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Sphingomonas parvus sp. nov. isolated from a ginseng-cultivated soil[§]

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Strain GP20-2^T was isolated from a soil cultivated with ginseng in Korea. The 16S rRNA gene sequence of this strain showed the highest sequence similarity with *Sphingomonas daechungensis* CH15-11^T (96.7%) and *Sphingomonas sediminicola* Dae 20^T (96.2%) among the type strains. The strain GP20-2^T was a strictly aerobic, Gram-negative, non-motile, rod-shaped bacterium that formed very tiny colonies, less than 0.3 mm in diameter after 10 days on R2A agar. The strain grew at 10–35°C (optimum, 35°C), at a pH of 5.0–8.0 (optimum, pH 6.0), and in the absence of NaCl. The DNA G+C content of strain GP20-2^T was 67.2 mol%. It contained ubiquinone Q-10 as the major isoprenoid quinone, and summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$, 49.8%) and $C_{16:0}$ (17.0%) as the major fatty acids. On the basis of evidence from our polyphasic taxonomic study, we concluded that strain GP20-2^T should be classified as a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas parvus* sp. nov. is proposed. The type strain is GP20-2^T (=KACC 12865^T =DSM 100456^T).

Keywords: *Sphingomonas,* strain GP20-2, novel species, polyphasic taxonomy

Introduction

The genus *Sphingomonas* was so named by Yabuuchi *et al.* (1990) because of the presence of unique sphingoglycolipids in its cellular lipid. Strains of the genus *Sphingomonas* are characterized as Gram-negative, non-sporulating, strictly aerobic, motile or non-motile, and chemoorganotrophic rods. Colony color varies from orange or yellow to white to non-pigmented. They have Q-10 as the predominant quinone, and *sym*-homospermidine as the major polyamine. The G+C content of their DNA varies between 62% and 68% and the major fatty acids are $C_{18:1}$, saturated $C_{16:0}$, and/or $C_{17:1}$. Phylogenetically, the genus *Sphingomonas* is a member of the α-4 subclass of *Proteobacteria* (Chen *et al.*, 2012).

The members of the genus *Sphingomonas* are free living in natural and man-made environments such as air (Busse *et al.*, 2003; Kim *et al.*, 2014), soil (Zhang *et al.*, 2010; Kim *et al.*, 2014), desert sand (An *et al.*, 2011), natural mineral water (Lee *et al.*, 2001), wastewater (Fujii *et al.*, 2001; Yoon *et al.*, 2009), tidal flat sediment (Roh *et al.*, 2009), seawater (Vancanneyt *et al.*, 2001), and plants (Takeuchi *et al.*, 1995; Xie and Yokota, 2006). Recent studies showed that the genus *Sphingomonas* is one of the dominant bacterial genera present on the phyllosphere and involved in plant protection against bacterial pathogens (Vorholt, 2012). At the time of this writing, the genus comprised 89 recognized species (www.bacterio.net).

During the course of an investigation of a bacterial community in soil cultivated with ginseng, one isolate was shown to represent a novel species of the genus *Sphingomonas* on the basis of phenotypic data and phylogenetic inference.

Materials and Methods

Bacterial strains

The soil sample was collected from a ginseng field in Yeongju region, Korea. It was diluted serially in 0.85% (w/v) saline solution, spread on R2A agar (Difco), and incubated for 7 days at 28°C. Bacterial colonies with unique morphologies were selected for the analysis of the 16S rRNA gene sequences and one of them, designated as strain GP20- 2^T, was selected for polyphasic characterization. Type strains of the genus *Sphingomonas* were obtained from the Korean Agricultural Culture Collection (KACC) for comparative taxonomic analysis.

Phylogenetic analysis

The 16S rRNA gene of the strain GP20-2^T was amplified by the polymerase chain reaction (PCR) and sequenced as described by Ahn *et al.* (2014). The near-complete 16S rRNA gene sequence of strain GP20-2^T (1,427 nt) and those of the selected type strains belonging to the genus *Sphingomonas* were aligned using the online tool SINA Alignment Service, version 1.2.11 (Pruesse *et al.*, 2012) (www.arb-silva.de/aligner). The aligned sequences were exported to the Molecular Evolutionary Genetics Analysis (MEGA) software program, version 6.06 (Tamura *et al.*, 2011) and maximum-likelihood, neighbor-joining, and maximum-parsimony trees were constructed. Nucleotide similarity values were calculated using the EzTaxon-e server (Kim *et al.*, 2012) (www.ezbiocloud.net/eztaxon).

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[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

Nucleotide sequence accession number

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GP20-2^T is KP025677.

