Sphingomonas glacialis sp. nov., a psychrophilic bacterium isolated from alpine glacier cryoconite

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A non-motile, rod-shaped, yellow bacterium, designated C16y^T, was isolated from alpine glacier cryoconite. Cells behaved Gram-positively, were aerobic and psychrophilic (good growth at 1–25 °C). Phylogenetic analysis based on 16S rRNA gene sequences showed that strain C16y^T was related to the genus *Sphingomonas* and had highest 16S rRNA gene sequence similarity with *Sphingomonas oligophenolica* JCM 12082^T (97.6 %) and *Sphingomonas echinoides* DSM 1805^T (97.2 %). DNA–DNA hybridization demonstrated that strain C16y^T could not be considered as a member of either *Sphingomonas oligophenolica* or *Sphingomonas echinoides*. Strain C16y^T contained Q-10 as the predominant ubiquinone and C_{18:1} and C_{16:0} were the dominant fatty acids. The polar lipid profile contained phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, sphingoglycolipid, five unidentified glycolipids, two unidentified aminophospholipids and two unidentified lipids. The major polyamines were the triamines *sym*-homospermidine and spermidine. The G + C content was 67.9 mol%. Combined data from phenotypic, phylogenetic and DNA–DNA relatedness studies demonstrated that strain C16y^T is a representative of a novel species of the genus *Sphingomonas*, for which we propose the name *Sphingomonas glacialis* sp. nov. The type strain is C16y^T (=DSM 22294^T =CGMCC 1.8957^T =CIP 110131^T).

The genus *Sphingomonas* was first proposed by Yabuuchi *et al.* (1990), who described five species and two genospecies. On the basis of phylogenetic, chemotaxonomic and physiological analyses, the genus *Sphingomonas* has been divided into four genera: *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* (Takeuchi *et al.*, 2001). These genera differ in their polyamine pattern, nucleotide signatures and some phenotypic characteristics (e.g. nitrate reduction) and form four 16S rRNA gene sequence clusters (I–IV) with distance matrix analysis (Takeuchi *et al.*, 2001). However, Yabuuchi *et al.* (2002) emphasized that the three genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, should be considered as later homotypic synonyms of species of the genus *Sphingomonas* because there was no phenotypic and phylogenetic evidence to support their proposal. So far, the nomenclature of Takeuchi *et al.* (2001) is generally used (Busse *et al.*, 2003). At the time of

writing, the genus *Sphingomonas sensu stricto* contains 45 species with validly published names. According to emended descriptions (Takeuchi *et al.*, 2001; Yabuuchi *et al.*, 2002; Busse *et al.*, 2003), this genus accommodates aerobic, Gram-stain 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.

Strain C16y^T was isolated from an alpine glacier cryoconite collected from the Stubai Glacier near Innsbruck in Tyrol, Austria (altitude 2900 m). The strain was isolated as described previously (Margesin *et al.*, 2002). Strain C16y^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 and *Sphingomonas*

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain C16y^T is GQ253122.

A supplementary figure and a supplementary table are available with the online version of this paper.

oligophenolica JCM 12082^T were routinely grown on R2A agar at 25 °C and used as reference strains.

DNA was extracted and purified as described by Sambrook *et al.* (1989). The gene encoding 16S rRNA was amplified by PCR with two universal primers (Zhang *et al.*, 2006). PCR products were cloned using the pGEM-T easy vector system (Promega). Sequencing reactions were performed as described by Zhang *et al.* (2006). Phylogenetic analysis carried out by using the program MEGA version 3.0 (Kumar *et al.*, 2004) showed that strain C16y^T was grouped with the members of the genus *Sphingomonas* and formed a distinct cluster with *Sphingomonas oligophenolica* JCM 12082^T (97.6 %) and *Sphingomonas echinoides* DSM 1805^T (97.2 %) in the neighbour-joining tree (Fig. 1). In addition, a similar tree topology was also found in the tree generated with the maximum-likelihood method in the PHYLIP 3.69 package (Felsenstein, 2009) after sequences were aligned using CLUSTAL X 1.8 (Thompson *et al.*, 1997). Nucleotide signatures specific to the 16S rRNA of strain C16y^T were 52:359 (C–G), 134 (A), 593 (G), 987:1218 (G–C) and 990:1215 (U–G), which are the same as those of the genus *Sphingomonas* (Cluster 1) reported by Takeuchi *et al.* (2001) except that there was A instead of G at position 134 (*Escherichia coli* numbering; Brosius *et al.*, 1978).

