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Sphingomonas hunanensis sp. nov., isolated from forest soil

Qi-Hui Chen · Jin-Hua Chen · Ying Ruan · Yu-Qin Zhang · Shu-Kun Tang · Zhu-Xiang Liu · Wen-Jun Li · Yi-Guang Chen

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Abstract A novel Gram-negative, catalase- and oxidase-positive, strictly aerobic, non spore-forming, rod-shaped bacterium, designated strain JSM 083058^T, was isolated from non-saline forest soil in Hunan Province, China. Growth occurred with 0–8% (w/v) NaCl (optimum, 0.5–3%) at pH 6.0–10.0 (optimum, pH 7.0) and at 5–35°C (optimum, 25–30°C). Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain JSM 083058^T fell within the cluster comprising species of the genus *Sphingomonas*, clustering with *Sphingomonas aestuarii* K4^T, with which it shared highest 16S rRNA gene sequence similarity (99.2%). The chemotaxonomic properties of strain JSM 083058^T were consistent with those of the genus *Sphingomonas*. The predominant respiratory quinone was ubiquinone Q-10, and the major cellular fatty acids were summed feature 8 (C18:1ω7*c*/C18:1ω6*c*), C16:0, summed feature 3 (C16:1ω7*c*/C16:1ω6*c*) and C17:1ω6*c*. The polar lipids consisted of diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and sphingoglycolipid. The genomic DNA G+C content of strain JSM 083058^T was 65.5 mol%. The combination of phylogenetic analysis, DNA–DNA relatedness, phenotypic characteristics and chemotaxonomic data supported the view that strain JSM 083058^T represents a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas hunanensis* sp. nov. is

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Q.-H. Chen · J.-H. Chen · Z.-X. Liu · Y.-G. Chen (✉)
College of Biology and Environmental Sciences, Jishou University, Jishou 416000, People's Republic of China
e-mail: mchenjsu@yahoo.com.cn

J.-H. Chen · Y. Ruan
Pre-State Key Laboratory for Germplasm Innovation and Resource Utilization of Crop, Bioscience and Biotechnology College, Hunan Agricultural University, Changsha 410128, People's Republic of China

Y.-Q. Zhang
Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, People's Republic of China

S.-K. Tang · W.-J. Li
The Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan Institute of Microbiology, Yunnan University, Kunming 650091, People's Republic of China

W.-J. Li
Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, People's Republic of China

proposed. The type strain is JSM 083058^T (=CCTCC AA 209011^T = DSM 22213^T).

Keywords *Sphingomonas hunanensis* sp. nov. · Non-saline soil

Introduction

The genus *Sphingomonas* was first proposed by Yabuuchi et al. (1990) and later emended by Takeuchi et al. (1993), Yabuuchi et al. (1999), Takeuchi et al. (2001), Yabuuchi et al. (2002) and Busse et al. (2003), respectively. Members of the genus *Sphingomonas* are Gram-negative, strictly aerobic, catalase-positive, non-fermentative, non spore-forming, motile or non-motile rods with yellow, orange or off-white or cream or red colonies, and are characterized chemotaxonomically by the presence of Q-10, 2-hydroxy fatty acids and sphingolipid and the absence of 3-hydroxy fatty acids (Takeuchi et al. 2001; Yabuuchi et al. 2002; Busse et al. 2003; Asker et al. 2007; Nigam et al. 2010). At the time of writing, the genus comprises more than 40 recognized species with validly published names, including the recently described species *Sphingomonas insulae* (Yoon et al. 2008), *Sphingomonas sanxanigenens* (Huang et al. 2009), *Sphingomonas aestuarii* (Roh et al. 2009), *Sphingomonas japonica* (Romanenko et al. 2009), *Sphingomonas hankookensis* (Yoon et al. 2009), *Sphingomonas changbaiensis* (Zhang et al. 2010) and *Sphingomonas histidinilytica* (Nigam et al. 2010). During an investigation of the diversity of halophilic and/or halotolerant bacteria distributed throughout non-saline environments (Chen et al. 2010a, b, c, d), a novel *Sphingomonas*-like strain, designated JSM 083058^T, was isolated from a non-saline forest soil sample collected in Hunan Province, China. The aim of this study was to determine whether strain JSM 083058^T represents a novel species within the genus *Sphingomonas* by a polyphasic approach.