Determination of DNA G+C content

The DNA G+C content was determined as described by Gonzalez and Saiz-Jimenez (2002) using the CFX96 real-time PCR system (Bio-Rad). Four bacterial strains were used to construct the calibration curve: *Bacillus cereus* KACC 11240^T, *Bacillus amyloliquefaciens* subsp. *plantarum* KACC 17177^T, *Pseudomonas stutzeri* KACC 10290^T, and *Micrococcus luteus* KACC 10488^T.

Morphological, physiological, and biochemical characterization

The cell morphology and motility of strain GP20-2^T was examined by using oil-immersion phase-contrast microscopy (Axioplan 2; Zeiss) with cells grown for 4 days on R2A agar at 30°C. In addition to R2A agar, growth of strain GP20-2^T was tested on trypticase soy agar (TSA) (Difco), nutrient agar (NA) (Difco), Luria-Bertani agar (LB) (Difco), and marine agar 2216 (MA) (Difco). Growth in the presence of 0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, and 3.0% NaCl (w/v), and at various temperatures (5–45°C at intervals of 5°C) was investigated by 2 weeks of incubation on R2A agar. The pH range for growth was observed for 10 days of incubation in R2A broth at a pH of 2.0–11.0 in increments of 1.0 units. In this experiment, no buffer was added due to the sensitivity of strain GP20-2^T to salt. Gram staining behavior was tested by the KOH test (Smibert and Krieg, 1994) and assessing L-alanine aminopeptidase activity (Bactident Aminopeptidase test kit; Merck). Catalase activity was detected by dispersing colonies in 3% (v/v) hydrogen peroxide and checking for bubble formation, and oxidase activity was determined by using the Oxidase Reagent (bioMérieux). Casein, carboxymethyl cellulose (CM-cellulose), starch, Tween 20/40/60/80, tyrosine, and lipid hydrolyses were examined on R2A plates containing milk powder [5% (w/v)], CM-cellulose [1% (w/v)], starch [1% (w/v)], Tween 20/40/60/80 [1% (w/v)], tyrosine [0.1% (w/v)], and tributyrin [1.0% (w/v)], respectively, for two weeks. Growth under anaerobic condition was tested by incubating R2A agar plates in AnaeroGen sachet pouches (Oxoid Ltd.) at 30°C for two weeks.

