Sphingomonas alpina sp. nov., a psychrophilic bacterium isolated from alpine soil

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An aerobic, Gram-negative-staining, motile, psychrophilic bacterium, designated strain S8-3^T, was isolated from alpine soil. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain S8-3^T was related to the genus *Sphingomonas* and had highest 16S rRNA gene sequence similarity with *Sphingomonas oligophenolica* S213^T (98.0 %). 16S rRNA gene sequence similarity between strain S8-3^T and *Sphingomonas paucimobilis* ATCC 29837^T (the type species of the genus *Sphingomonas*) was 93.0 %. Strain S8-3^T contained Q-10 as the ubiquinone and C_{18:1}ω7c (65.0 %) and C_{14:0} 2-OH (13.4 %) as the dominant fatty acids (>10 %). The major polyamines were the triamine sym-homospermidine and spermidine. The polar lipid profile contained sphingoglycolipid, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylmonomethylethanolamine. The DNA G + C content was 64.1 mol%. Combined data from phenotypic, phylogenetic and DNA–DNA relatedness studies demonstrated that strain S8-3^T is a representative of a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas alpina* sp. nov. is proposed. The type strain is S8-3^T (=DSM 22537^T=LMG 26055^T).

The genus *Sphingomonas* was first proposed by Yabuuchi *et al.* (1990) and has been emended subsequently by Takeuchi *et al.* (1993, 2001), Yabuuchi *et al.* (2002) and Busse *et al.* (2003) to accommodate aerobic, Gram-negative and non-spore-forming bacteria that are characterized chemotaxonomically by the absence of 3-hydroxy fatty acids and by the presence of 2-hydroxymyristic acid (C_{14:0} 2-OH), Q-10 as the respiratory quinone and sym-homospermidine as the key polyamine. So far, the nomenclature of Takeuchi *et al.* (2001) is generally used (Busse *et al.*, 2003).

Strain S8-3^T was isolated from alpine soil collected in the Hohe Tauern/Grossglockner area (Pifflalpe) on the north slope at 1500 m above sea level (Margesin *et al.*, 2009). The strain was isolated as previously described (Zhang *et al.*, 2010). One of the pure cultures was yellow-pigmented and was designated S8-3^T. Strain S8-3^T was routinely cultured on R2A agar (containing 0.05 % yeast extract, 0.05 %

peptone, 0.05 % Casamino acids, 0.05 % glucose, 0.05 % starch, 0.03 % sodium pyruvate, 0.03 % K₂HPO₄, 0.005 % MgSO₄, 1.5 % agar; pH 7; Reasoner & Geldreich, 1985) at 20 °C, and stored as a suspension in skimmed milk (10 %, w/v) at –80 °C. *Sphingomonas echinoides* DSM 1805^T, *Sphingomonas oligophenolica* S213^T and *Sphingomonas glacialis* C16y^T (=DSM 22294^T; Zhang *et al.*, 2011) were routinely grown on R2A agar at 20 °C and used as reference strains.

DNA of strain S8-3^T was extracted and purified as described by Sambrook *et al.* (1989). The 16S rRNA gene was amplified by PCR (Margesin *et al.*, 2012) with a pair of universal primers, 27F (5'-AGAGTTTGATCCTGGCTC-AG-3') and 1541R (5'-AAGGAGGTGATCCAGCCGCA-3'). The amplification product was cloned into vector pGEM-T Easy (Promega), and the recombinant plasmid was propagated in *Escherichia coli* according to the manufacturer's instructions (Zhang *et al.*, 2011). After aligning sequences retrieved from GenBank and EMBL databases using CLUSTAL_X version 1.8 (Thompson *et al.*, 1997), phylogenetic analysis was carried out using MEGA version 5.0 (Tamura *et al.*, 2011). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms in MEGA5 software (<http://www.megasoftware.net/>). The neighbour-joining tree (Fig. 1) showed that strain S8-3^T grouped with the genus *Sphingomonas* and formed a distinct cluster

Abbreviations: APL, aminophospholipids; DPG, diphosphatidylglycerol; GL, glycolipids; L, polar lipids; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipids; PME, phosphatidylmonomethylethanolamine; SGL, sphingoglycolipid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Sphingomonas alpina* S8-3^T is GQ161989.

Three supplementary figures and a supplementary table are available with the online version of this paper.