Materials and methods

Organism and culture conditions

Strain JSM 083058^T was isolated from a non-saline forest soil sample using the dilution plating technique on marine agar 2216 (MA; Difco) supplemented with 5% (w/v) NaCl and cultivated at 28°C for 2 weeks. After primary isolation and purification, the isolate was maintained as serial transfers on slants of nutrient agar (NA; Atlas and Parks 1993) supplemented with 1% (w/v) NaCl, otherwise lyophilized cultures at 4°C and also deep-frozen at −80°C in 20% (v/v) glycerol. For comparison, four type strains, *S. aestuarii* DSM 19475^T, *Sphingomonas asaccharolytica* DSM 10564^T, *Sphingomonas mali* DSM 10565^T and *Sphingomonas pruni* DSM 10566^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Unless indicated otherwise, morphological, physiological, molecular and chemotaxonomic studies were performed with cells grown on NA (pH 7.0) supplemented with 1% (w/v) NaCl at 30°C.

Phenotypic characterization

Cell morphology was examined by using light microscopy (model DM3000; Leica). The Gram staining and the KOH lysis test were carried out according to Smibert and Krieg (1994) and Gregersen (1978), respectively. Growth in the absence of NaCl was investigated in nutrient broth (NB) prepared according to the formula of Atlas and Parks (1993) except that NaCl was excluded. Tolerance of NaCl was tested in NB at different NaCl concentrations [0.1 and 0.5% (w/v), and 1–30% (w/v) in increments of 1%]. Growth was tested at various temperatures (4, 5–55°C, in increments of 5°C) and at different pH (5.0–11.0, in increments of 0.5 pH units) in NB supplemented with 1% (w/v) NaCl. The buffer solutions described by Chen et al. (2007) were used for pH experiments. Methyl red and Voges–Proskauer tests and determination of H_2S production from L-cysteine, hydrolysis of aesculin, indole production, oxidation/fermentation of glucose, nitrate reduction and activities of phenylalanine deaminase and urease were performed as recommended by Smibert and Krieg (1994). Arginine dihydrolase activity was tested as described by Møller (1955). Hydrolysis of casein, cellulose, gelatin, PNPG, starch, Tween 20, 40, 60 and 80 was determined as described by Cowan and Steel (1965). Growth under anaerobic conditions was determined on MA supplemented with 0.5% (w/v) glucose and with or without 0.1% (w/v) nitrate using the GasPak Anaerobic Systems (BBL) according to the manufacturer's

instructions. Determination of acid production from carbohydrates and utilization of carbon and nitrogen sources was performed as recommended by Ventosa et al. (1982). Observation of motility and tests of catalase and oxidase activities were detected as described previously (Chen et al. 2007). Other enzymic activities were assayed using API ZYM strips (bioMérieux) according to the manufacturer's instructions.

Determination of 16S rRNA gene sequence, phylogenetic analysis and DNA–DNA hybridization

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Cui et al. (2001). Pairwise sequence similarities were calculated using a global alignment algorithm, implemented at the EzTaxon server (Chun et al. 2007). Phylogenetic analysis was performed using the software package MEGA version 3.1 (Kumar et al. 2004) after multiple alignment of sequence data by CLUSTAL_X (Thompson et al. 1997). Distances were calculated using distance options according to Kimura's two-parameter model (Kimura 1980) and clustering was performed with the neighbour-joining method (Saitou and Nei 1987). Maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Kluge and Farris 1969) trees were generated using the treeing algorithms contained in the PHYLIP package (Felsenstein 2002). Bootstrap analysis was used to evaluate the tree topology by means of 1000 resamplings (Felsenstein 1985). DNA–DNA hybridization experiments were performed using the optical renaturation method (De Ley et al. 1970; Huß et al. 1983; Jahnke 1992). Every hybridization experiment was performed with three replications and the relatedness value was expressed as the mean of the three values.