Other biochemical characteristics were determined using

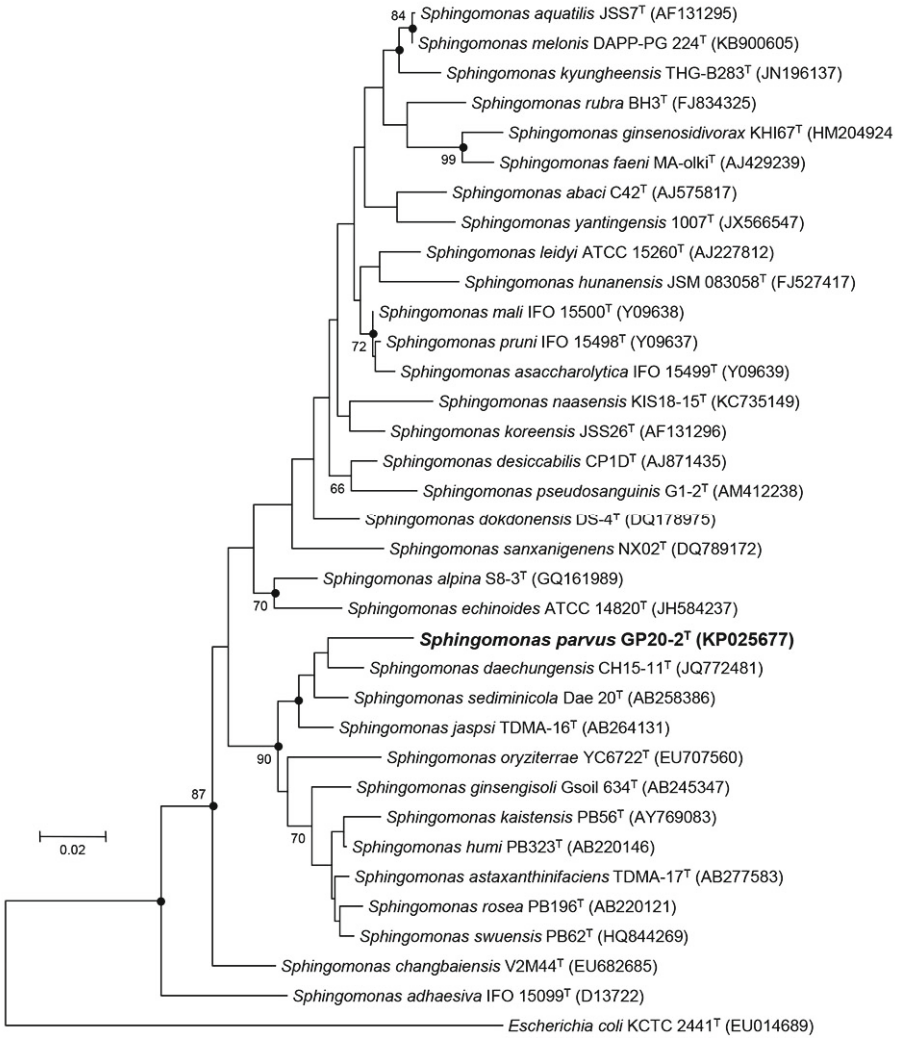

Fig. 1. Maximum-likelihood tree based on a comparative analysis of 16S rRNA gene sequences showing the relationship between strain GP20-2^T and selected type strains belonging to the genus *Sphingomonas*. Bootstrap values (>70%) based on 500 resamplings are shown at branching points. The dots indicate that the corresponding branches were also recovered in the neighbor-joining and maximum-parsimony trees. The scale bar indicates 0.02 estimated change per nucleotide. *Escherichia coli* KCTC 2441^T was used as an outgroup.

the API ZYM, API 20NE, and ID 32GN systems according to the instructions of the manufacturer (bioMérieux). The API ZYM test strip was read after a 4 h incubation at 37°C, while the API 20NE and ID 32GN strips were examined after being incubated for two weeks at 30°C.

Chemotaxonomy

Cells of strain GP20-2^T and the selected type strains of the genus *Sphingomonas* were grown on R2A agar for 4 days at 30°C and fatty acid methyl esters were extracted and prepared according to the standard protocol of the Microbial Identification System (Microbial Identification Inc.) as previously described (Ahn *et al.*, 2014). The extracted compounds were then analyzed using the TSBA6 database, version 6.10 (Microbial Identification Inc.). Polar lipids were extracted and separated by two-dimensional silica-gel thin-layer chromatography as described by Tindall (1990a and 1990b). Respiratory quinones were extracted and identified according to the methods of Nakagawa and Yamasato (1993). Isoprenoid quinones were extracted from 300 mg freeze-dried cells with chloroform/methanol (2:1, v/v), separated by using hexane and eluted with hexane/diethyl ether (98:2, v/v). The eluent was evaporated and quinones were dissolved in acetone and analyzed using high pressure liquid chromatography (L-5000, Hitachi) with a reversed-phase column (YMC Pack ODS-A, YMC).

Results and Discussion

Nucleic acid characteristics

Analyses of the 16S rRNA gene sequences showed that strain GP20-2^T shared the highest sequence similarity with *Sphingomonas daechungensis* CH15-11^T (96.7%) and *Sphingomonas sediminicola* Dae 20^T (96.2%) among the type strains. The phylogenetic analysis based on the maximum-likelihood tree showed that the strain GP20-2^T formed a clade with *Sphingomonas daechungensis* CH15-11^T, *Sphingomonas sediminicola* Dae 20^T, and *Sphingomonas jaspsi* TDMA-16^T, which was also supported by the neighbor-joining and maximum-parsimony trees (Fig. 1). The DNA G+C content of strain GP20-2^T was 67.2 mol%.

Morphological, physiological, and biochemical characteristics

Following growth on R2A agar at 30°C for 10 days, strain

Table 1. Differentiation between strain GP20-2^T and selected type strains of *Sphingomonas* species

Strains: 1, GP20-2^T; 2, *S. daechungensis* KACC 18115^T; 3, *S. sediminicola* KACC 15039^T; 4, *S. jaspsi* KACC 13230^T; 5, *S. kaistensis* KACC 14319^T. +, Positive; -, negative; w, weakly positive.

All strains are positive for hydrolysis of lipid and esculin, but negative for hydrolysis of casein, tyrosine, and Tweens 20/40/60/80. All strains are positive for assimilation of glycogen, but negative for indole production, glucose fermentation, and assimilation of L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, capric acid, malic acid, trisodium citrate, phenylacetic acid. L-rhamnose, D-ribose, inositol, D-saccharose, itaconic acid, suberic acid, sodium malonate, sodium acetate, lactic acid, L-alanine, potassium 5-ketogluconate, 3-hydroxybenzoic acid, L-serine, salicin, D-melibiose, L-fucose, D-sorbitol, propionic acid, valeric acid, L-histidine, potassium 2-ketogluconate, 4-hydroxybenzoic acid, and L-proline. All strains are positive for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and α-glucosidase but negative for lipase (C14), crystine arylamidase, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase.

