16:1}\omega$6*c*) as the major fatty acids. However, in addition to a lack of 16S rRNA gene sequence similarity with members of the genus *Sphingobacterium* with validly published names, obvious phenotypic and chemotaxonomic characteristics also differentiate the novel isolate (Tables 1 and 2). So, strain GJ30-7^T should be assigned to the genus *Sphingobacterium* as the type strain of a novel species, for which the name *Sphingobacterium jejuense* sp. nov. is proposed.

Description of *Sphingobacterium jejuense* sp. nov.

Sphingobacterium jejuense (je.ju.en′se. N.L. neut. adj. *jejuense* pertaining to Jeju Island, Korea, geographical origin of the type strain of the species).

Cells are Gram-stain-negative, strictly aerobic, non-motile, short rods (0.3–0.7×1.5–3 μm). Colonies on R2A agar

Table 2. Cellular fatty acid profiles of strain GJ30-7^T and phylogenetically related species of the genus *Sphingobacterium*

Strains; 1, GJ30-7^T; 2, *S. psychroaquaticum* KACC 18188^T (this study); 3, *S. thermophilum* KCTC 23708^T (this study); 4, *S. spiritivorum* KACC 10895^T (this study); 5, *S. arenae* 5KCTC 32294^T (data from Jiang *et al.*, 2014); 6, *S. gobiense* ACCC 05757^T (data from Zhao *et al.*, 2014); 7, *S. yanglingense* JCM 30166^T (data from Peng *et al.*, 2014). Only data where cellular fatty acids accounted for more than 1 % of the total in each strain is shown. TR: Traces (<1 %); –, not detected/no data available.

Fatty acids	1	2	3	4	5	6	7
Saturated							
$C_{14:0}$	TR	1.0	TR	3.6	1.37	TR	2.4
$C_{16:0}$	2.1	4.3	–	7.0	7.26	3.3	3.0
Branched							
iso-$C_{15:0}$	38.2	26.5	51.7	28.9	27.1	32.6	37.8
iso-$C_{15:0}$ 3-OH	3.2	–	–	2.8	3.0	2.4	2.7
anteiso-$C_{15:0}$	TR	–	1.2	TR	TR	TR	3.6
$C_{16:0}$ 3-OH	TR	4.6	–	5.0	TR	2.2	7.2
iso-$C_{17:0}$ 3-OH	14.6	10.2	13.5	9.1	15.4	18.1	8.6
Unsaturated							
iso-$C_{15:1}$ G	–	TR	1.2	–	–	–	–
iso-$C_{15:1}$ F	1.8	–	–	TR	–	–	–
$C_{16:1}\omega$5c	1.7	1.4	–	TR	TR	1.1	–
Summed features*							
1. iso-$C_{15:1}$ H and /or $C_{13:0}$ 3-OH	1.6	–	1.3	TR	TR	TR	–
3. $C_{16:1}\omega$7*c* and /or $C_{16:1}\omega$6*c*	20.9	47.6	21.3	38.3	37.8	36.2	34.6
4. iso-$C_{17:1}$ I and /or anteiso-$C_{17:1}$ B	2.7	–	–	TR	–	TR	–
9. iso-$C_{17:1}\omega$9*c* and /or $C_{16:0}$ 10-methyl	10.4	TR	4.9	1.1	–	1.2	–

*Summed features represent groups of two or three fatty acids that could not be separated by gas chromatography (GLC) with the MIDI system.

plates usually become yellowish after several days at room temperature; they are smooth, opaque and circular with regular margins, and 0.5–1.5 mm in diameter. Growth occurs at 20–40 °C (optimum, 30 °C), pH 5.5–8.5 (optimum, pH 6–7) and in 0.5–1.5 % (w/v) NaCl (optimum, 0 % NaCl). Do not hydrolyze casein, starch and CM-cellulose, but hydrolyzes DNase agar. In API kits, positive for alkaline phosphatase, esterase, esteralse lipase, esculin hydrolysis, α-glucosidase, β-glucosidase, naphtol-AS-BI-phosphohydrolase, acid phosphatase, leucine arylamidase, cystine arylamidase, trypsin and 4-nitrophenyl-β-D-galactopyranoside. D-Glucose, salicin, D-melibiose, D-mannose, *N*-acetyl-glucosamine, D-maltose, glycogen and L-proline are assimilated, but L-rhamnose, D-ribose, D-saccharose, sodium acetate, lactic acid, potassium 5-ketogluconate, 4-hydroxybenzoic acid, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid are not. MK-7 is the predominant respiratory quinone and iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and summed feature 9 (iso-$C_{17:1}\omega9c$ and/or $C_{16:0}$ 10-methyl) are the major cellular fatty acids. Five unknown polar lipids (L1-L6 except L4) and two unidentified phospholipids are also present.

The type strain, isolated from compost collected in Jeju island, Republic of Korea, is GJ30-7^T (=KACC 18625^T=JCM 30948^T). The G+C content of the genomic DNA of the type strain is 43 mol%.

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