Sphingomonas changbaiensis sp. nov., isolated from forest soil

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Bacterial strain V2M44^T was isolated from forest soil from the Changbai Mountains, Heilongjiang Province, China. Cells of strain V2M44^T were Gram-negative, rod-shaped and 0.3–0.4 µm in diameter and 1.5–2.5 µm long. The cells were strictly aerobic and were motile by means of peritrichous flagella. Growth occurred at 20–33 °C (optimum, 29–31 °C), at pH 5–8 (optimum, pH 6.0–7.0) and in the presence of 0–0.1 % (w/v) NaCl (optimum, 0.05 %). Strain V2M44^T contained C_{17:1}ω6c (38.9 %) and summed feature 7 (comprising C_{18:1}ω7c, C_{18:1}ω9t and/or C_{18:1}ω12t, 31.5 %) as the major cellular fatty acids and Q-10 as the major respiratory quinone. The major polar lipids were sphingoglycolipid, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol along with two unidentified phospholipids and four unidentified glycolipids. The major component in the polyamine pattern was *sym*-homospermidine. The DNA G + C content was 65.8 mol%. Analysis of 16S rRNA gene sequences indicated that strain V2M44^T was phylogenetically related to members of the genus *Sphingomonas*, with sequence similarities ranging from 92.0–95.8 %. Based on these results, it is concluded that strain V2M44^T represents a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas changbaiensis* sp. nov. is proposed. The type strain is V2M44^T (=CGMCC 1.7057^T=NBRC 104936^T).

The genus *Sphingomonas* was proposed by Yabuuchi *et al.* (1990) and has been subsequently emended by Takeuchi *et al.* (2001), Yabuuchi *et al.* (2002) and Busse *et al.* (2003). On the basis of phylogenetic analysis of 16S rRNA gene sequences, polyamine patterns and polar lipid profiles, Takeuchi *et al.* (2001) proposed that the genus *Sphingomonas* should be classified into four genera *Sphingomonas*, *Novosphingobium*, *Sphingobium* and *Sphingopyxis*, however, the phenotypic evidence to support this proposal was weak (Yabuuchi *et al.*, 2002). According to Busse *et al.* (2003), species of the genus *Sphingomonas* comprising cluster I as proposed by Takeuchi *et al.* (2001), with *sym*-homospermidine as the major polyamine pattern, should be considered as members of *Sphingomonas sensu stricto*, with *Sphingomonas paucimobilis* as the type species. Members of this genus are off-white, yellow or orange-pigmented, non-fermentative, Gram-negative, non-motile or motile rods with a single polar

flagellum or peritrichous flagella and are characterized by the presence of *sym*-homospermidine, a unique sphingoglycolipid, Q-10, 2-hydroxymyristic acid and the absence of 3-hydroxy fatty acids. In this study, we report the isolation and identification of a novel isolate, strain V2M44^T, from a sample of forest soil.

In an attempt to study the cultured microbial diversity in forest soil from the Changbai Mountains, Heilongjiang Province, China, bacterial strains were isolated by using a serial dilution method (Sait *et al.*, 2002). V2M isolation medium was modified from VL55 medium (Sait *et al.*, 2002) by the addition of (I⁻): 5 ml amino acid mixture (Davis *et al.*, 2005), 1 ml vitamin solution 1 (Janssen *et al.*, 1997), 3 ml vitamin solution 2 (Janssen *et al.*, 1997), 100 µl solution 1 and 5 ml solution 2. Gellan (1.5 %) was used as the solidifying agent. Solution 1 contained the following ingredients (I⁻): 4 mg SrCl₂, 3 mg NaSiO₃·9H₂O, 3 mg TiO₂, 2 mg Cd(NO₃)₂·4H₂O and 2 mg SnCl₂·H₂O. Solution 2 contained (I⁻): 30 mg DL-glyceraldehyde, 20 mg chromotropic acid, 70 mg gallic acid, 30 mg ascorbic acid, 10 mg sodium thioglycolate, 50 mg sodium succinate, 50 mg DL-malic acid, 50 mg saligenin, 50 mg sorbitol, 40 mg salicin, 20 mg barbitol, 20 mg aconitic acid, 30 mg creatine and 30 mg sodium deoxycholate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain V2M44^T is EU682685.