Characteristics	1	2	3	4	5
Reduction of nitrate	-	+	+	-	-
Hydrolysis of					
Starch	-	+	+	-	+
CM-Cellulose	-	+	-	-	-
Urea	+	+	-	-	+
Gelatin	+	+	+	w	+
4-Nitrophenyl-β-D-galactopyranoside	-	+	-	-	-
Assimilation of					
D-Glucose	-	-	-	+	-
D-Maltose	-	-	-	+	+
Potassium gluconate	-	-	-	+	-
Adipic acid	-	-	-	+	-
3-Hydroxybutyric acid	-	-	-	+	-
Enzyme activity					
Arginine dihydrolase	+	+	-	-	-
Esterase (C4)	+	+	-	+	+
Valine arylamidase	+	+	+	+	-
Trypsin	+	-	-	+	-
α-Chymotrypsin	-	-	+	-	+
β-Galactosidase	-	+	-	-	-
β-Glucosidase	-	+	-	-	-
DNA G+C content (mol%)	67.2	65.6[a]	67.9[b]	63.3[c]	69.1-69.9[d]

[a] Data was taken from Huy *et al.* (2014).
[b] Data was taken from An *et al.* (2013).
[c] Data was taken from Asker *et al.* (2007).
[d] Data was taken from Kim *et al.* (2007).

GP20-2^T formed light-orange, glistering, and tiny (0.18–0.27 mm in diameter) colonies. Strain GP20-2^T showed a significantly slower growth rate on R2A agar at 30°C compared with those of the type strains of the genus *Sphingomonas* tested in this study. Cells of strain GP20-2^T were strictly aerobic, Gram-negative, and non-motile rods that were 0.32–0.47 μm wide and 1.24–1.79 μm long. In addition to R2A agar, strain GP20-2^T grew on NA, but did not grow on TSA, LB, and MA. The temperature range for growth was 10–35°C, with optimum growth at 35°C. The addition of NaCl to R2A agar to a final concentration of 0.5–3.0% (w/v) inhibited the growth of strain GP20-2^T, and similar results were also observed for closely related strains (Asker *et al.*, 2007; An *et al.*, 2013; Huy *et al.*, 2014). The pH range for growth was 5.0–8.0, with optimum growth at pH 6.0. Cells were positive for catalase and oxidase activities. Other phenotypic characteristics distinguishing strain GP20-2^T from its close relatives in the genus *Sphingomonas* are shown in Table 1.

Chemotaxonomy

Like other type strains of the genus *Sphingomonas*, ubiquinone Q-10 was the major isoprenoid quinone of strain GP20-2^T. The major fatty acids in strain GP20-2^T were summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$, 49.8%) and $C_{16:0}$ (17.0%). The complete fatty acid composition is provided in Table 2. The abundances of $C_{18:0}$ and $C_{18:1}$ $\omega7c$ 11-methyl in the cellular fatty acids of strain GP20-2^T were higher than those of the other type strains, while the abundances of $C_{18:1}$ 2-OH and summed feature 3 were lower. The polar lipids found in strain GP20-2^T were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, sphingolipid, an unidentified phospholipid, an unidentified aminoglycolipid, and some unidentified glycolipids (Supplementary data Fig. S1). This polar lipid profile is similar to that of *S. daechungensis* (Huy *et al.*, 2014), the type strain most closely related to strain GP20-2^T, but is different in that the strain GP20-2^T contained both an unidentified phospholipid and aminoglycolipid, and did not contain phosphatidylcholine.

Taxonomic conclusion

In conclusion, strain GP20-2^T is similar to other type strains of the genus *Sphingomonas,* because it hydrolyzed esculin and contained ubiquinone Q-10 as the major isoprenoid quinone, and summed feature 8 and $C_{16:0}$ as the major fatty acids, but GP20-2^T can be distinguished from the other type strains of its genus by its low 16S rRNA gene sequence similarity and different metabolic profile. Thus, strain GP20-2^T represents a novel species in the genus *Sphingomonas*, for which the name *Sphingomonas parvus* sp. nov. is proposed.

Description of *Sphingomonas parvus* sp. nov.

Sphingomonas parvus (par´pus. L. masc. adj. *parvus* small, referring to its small colonies on agar media).