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic positions of strain S8-3^T, some members of the genus *Sphingomonas* and representatives of other related taxa. Numbers at nodes represent percentages of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets. Asterisks indicate the nodes that were also found in the maximum-likelihood tree. GenBank accession numbers of 16S rRNA sequences are given in parentheses. Bar, 0.02 substitutions per site.

with *S. oligophenolica* S213^T (98.0 % pairwise 16S rRNA gene sequence similarity), *S. echinoides* DSM 1805^T (96.8 %) and *S. glacialis* C16y^T (96.5 %), whereas it shared only 93.0 % with the type species of the genus *Sphingomonas paucimobilis*. A similar topology was found in the maximum-likelihood tree (Fig. S1, available in IJSEM online). Strain S8-3^T exhibited 16S rRNA nucleotide signatures that were the same as those of the genus *Sphingomonas* (cluster I) reported by Takeuchi *et al.* (2001), 52:359 (C–G), 134 (G), 593 (G), 987:1218 (G–C) and 990:1215 (U–G) (*E. coli* numbering; Brosius *et al.*, 1978).

Cell morphology was examined by phase-contrast microscopy ($\times 1000$; Leitz Diaplan) and by transmission electron

microscopy (Libra 120 EFTEM; Zeiss) of cells grown on R2A agar at 20 °C. Motility was examined by microscopy ($\times 1000$) and by the API M system (bioMérieux). Gram-reaction was tested by Gram-staining and was confirmed by KOH lysis (Süßmuth *et al.*, 1987). Catalase activity was determined by bubble production in 3 % (v/v) H₂O₂ and cytochrome *c* oxidase activity was determined using 1 % (w/v) *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Physiological and biochemical characteristics and enzyme activities were determined with the API 20 E, API 20 NE and API ZYM systems (bioMérieux) at 20 °C. Amylase, lipase and protease were also tested on R2A agar supplemented with the appropriate substrates (Margesin *et al.*, 2003). Growth at 1–42 °C was assessed on R2A agar

and in R2A broth at 150 r.p.m. Growth at pH 5–11 (using buffered medium) and with 0–10 % (w/v) NaCl was determined on R2A agar. Growth on different media [R2A, NA (nutrient agar), TSA (trypticase soy agar; 1.5 % casein peptone, 0.5 % soy peptone, 0.5 % sodium chloride, 1.5 % agar; pH 7)] was assessed on agar plates. Susceptibility to antibiotics was determined on R2A agar supplemented with various antibiotics and incubated at 20 °C. All tests were carried out simultaneously with strain S8-3^T and the three reference strains using 2–3 replicates for each test. The morphological, physiological and biochemical characteristics of strain S8-3^T are given in the species description and the features that differentiate strain S8-3^T from the reference strains are given in Table 1.

The respiratory quinones were extracted and purified according to Collins (1985) and were analysed by HPLC

Table 1. Phenotypic characteristics that differentiate strain S8-3^T from the type strains of its closest phylogenetic neighbours

Strains: 1, *Sphingomonas alpina* sp. nov. S8-3^T; 2, *S. oligophenolica* S213^T; 3, *S. echinoides* DSM 1805^T; 4, *S. glacialis* C16y^T. All data were obtained in this study. All strains are positive for catalase, cytochrome *c* oxidase, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase and β -galactosidase. All strains are negative in tests for hydrolysis of starch, indole production, H₂S production, citrate utilization, urease, lysine dihydrolase, ornithine dihydrolase, arginine dihydrolase, tryptophan deaminase, α -mannosidase, fermentation of glucose, assimilation of D-mannitol, capric acid, adipic acid, trisodium citrate and phenylacetic acid and sensitivity (100 μ g ml⁻¹) to kanamycin and tetracycline. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4
Growth on R2A agar at/with:				
30 °C	w	+	+	w
pH 5	–	–	+	+
pH 8	+	–	+	+
1 % (w/v) NaCl	+	–	+	+
Nitrate reduction	–	+	–	–
Hydrolysis of gelatin	+	+	–	–
Enzymes (API ZYM)				
Lipase (C14)	+	–	–	–
N-Acetyl- β -glucosaminidase	–	+	+	–
α -Fucosidase	–	+	–	–
Assimilation of (API 20 NE):				
D-Glucose	+	–	+	+
D-Mannose	+	–	–	–
Potassium gluconate	–	w	+	–
Malic acid	+	–	w	w
Resistance to (100 μ g ml ⁻¹):				
Penicillin	+	–	+	+
Ampicillin	+	–	+	–
Streptomycin	+	–	+	w
Rifampicin	+	–	–	–
Chloramphenicol	+	–	–	–

(Wu *et al.*, 1989), using Q-10 from *S. echinoides* DSM 1805^T and *S. oligophenolica* S213^T as a reference. The ubiquinone found in strain S8-3^T was Q-10.