Figures showing additional phylogenetic trees, scanning and transmission electron micrographs of cells of strain V2M44^T and the results of the polar lipid analysis are available as supplementary figures. A supplementary table detailing the cellular fatty acid contents of strain V2M44^T is also available with the online version of this paper.

Strain V2M44^T was isolated from several V2M plates inoculated with the 10⁻⁷ dilution after one week of incubation at 30 °C. Each plate displayed several tiny yellow colonies of strain V2M44^T.

The nearly complete 16S rRNA gene of strain V2M44^T (1477 bp) was amplified and sequenced according to the method described by Zhang *et al.* (2003). DNA BLAST searches (Altschul *et al.*, 1990) of 16S rRNA gene similarity using the NCBI database showed that strain V2M44^T was phylogenetically related to members of the genus *Sphingomonas*. Alignments of the 16S rRNA gene sequences of members of the genus *Sphingomonas* were performed with the CLUSTAL_X program (version 1.64b, Thompson *et al.*, 1997) and the alignment positions with insertions or deletions were excluded from calculations. Phylogenetic trees based on 16S rRNA gene sequence analysis were constructed by the neighbour-joining and maximum-parsimony methods with Kimura's two-parameter calculation model in MEGA version 3.1 (Kumar *et al.*, 2004), and by the maximum-likelihood method in fastDNAmI version 1.2.2 (Olsen *et al.*, 1994; Felsenstein, 1981). 16S rRNA gene sequence analysis indicated that strain V2M44^T was phylogenetically related to members of the genus *Sphingomonas*, with sequence similarities ranging from 92.0 to 95.8 %. In particular, strain V2M44^T was closely related to *Sphingomonas mali* NBRC 15500^T (95.8 %), *Sphingomonas kaistensis* PB56^T (95.8 %), *Sphingomonas oligophenolica* S213^T (95.8 %), *Sphingomonas dokdonensis* DS-4^T (95.6 %), *Sphingomonas pituitosa* EDIV^T (95.6 %), *Sphingomonas pruni* NBRC 15498^T (95.6 %), *Sphingomonas aerolata* NW12^T (95.4 %), *Sphingomonas asaccharolytica* NBRC 15499^T (95.3 %), *Sphingomonas azotifigens* Y39^T (95.3 %), *Sphingomonas trueperi* ATCC 12417^T (95.3 %), *Sphingomonas desiccabilis* CP1D^T (95.2 %), *Sphingomonas molluscorum* KMM 3882^T (95.2 %), *Sphingomonas echinoides* DSM 1805^T (95.1 %), *Sphingomonas astaxanthinifaciens* TDMA-17^T (95.1 %), *Sphingomonas aquatilis* JSS7^T (95.0 %), and *Sphingomonas koreensis* JSS26^T (95.0 %). The phylogenetic tree (Fig. 1) also indicated that strain V2M44^T clustered with species of the genus *Sphingomonas* and the topologies of the maximum-parsimony tree and maximum-likelihood tree were essentially the same (see Supplementary Figs S1 and S2 in IJSEM Online).

The morphological, physiological and biochemical characteristics of strain V2M44^T were investigated using routine cultivation on diluted tryptic soy broth (D-TSB) medium at 30 °C. The D-TSB medium was 1/5 strength tryptic soy broth agar and contained 0.6 g of Bacto tryptic soy broth dissolved in 100 ml water. Growth was also assessed in tryptic soy broth (TSB), 1/2, 1/10 and 1/20 strength TSB, as well as in Luria–Bertani broth (LB), 1/10 strength LB, nutrient broth (NB, Yabuuchi *et al.*, 2002) and 1/2 strength NB. NB contained 0.5 g Bacto peptone and 0.3 g Bacto yeast extract in 100 ml water. The Gram stain was performed according to Gerhardt *et al.* (1994) with cells grown on D-TSB agar at 30 °C for 3 days. Morphological observations were performed by transmission (H-600; Hitachi) and scanning