Chemotaxonomy

For chemical studies, the organism was inoculated in NB supplemented with 1% (w/v) NaCl in flasks on a rotary shaker (with shaking at 180 rpm) at 30°C for 3 days. The biomass was harvested by centrifugation, washed twice with distilled water. Isoprenoid quinones were analysed by HPLC as described by Groth et al. (1996). Polar lipids were extracted according to the method of Minnikin et al. (1979) and were identified by two-dimensional TLC, and total lipid material and specific functional groups were detected by spraying the plate with appropriate detection reagents (Dittmer and Lester 1964; Vaskovsky et al. 1975; Ryu and MacCoss 1979; Collins and Jones 1980; Tindall 1990). The designations of all spots were referred to Busse et al. (1999). Fatty acids were determined for the new isolate as well as for strain *S. aestuarii* DSM 19475^T, according to Sasser (1990) using the Microbial Identification System (Microbial ID) with cells grown in NB supplemented with 1% (w/v) NaCl in flasks on a rotary shaker (with shaking at 200 rpm) at 30°C for 2 days. Genomic DNA was isolated according to Hopwood et al. (1985) and the G+C content was determined using the HPLC method (Mesbah et al. 1989).

Results and discussion

Phenotypic characteristics

Detailed phenotypic properties that differentiate strain JSM 083058^T from related *Sphingomonas* species are summarized in Table 1 and also mentioned in the species description below. The phenotypic properties of strain JSM 083058^T were similar to those of the related *Sphingomonas* species (Table 1). Colonies of strain 083058^T were yellow-pigmented, like those of related *Sphingomonas* species. It was Gram-negative, strictly aerobic, and formed non-motile, non spore-forming, rod-shaped cells. The organism hydrolysed aesculin, PNPG, tween 20 and 40 but not casein, cellulose and gelatin, produced catalase and oxidase but not arginine dihydrolase and phenylalanine deaminase. It was negative for glucose fermentation, nitrate reduction and methyl red and Voges–Proskauer tests. H_2S and indole were not produced (Table 1). However, strain JSM 083058^T differed clearly from its related *Sphingomonas* species by the strain's ability to tolerate up to 8% (w/v) NaCl and down to 5°C, as well as by its positive reaction for urease activity (Table 1).

Phylogenetic analysis based on 16S rRNA gene sequence comparison and DNA–DNA relatedness.

An almost-complete 16S rRNA gene sequence (1,434 bp) was determined. The GenBank/EMBL/

Table 1 Characteristics distinguishing strain JSM 083058^T from phylogenetically related *Sphingomonas* species

Characteristic	1	2	3	4	5
Motility	−	−	+	+	+
Urease	+	−	−	−	−
Oxidative acid production from glucose	−	+	+	−	−
Growth condition					
NaCl range (% w/v)	0–8	0–5	0–3	0–4	0–4
NaCl optimum (% w/v)	0.5–3	0–1	0–1	0–1	0
pH range	6.0–10.0	6.5–10.5	6.0–10.0	6.5–10.0	6.0–10.5
pH optimum	7.0	7.0–7.5	7.0	7.0–7.5	7.0–7.5
Temperature range (°C)	5–35	10–40	10–35	10–40	10–40
Temperature optimum (°C)	25–30	30	25–30	25–30	30
Hydrolysis of					
Starch	+	−	+	−	+
Tween 60	+	−	−	+	+
Tween 80	−	−	+	+	−
Source[a]	Non-saline forest soil	Tidal flat sediment	Root of plant	Root of plant	Root of plant