For fatty acid methyl ester analysis, strain S8-3^T and the reference strains (*S. echinoides* DSM 1805^T, *S. oligophenolica* S213^T and *S. glacialis* C16y^T) were grown on R2A agar at 20 °C for 2 days. All type strains included in the fatty acid analyses exhibited similar growth and sufficient cells of comparable physiological age could be harvested from the third streak quadrant of the R2A agar plates after cultivation under the same conditions. The fatty acid methyl esters were extracted and prepared according to the standard protocol of the Sherlock Microbial Identification System (MIDI, version 6.1) (Sasser, 1990), using a gas chromatograph (6890N; Agilent), and were identified using the TSBA (version 4.10) database. Fatty acid analyses were carried out by the Identification Service of the DSMZ (Braunschweig, Germany). The predominant cellular fatty acids (>5 %) of strain S8-3^T were C_{18:1 ω 7c} (65.0 %), C_{14:0} 2-OH (13.4 %), summed feature 3 comprising C_{16:1 ω 7c} and/or iso-C_{15:0} 2-OH (7.9 %), C_{16:0} (6.9 %), 11-methyl C_{18:1 ω 7c} (3.5 %) and C_{16:1 ω 5c} (1.6 %) (Table S1). Since the fatty acid profile of strain S8-3^T did not contain other branched fatty acids, which is in agreement with all species of the family *Sphingomonadaceae*, it is most likely that summed feature 3 is representing exclusively C_{16:1 ω 7c} and not iso-C_{15:0} 2-OH. The fatty acid profile of strain S8-3^T was in agreement with those of other members of the genus *Sphingomonas* (Busse *et al.*, 1999, 2003, 2005; Denner *et al.*, 1999; Kämpfer *et al.*, 2007; Ohta *et al.*, 2004; Zhang *et al.*, 2011).

For polyamine analysis, strain S8-3^T was grown in PYE medium (containing 0.3 % yeast extract, 0.3 % peptone; pH 7.2) at 20 °C. Polyamines were extracted as described by Busse & Auling (1988) and analysed as described by Busse *et al.* (1997) using the HPLC equipment reported by Stolz *et al.* (2007). The polyamine pattern of strain S8-3^T consisted of [μ mol (g dry mass)⁻¹] the predominant triamine *sym*-homospermidine (36.1), spermidine (11.2) and minor amounts of spermine (2.3), cadaverine (0.1) and putrescine (0.1). The presence of *sym*-homospermidine as a major compound in the polyamine pattern has been considered as a diagnostic feature of the genus *Sphingomonas* (Busse *et al.*, 1999; Takeuchi *et al.*, 2001) and species recently assigned to this genus also share this trait (Busse *et al.*, 2003, 2005; Kämpfer *et al.*, 2007; Nigam *et al.*, 2010). However, it has been reported that species of the related genus *Sphingosinicella* also contain *sym*-homospermidine as the major compound (Geueke *et al.*, 2007) in the polyamine pattern, which hampers the classification of novel strains within this family.

Polar lipid profiles were analysed according to Tindall (1990a, b). Molybdatophosphoric acid, molybdenum blue, ninhydrin, α -naphthol and Dragendorff reagent were used to detect total lipids, phospholipids, aminolipids, glycolipids and choline-containing lipids, respectively. The polar lipid profile of strain S8-3^T contained sphingoglycolipid

(SGL), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME) and two unidentified aminophospholipids (APL1–2), two unidentified glycolipids (GL1–2), seven unidentified phospholipids (PL1–7) and eight unidentified polar lipids (L1–8) (Fig. S2). PE, PG, DPG and SGL are found in all sphingomonads (Busse *et al.*, 1999) and were consequently also detected in the polar lipid profile of strain S8-3^T. Like *S. echinoides* DSM 1805^T and *S. glacialis* C16y^T, strain S8-3^T contained PG, DPG, SGL, PE and APL in the polar lipid profile (Denner *et al.*, 1999; Zhang *et al.*, 2011). However, the presence of PME, GL1–2 and many unidentified polar lipids (L) distinguished strain S8-3^T from *S. echinoides* DSM 1805^T. The presence of PME, GL1 and many unidentified polar lipids, as well as the absence of additional glycolipids (designated GL1 and GL3–5 in *S. glacialis*), distinguished strain S8-3^T from *S. glacialis* C16y^T (Zhang *et al.*, 2011).

The DNA G + C content of strain S8-3^T, as determined by using standard HPLC analysis (Mesbah *et al.*, 1989), was 64.1 mol%. DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) using a UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Cary 100 Bio; Varian). Strain S8-3^T showed relatively low levels of DNA–DNA relatedness of 13.7% and 16.8% with *S. oligophenolica* S213^T and *S. echinoides* DSM 1805^T, respectively. Both the DNA G + C content and the DNA–DNA relatedness were determined by the Identification Service of the DSMZ (Braunschweig, Germany).