(Quanta 200; FEI) electron microscopy. The presence of flagella, gliding motility and growth under anaerobic conditions were examined according to Dong & Cai (2001). The growth temperature range was determined with a TN3F temperature gradient incubator (Advantec) at 12, 13, 14, 16, 17.5, 19, 21, 22, 24, 27, 28, 29, 31, 34, 36, 38, 41, 45, 49, 51 and 56 °C. The pH range for growth was tested at pH 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.1 and 9.8 by using D-TSB in which the pH was adjusted by the addition of 5 M NaOH or HCl and verified after autoclaving. Tolerance to NaCl was examined in D-TSB supplemented with 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0 and 2.0 % (w/v) NaCl. Tests for catalase and oxidase activities and for the hydrolysis of casein, starch, Tween 20 and Tween 80 were performed according to Dong & Cai (2001). In addition, strain V2M44^T was characterized by using API 20NE, API 50CH and API ZYM identification systems (bioMérieux) at 30 °C.

Cells of strain V2M44^T were Gram-negative, rod-shaped and 0.3–0.4 µm in diameter and 1.5–2.5 µm in length. Cells were strictly aerobic and were motile by means of peritrichous flagella (see Supplementary Fig. S3 in IJSEM Online). Detailed physiological and biochemical characteristics of strain V2M44^T are presented in the species description and the properties that distinguish the novel strain from closely related species are listed in Table 1.

The cell mass of strain V2M44^T and *S. mali* JCM 10193^T used for chemotaxonomic analyses was produced on D-TSB agar at 30 °C for 3 days. Cellular fatty acids were extracted, methylated and analysed by the Sherlock Microbial Identification System following the manufacturer's instructions. Isoprenoid quinones were extracted and purified according to the method of Collins (1985) and analysed by HPLC (Wu *et al.*, 1989; Hu *et al.*, 2004). Polyamines were analysed as described previously (Flores & Galston, 1982), with strain *S. mali* JCM 10193^T (Takeuchi *et al.*, 1995) as the reference strain in a parallel experiment. Polar lipids were extracted according to the modified method of Kamekura (1993) and separated by TLC on Merck Kieselgel 60-HPTLC by two-dimensional development as described by Ross *et al.* (1985). Phospholipids were detected with the Zinzadze reagent of Dittmer & Lester (1964). Glycolipids were detected by spraying the plate with 0.5 % 1-naphthol in methanol/water (1:1 v/v) and then with sulfuric acid/ethanol (1:1 v/v), followed by heating at 120 °C for 5–10 min (Xin *et al.*, 2001). Whole lipid profiles were detected by spraying with molybdatophosphoric acid (10 g molybdatophosphoric acid hydrate in 100 ml ethanol) followed by heating at 120–160 °C (Worliczek *et al.*, 2007).

The predominant isoprenoid quinone of strain V2M44^T was ubiquinone-10 (Q-10). The fatty acid profile of strain V2M44^T comprised C_{17:1}ω6c (38.9 %), summed feature 7 (comprising C_{18:1}ω7c, C_{18:1}ω9t and/or C_{18:1}ω12t, 31.5 %), C_{16:0} (5.3 %) and C_{17:1}ω8c (5.2 %). The hydroxy fatty acids were C_{15:0} 2-OH (4.4 %), C_{16:0} 2-OH (1.8 %) and C_{14:0} 2-OH (1.3 %). No 3-hydroxy fatty acids were detected in this strain. The fatty acid composition of the

Fig. 1. Phylogenetic tree constructed with the neighbour-joining method according to the 16S rRNA gene evolutionary distance between strain V2M44^T and the type strains of the genera *Sphingomonas*, *Sphingobium*, *Sphingopyxis*, *Sphingosinicella* and *Novosphingobium*. The topology shows that strain V2M44^T and other species of the genus *Sphingomonas* are clustered together. *Rhodospirillum rubrum* ATCC 11170^T was used as the outgroup. GenBank accession numbers are given in parentheses. The numbers at the nodes represent the confidence levels from bootstrap sampling (100 replicates). Bar, 0.01 evolutionary distance (K_{nuc}).