All strains are Gram-negative, catalase- and oxidase-positive, nonspore-forming, non-motile, aerobic rods. All strains form yellow colonies, with growth in the presence of 0–5% (w/v) NaCl at pH 6.5–10.0 and at 10–35°C. All strains are positive for aesculin hydrolysis, PNPG, tween 20 and 40, but negative for glucose fermentation, nitrate reduction, H_2S and indole production, methyl red and Voges–Proskauer test, hydrolysis of casein, cellulose and gelatin and activity of arginine dihydrolase and phenylalanine deaminase. All data were obtained from this study unless indicated otherwise

Strains: *1 S. hunanensis* sp. nov. JSM 083058^T, *2 S. aestuarii* DSM 19475^T, *3 S. asaccharolytica* DSM 10564^T, *4 S. mali* DSM 10565^T, *5 S. pruni* DSM 10566^T

"+" Positive, "−" negative or not detected

[a] Data for reference strains were from Roh et al. (2009) and Takeuchi et al. (1995)

DDBJ accession number for the 16S rRNA gene sequence of strain JSM 083058^T is FJ527417. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain JSM 083058^T should be assigned to the genus *Sphingomonas*, being most closely related to *S. aestuarii* K4^T (sequence similarity 99.2%; Roh et al. 2009), followed by *S. asaccharolytica* Y-345^T (97.6%; Takeuchi et al. 1995), *S. mali* Y-351^T (97.3%; Takeuchi et al. 1995) and *S. pruni* Y-250^T (97.1%; Takeuchi et al. 1995); lower than 97.0% sequence similarity was observed with other *Sphingomonas* species. The neighbour-joining phylogenetic tree further confirmed that strain JSM 083058^T was phylogenetically closely related to members of the genus *Sphingomonas* and formed a robust lineage with *S. aestuarii* K4^T supported by a significant bootstrap resampling value (100%) (Fig. 1). Similar tree topologies were also found in the trees maximum-likelihood and maximum-parsimony methods (Supplementary Fig. S1, available in Antonie van Leeuwenhoek Online). Levels of DNA–DNA relatedness between the novel isolate and the type strains of *S. aestuarii*, *S. asaccharolytica*, *S. mali* and *S. pruni* were 35.3, 25.8, 20.4 and 12.9%, respectively, values that are well below the threshold value (70%) recommended by Wayne et al. (1987) for the definition of members of a species. Therefore, it would appear that, on the basis of the phylogenetic and DNA–DNA hybridization data, strain JSM 083058^T warrants novel genomic species status in the genus *Sphingomonas* according to accepted criteria (Wayne et al. 1987; Stackebrandt and Goebel 1994).

Chemotaxonomic characteristics

The chemotaxonomic data for strain JSM 083058^T were consistent with its assignment to the genus *Sphingomonas*. The predominant respiratory quinone ubiquinone Q-10 (95.5%), which is characteristic of *Sphingomonas* species, but Q-9 (4.5%) was also present in minor amounts. The polar lipids of this strain consisted of diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol

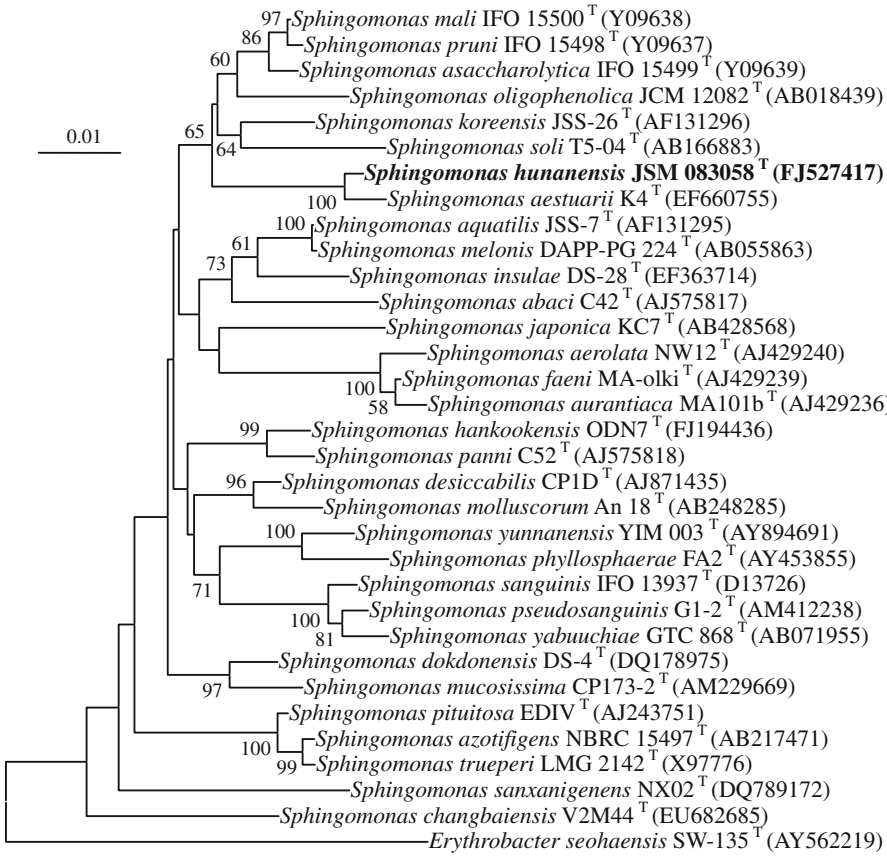

Fig. 1 Phylogenetic tree showing the phylogenetic positions of strain JSM 083058^T and related taxa based on 16S rRNA gene sequence analysis reconstructed using the neighbour-joining method. *Numbers* at nodes are bootstrap percentages (>50%) based on a neighbour-joining analysis of 1,000 resampled datasets. *Bar*, 1 substitution per 100 nucleotides

and sphingoglycolipid. The fatty acid profile of strain JSM 083058^T was similar to that of strain *S. aestuarii* DSM 19475^T, although there were differences in the proportions of some components (Table 2). Major fatty acids (>10% of the total) of strain JSM 083058^T were summed feature 8 (C18:1ω7*c*/C18:1ω6*c*; 29.9%), C16:0 (26.7%), summed feature 3 (C16:1ω7*c*/C16:1ω6*c*; 12.9%) and C17:1ω6*c* (10.0%). Minor amounts of 2-hydroxy fatty acids C14:0 2-OH (2.1%) and C15:0 2-OH (0.7%) were also present. No 3-hydroxy fatty acids were identified. This composition profile is characteristic of members of the genus *Sphingomonas* (Takeuchi et al. 2001; Roh et al. 2009). The DNA G+C content of strain JSM 083058^T was 65.5 mol%.

In conclusion, the phylogenetic analysis based on 16S rRNA gene sequences, the DNA–DNA relatedness results and the phenotypic and chemotaxonomic data presented here allowed us to assign the novel isolate to a novel species, for which we propose the name *Sphingomonas hunanensis* sp. nov.

Description of *Sphingomonas hunanensis* sp. nov.

Sphingomonas hunanensis (hu.nan.en'sis. N.L. fem. adj. *hunanensis* pertaining to Hunan Province, China, the source of the sample from which the type strain was isolated).

Cells are Gram-negative, catalase- and oxidase-positive, nonspore-forming, non-motile, strictly aerobic rods, ~0.4–0.7 μm wide and 1.2–2.5 μm long. Colonies are yellow-pigmented, circular, somewhat convex and non-translucent, have glistening surfaces and entire margins and are 1–2 mm in diameter after incubation at 28°C for 3–5 days on nutrient agar supplemented with 1% (w/v) NaCl. No diffusible pigments are produced. Growth occurs with 0–8%

Table 2 Fatty acid compositions of strains JSM 083058^T and *Sphingomonas aestuarii* DSM 19475^T