The results of the analyses of 16S rRNA gene sequences, polyamine patterns, the quinone system, cellular fatty acids and polar lipids demonstrate that strain S8-3^T is a psychrophilic representative of the genus *Sphingomonas* and shares the ability to grow well over a temperature range of 1–25 °C with its phylogenetic neighbour *S. glacialis* C16y^T. We use the term psychrophile as a general term that describes a micro-organism that grows in a cold environment (Margesin *et al.*, 2008), since the use of growth rates to define the optimum growth temperature as described by Morita (1975) has been shown to be ambiguous and inappropriate (Cavicchioli, 2006; Margesin, 2009). Strain S8-3^T could be easily differentiated from its closest phylogenetic neighbours (*S. echinoides* DSM 1805^T, *S. oligophenolica* S213^T and *S. glacialis* C16y^T) by its ability to produce lipase (C14) and to assimilate D-mannose and by its resistance (µg ml⁻¹) to rifampicin (30–100) and chloramphenicol (100). In addition, strain S8-3^T could be differentiated from *S. oligophenolica* S213^T by its ability to grow at pH 8–10 and to assimilate malic acid, by its resistance to a number of antibiotics (Table 1) as well as by its weak growth at 30 °C and its inability to reduce nitrate and to produce *N*-acetyl-β-glucosaminidase and α-fucosidase. The ability to hydrolyse gelatin and the inability to grow at pH 5 distinguished strain S8-3^T from *S. echinoides*

DSM 1805^T and *S. glacialis* C16y^T. Therefore, on the basis of phenotypic, phylogenetic and genomic evidence, strain S8-3^T is a representative of a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas alpina* sp. nov. is proposed.

Description of *Sphingomonas alpina* sp. nov.

Sphingomonas alpina (al.pi'na. L. fem. adj. *alpina* of or pertaining to the Alps, Alpine, referring to the isolation of this strain from an alpine environment).

Cells are aerobic, Gram-negative-staining, motile (polar flagellation, Fig. S3), short rods (0.6–0.8 × 1.1–1.4 µm after 6 days at 20 °C on R2A agar). Colonies on R2A agar are yellow, convex and round with entire margins (2 mm in diameter after 6 days at 20 °C). Grows well at 1–25 °C, weakly at 30 °C and does not grow at 37 °C. Grows at pH 6–10 (optimum, pH 7–8) and with 0–1% (w/v) NaCl. Grows on R2A agar, NA and TSA. Positive for catalase and cytochrome *c* oxidase. Negative result in tests for nitrate reduction, indole production, H₂S production and utilization of citrate. Negative for hydrolysis of starch and protein (skimmed milk), urease, lysine dihydrolase, ornithine dihydrolase, arginine dihydrolase, tryptophan deaminase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase, α-galactosidase and α-glucosidase. Positive result for aesculin hydrolysis, gelatin hydrolysis, alkaline phosphatase, acidic phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, cystine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, β-glucosidase, β-galactosidase and β-glucuronidase. Assimilates D-glucose, L-arabinose, D-mannose, maltose and malic acid. Does not assimilate D-mannitol, potassium gluconate, capric acid, adipic acid, trisodium citrate or phenylacetic acid. Negative result for fermentation of D-glucose, D-mannitol, sucrose, inositol, sorbitol, L-rhamnose, melibiose, amygdalin and L-arabinose. Resistant to (100 µg ml⁻¹) penicillin, ampicillin, streptomycin, rifampicin, chloramphenicol, cyclosporin A and trimethoprim; and sensitive to kanamycin and tetracycline. The predominant cellular fatty acids are C_{18:1ω7c} (65.0%), C_{14:0} 2-OH (13.4%), summed feature 3 comprising C_{16:1ω7c} and/or iso-C_{15:0} 2-OH (7.9%), C_{16:0} (6.9%) and 11-methyl C_{18:1ω7c} (3.5%). The ubiquinone is Q-10. The polar lipid profile contains SGL, PG, DPG, PE, PME and two unidentified APL (APL1–2), two unidentified GL (GL1–2), seven unidentified PL (PL1–7) and eight unidentified polar lipids (L1–8). The major polyamines are *sym*-homospermidine and spermidine.

The type strain, S8-3^T (=DSM 22537^T=LMG 26055^T), was isolated from alpine soil in the Hohe Tauern, Austria. The DNA G + C content of the type strain is 64.1 mol% (HPLC).

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