strain V2M44^T is given in more detail in Supplementary Table S1 (available in IJSEM Online). The major polyamine was *sym*-homospermidine. The major polar lipids were sphingoglycolipid, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, two unidentified phospholipids and four unidentified glycolipids (see Supplementary Fig. S4). Phosphatidyltrimethylethanolamine and phosphatidylcholine were not detected, distinguishing strain V2M44^T from *S. pituitosa*, *S. pruni*, *S. asaccharolytica* and *S. trueperi* (Denner *et al.*, 2001; Kämpfer *et al.*, 1997). The chemotaxonomic features of strain V2M44^T, including Q-10 as the major isoprenoid quinone, C_{17:1}ω6c and summed feature 7 as the major fatty acids, the absence of 3-hydroxy fatty acids and

the presence of sphingoglycolipid are as expected for species closely related to the genus *Sphingomonas* (Busse *et al.*, 2003; Denner *et al.*, 1999, 2001; Kämpfer *et al.*, 1997; Lee *et al.*, 2001; Reddy & Garcia-Pichel, 2007; Romanenko *et al.*, 2007; Takeuchi *et al.*, 1995; Xie & Yokota, 2006; Yoon *et al.*, 2006).

The DNA base composition was determined by thermal denaturation (Marmur & Doty, 1962) and *Escherichia coli* K-12^T was used as the reference. The DNA G + C content of strain V2M44^T was 65.8% (T_m), which is within the range of values (60.7–69.9%) previously reported for the genus *Sphingomonas*.

Based on the above phenotypic and phylogenetic studies, it is clear that strain V2M44^T is a member of the genus

Table 1. Phenotypic characteristics that differentiate strain V2M44^T from related members of the genus *Sphingomonas*

Taxa: 1, V2M44^T; 2, *S. mali* NBRC 15500^T (data from Takeuchi *et al.*, 1995); 3, *S. kaistensis* PB56^T (Kim *et al.*, 2007); 4, *S. oligophenolica* S213^T (Ohta *et al.*, 2004); 5, *S. dokdonensis* DS-4^T (Yoon *et al.*, 2006); 6, *S. pituitosa* EDIV^T (Denner *et al.*, 2001); 7, *S. pruni* NBRC 15498^T (Takeuchi *et al.*, 1995); 8, *S. aerolata* NW12^T (Busse *et al.*, 2003); 9, *S. asaccharolytica* NBRC 15499^T (Takeuchi *et al.*, 1995); 10, *S. azotifigens* Y39^T (Xie & Yokota, 2006); 11, *S. trueperi* ATCC 12417^T (Kämpfer *et al.*, 1997); 12, *S. desiccabilis* CP1D^T (Reddy & Garcia-Pichel, 2007); 13, *S. molluscorum* KMM 3882^T (Romanenko *et al.*, 2007); 14, *S. echinoides* DSM 1805^T (Denner *et al.*, 1999); 15, *S. astaxanthinifaciens* TDMA-17^T (Asker *et al.*, 2007); 16, *S. aquatilis* JSS7^T (Lee *et al.*, 2001); 17, *S. koreensis* JSS26^T (Lee *et al.*, 2001). All strains are negative for indole production and arginine dihydrolase activity; all strains are positive for catalase activity. +, Positive; –, negative; (+), weakly positive; ND, not determined or no data; Y, yellow; LY, light yellow; DY, dark yellow; O, orange; OR, orange red; PY, pale yellow.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Colony colour	Y	LY	OR	PY	Y	DY	LY	O	LY	Y	Y	Y	Y	Y	DY	Y	Y
Motility	+	+	–	–	+	+	+	+	+	+	+	–	–	+	+	+	+
Oxidase activity	+	+	+	+	+	–	ND	+	ND	+	+	+	+	+	–	+	+
Nitrate reduction	–	–	–	+	–	–	–	–	–	–	ND	+	+	ND	–	–	–
Urease activity	+	–	–	–	–	–	–	–	–	ND	–	–	–	–	–	–	–
Gelatin hydrolysis	+	–	–	+	+	–	–	–	–	–	+	+	–	–	+	–	–
Aesculin hydrolysis	+	+	–	ND	+	+	(+)	+	+	+	+	–	+	+	+	–	–
β -Galactosidase	–	+	–	ND	–	+	+	–	+	ND	+	+	+	+	–	+	+
Assimilation of:																	
N-Acetyl-D-glucosamine	–	+	+	ND	–	+	+	+	+	ND	+	ND	+	+	–	+	+
Adipate	+	+	+	ND	+	–	–	(+)	–	–	–	ND	–	–	–	–	–
L-Arabinose	–	(+)	–	+	–	+	+	–	(+)	+	+	+	+	+	–	+	–
Caprate	–	–	–	ND	–	–	–	–	–	ND	–	ND	–	ND	–	+	–
Citrate	–	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–
Gluconate	+	+	(+)	+	–	–	–	+	–	–	–	–	+	–	–	–	–
D-Glucose	(+)	+	+	+	+	+	+	+	(+)	+	+	+	+	(+)	–	+	+
Malate	(+)	–	+	–	–	+	+	+	–	+	+	ND	–	+	–	–	+
Maltose	–	–	+	+	–	+	+	+	+	+	+	+	+	(+)	–	+	+
D-Mannitol	–	–	+	ND	–	–	–	–	–	ND	–	–	–	–	–	–	–
D-Mannose	–	+	+	+	+	+	+	+	(+)	ND	+	+	+	–	–	–	–
Phenylacetate	–	ND	–	–	–	–	–	–	ND	ND	–	ND	–	–	–	–	–
DNA G + C content (mol%)	65.8	65.4–65.9	69.9	64.2	66.9	64.5	65.4	65.4	64.8	68.0	65.6	ND	68.3	65.8	63.3	63.0	66.0