Fatty acid	*Sphingomonas hunanensis* JSM 083058^T	*Sphingomonas aestuarii* DSM 19475^T
C10:0	0.1	0.1
C12:0	0.4	0.2
C14:0	4.7	2.0
C15:1ω6*c*	–	0.3
C15:0 anteiso	0.1	–
C14:0 2-OH	2.1	2.2
C15:0 2-OH	0.7	0.8
C16:1ω5*c*	0.8	1.0
C16:0	26.7	18.6
C17:1ω6*c*	10.0	21.2
C17:1ω8*c*	0.9	2.6
C17:0	2.1	1.6
C18:1ω5*c*	0.3	0.4
C18:1ω9*c*	0.6	0.6
C18:0	0.6	0.6
C18:1ω7*c* 11-methyl	4.6	5.8
Summed feature 3[a]	12.9	14.7
Summed feature 4[a]	1.4	–
Summed feature 8[a]	29.9	26.8

Values are percentages of total fatty acid content; all data are from the present study. "–" Not detected

[a] Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed features: *3* contained C16:1ω7*c* and/or C16:1ω6*c*; *4* contained C17:1 iso I and/or anteiso B; *8* contained C18:1ω7*c* and/or C18:1ω6*c*

(w/v) NaCl (optimum 0.5–3%) and at pH 6.0–10.0 (optimum pH 7.0) and at 5–35°C (optimum 25–30°C). Nitrate is not reduced. Negative for tests of methyl red, oxidation/fermentation of glucose, Voges–Proskauer, H_2S and indole production. Aesculin, PNPG, starch, Tween 20, 40 and 60 are hydrolyzed, but cellulose, casein, gelatin and Tween 80 are not. Acid is oxidatively produced from D-fructose, D-galactose, maltose, melibiose, trehalose and D-mannitol, but not from L-arabinose, cellobiose, D-glucose, glycogen, lactose, D-mannose, melezitose, raffinose, L-rhamnose, D-ribose, ucrose, D-xylose, *N*-acetylglucosamine, amygdalin, D-salicin, adonitol, dulcitol, glycerol, *myo*-inositol or D-sorbitol. The following compounds are utilized as sole sources of carbon and energy or sole sources of carbon, nitrogen and energy: cellobiose, D-fructose, D-glucose, maltose, sucrose, *N*-acetylglucosamine and L-glutamic acid; the following are not utilized: L-arabinose, dextrin, D-galactose, glycogen, lactose, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, starch, trehalose, D-xylose, D-salicin, adonitol, D-arabitol, glycerol, *myo*-inositol, D-mannitol, D-sorbitol, acetate, butyrate, citrate, gluconate, malate, propionate, succinate, L-alanine, L-arginine, L-asparagine, glycine, L-histidine, hydroxy-L-proline, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-serine and L-valine. Constitutive enzymes expressed are *N*-acetyl-β-glucosaminidase, esterase (C4), α- and β-galactosidase, β-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase, naphthol-AS-BI-phosphohydrolase and urease; acid phosphatase, alkaline phosphatase, α-chymotrypsin, esterase lipase (C8), leucine arylamidase, arginine dihydrolase, cystine arylamidase, α-fucosidase, α-glucosidase, phenylalanine deaminase, trypsin and valine arylamidase are not observed. The predominant respiratory quinone is Q-10 and the polar lipids consist of diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and sphingoglycolipid. Major fatty acids are summed feature 8 (C18:1ω7*c*/C18:1ω6*c*), C16:0, summed feature 3 (C16:1ω7*c*/C16:1ω6*c*) and C17:1ω6*c*. Minor amounts of 2-hydroxy fatty acids C14:0 2-OH and C15:0 2-OH were also present. No 3-hydroxy fatty acids were identified. The DNA G + C content of the type strain is 65.5 mol% (HPLC method).

The type strain is JSM 083058^T (=CCTCC AA 209011^T = DSM 22213^T), which is the isolate on which the species description is based. It was isolated from non-saline forest soil in Hunan Province, China.

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