Cells are strictly aerobic, Gram-staining-negative, non-motile, non-spore-forming rods, 0.3–0.5 μm wide and 1.2–1.8 μm long. It grows at 10–35°C (optimum, 35°C), at pH 5.0–8.0 (optimum, pH 6.0) and do not grow in the presence of 0.5% NaCl. Forms light-orange, glistering, and tiny colonies (0.18–0.27 mm in diameter) on R2A medium. Growth occurs on R2A and NA media. Positive for catalase and oxidase. Lipid is hydrolysed, but starch, casein, tyrosine, CM-cellulose, and Tween 20/40/60/80 are not hydrolysed. Positive for arginine dihydrolase, urease, esculin hydrolysis and gelatine hydrolysis, but negative for nitrate reduction, indole production, glucose fermentation, β-galactosidase, and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid (API 20NE). Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and α-glucosidase, but negative for lipase (C14), crystine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase activities (API ZYM). Positive for assimilation of glycogen, but negative for assimilation of L-rhamnose, *N*-acetyl-glucosamine, D-ribose, inositol, D-saccharose, itaconic acid, suberic acid, sodium malonate, sodium acetate, lactic acid, L-alanine, potassium 5-ketogluconate, 3-hydroxybenzoic acid, L-serine, salicin, D-melibiose, L-fucose, D-sorbitol, propionic acid, valeric acid, L-histidine, potassium 2-ketogluconate, 3-hydroxybutyric acid, 4-hydroxybenzoic acid, and L-proline. The major isoprenoid quinone is ubiquinone Q-10. The cellar polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol,

Table 2. Cellular fatty acid composition of strain GP20-2^T and selected type strains of *Sphingomonas* species

Strains: 1, GP20-2^T; 2, *S. daechungensis* KACC 18115^T; 3, *S. sediminicola* KACC 15039^T; 4, *S. jaspsi* KACC 13230^T; 5, *S. kaistensis* KACC 14319^T. All data are from this study. All strains were grown on R2A agar at 30°C for 4 days. Values are percentages of total fatty acids. -, Not detected.

Fatty acid	1	2	3	4	5
$C_{13:0}$ 2-OH	-	-	0.2	-	-
$C_{14:0}$	-	0.3	0.8	0.3	0.2
$C_{14:0}$ 2-OH	-	-	0.2	0.8	0.3
$C_{15:0}$ 2-OH	-	-	-	0.2	-
iso-$C_{15:1}$ G	-	-	0.1	-	-
$C_{15:1}$ $\omega6c$	-	-	-	0.2	-
$C_{16:0}$	17.0	7.9	15.4	8.3	6.6
$C_{16:0}$ 2-OH	-	0.5	0.3	0.3	-
iso-$C_{16:0}$ 3-OH	-	-	-	-	1.0
$C_{16:1}$ $\omega5c$	1.2	3.2	2.6	1.8	2.0
$C_{17:0}$	2.4	0.9	0.7	2.1	0.6
$C_{17:1}$ $\omega6c$	9.2	16.4	4.2	17.9	5.3
$C_{17:1}$ $\omega8c$	2.1	2.7	0.7	2.6	1.6
$C_{18:0}$	7.3	0.7	2.1	1.1	0.4
$C_{18:1}$ 2-OH	1.8	6.7	5.6	6.2	3.5
$C_{18:1}$ $\omega5c$	1.2	1.3	0.3	0.9	0.8
$C_{18:1}$ $\omega7c$ 11-methyl	7.0	1.4	2.1	-	2.3
$C_{18:1}$ $\omega9c$	-	-	0.9	-	-
$C_{19:0}$ cyclo $\omega8c$	-	-	0.5	-	-
Summed feature 3[a]	1.1	2.6	5.6	16.6	31.1
Summed feature 5[a]	-	0.2	-	-	-
Summed feature 8[a]	49.8	55.1	58.1	40.8	44.3

[a] Summed features are groups of two or three fatty acids that cannot be separated by the MIDI system. Summed feature 3 comprised $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$; summed feature 5 comprised $C_{18:2}\omega6,9c$ and/or $C_{18:0}$; summed feature 8 comprised $C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$.

sphingolipid, an unidentified phospholipid, an unidentified aminoglycolipid, and unidentified glycolipids. The predominant fatty acids are summed feature 8 ($C_{18:1}\omega6c$ and/ or $C_{18:1}\omega7c$, 49.8%) and $C_{16:0}$ (17.0%). The DNA G+C content of the type strain is 67.2 mol%. The type strain, GP20-2^T (=KACC 12865^T = DSM 100456^T), was isolated from a soil cultivated with ginseng in Yeongju region, Korea.

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