Sphingomonas. Strain V2M44^T exhibited a range of phenotypic characteristics that differentiated it from the currently recognized species of the genus *Sphingomonas* (Table 1), such as urease and β -galactosidase activities, assimilation of various carbon sources and the polar lipid profile. Therefore, it is concluded that strain V2M44^T represents a novel species of the genus *Sphingomonas* for which the name of *Sphingomonas changbaiensis* sp. nov. is proposed.

Description of *Sphingomonas changbaiensis* sp. nov.

Sphingomonas changbaiensis (chang.bai.en'sis. N.L. fem. adj. *changbaiensis* pertaining to the Changbai mountains, in the north-east of China, from where the type strain was isolated).

Strictly aerobic, Gram-negative, heterotrophic, oxidase- and catalase-positive. Cells are rods of 0.3–0.4 μ m in diameter and 1.5–2.5 μ m in length and are motile by means of peritrichous flagella. Colonies on D-TSB agar are circular,

entire and yellow. No growth is detected on TSB agar, 1/2 strength TSB agar, LB agar, 1/10 strength LB agar, NB agar or 1/2 strength NB agar. Growth occurs on 1/5 and 1/10 strength TSB agar. Growth occurs at 20–33 °C (optimum, 29–31 °C), at pH 5–8 (optimum, pH 6.0–7.0) and in the presence of 0–0.1 % (w/v) NaCl (optimum, 0.05 %). Nitrate is not reduced. Gelatin, aesculin, Tween 20 and Tween 80 are hydrolysed, but casein and starch are not hydrolysed. Urease activity is present, but arginine dihydrolase activity is absent. Indole and H₂S are not produced. Assimilates D-glucose, gluconate, adipate and malate, but not the following compounds: D-mannitol, maltose, L-arabinose, D-mannose, N-acetyl-D-glucosamine, caprate, citrate and phenylacetate. In the API 50 CH test system, acids are produced from glycerol, D-fructose, N-acetylglucosamine, aesculin and ferric citrate. All other API 50 CH test results are negative. In the API ZYM test system, alkaline phosphatase, esterase, lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, α -chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are present. Esterase (C4), lipase (C14), trypsin, N-acetyl- β -glucosami-

nidase, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, α -glucosidase, α -mannosidase and α -fucosidase activities are absent. The predominant cellular fatty acids are C_{17:1}ω6c, summed feature 7 (comprising C_{18:1}ω7c, C_{18:1}ω9t and/or C_{18:1}ω12t), C_{16:0}, C_{17:1}ω8c and C_{15:0} 2-OH. The major isoprenoid quinone is Q-10. The major polyamine is *sym*-homospermidine. The major polar lipids are sphingoglycolipid, phosphatidylethanolamine and phosphatidylglycerol.

The type strain, V2M44^T (=CGMCC 1.7057^T=NBRC 104936^T), was isolated from forest soil. The DNA G+C content of the type strain is 65.8 mol% (*T*_m).

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