Cell morphology was examined by phase-contrast microscopy (×1000; Leitz Diaplan). Cell motility was evaluated on soft agar at the DSMZ, Braunschweig, Germany. Gram-reaction was tested by classical Gram-staining and was confirmed by KOH lysis. Catalase activity was determined by bubble production in 3 % (v/v) H₂O₂ and 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 25 °C. β-Galactosidase, esterase lipase (C8), amylase and protease activities 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 5–9 and with 0–5 % (w/v) NaCl was determined on R2A agar. Susceptibility to antibiotics was determined on R2A agar supplemented with various antibiotics and incubated at 25 °C. All tests were carried out simultaneously with strain C16y^T and the two reference strains. The morphological, physiological and biochemical characteristics of strain C16y^T are given in the species description and the features that differentiate strain C16y^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 (Wu *et al.*, 1989), using Q-10 from *Sphingomonas oligophenolica* JCM 12082^T as a reference. The predominant ubiquinone of strain C16y^T was Q-10. For fatty acid methyl ester analysis, strain C16y^T and the reference strains were grown on R2A agar at 20 °C for 3 days. The fatty acid methyl esters were extracted and prepared according to the

standard protocol of the Microbial Identification system (Sasser, 1990). The predominant cellular fatty acids (>5 %) of strain C16y^T were summed feature 7 (comprising one or more of C_{18:1ω7c}, C_{18:1ω9t} and C_{18:1ω12t}; 61.4 %), C_{16:0} (8.2 %), summed feature 4 (comprising C_{16:1ω7c} and/or iso-C_{15:0} 2-OH; 6.8 %) and C_{14:0} 2-OH (5.9 %) (Supplementary Table S1, available in IJSEM Online). The fatty acid profile of strain C16y^T was in agreement with those of other members of the genus *Sphingomonas* (Denner *et al.*, 1999; Busse *et al.*, 2003; Ohta *et al.*, 2004; Busse *et al.*, 2005).

The cellular polar lipids were extracted and analysed on silica gel plates (Kieselgel 60 F; Merck) by TLC (Kates, 1986). Ethanolic molybdatophosphoric acid, α-naphthol-sulphuric acid reagent, Dragendorff reagent, ninhydrin and molybdenum blue reagent were used for the detection of total lipids, glycolipids, quaternary nitrogen and aminophospholipids, respectively. The polar lipid profile contained phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, sphingoglycolipid, five unidentified glycolipids, two unidentified aminophospholipids and two unidentified lipids (Supplementary Fig. S1). Although the polar lipids of the type strains of both *Sphingomonas echinoides* and *Sphingomonas oligophenolica* have been analysed (Denner *et al.*, 1999; Ohta *et al.*, 2004), only the polar lipid profile of *Sphingomonas echinoides* DSM 1805^T was accessible for visual comparison. The polar lipid profiles of *Sphingomonas echinoides* DSM 1805^T and C16y^T exhibited some striking differences. The polar lipid profile of strain C16y^T did not contain the lipids L1 and PL1 of *Sphingomonas echinoides* DSM 1805^T and the lipids GL1, GL5 and L2 of strain C16y^T also distinguished it from *Sphingomonas echinoides* DSM 1805^T.

For polyamine analysis, strain C16y^T was grown in PYE medium (containing 0.3 % yeast extract, 0.3 % peptone; pH 7) 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 C16y^T consisted of [μmol (g dry mass)⁻¹] *sym*-homospermidine (38.9), spermidine (13.0), spermine (3.4), 1,3-diaminopropane (1.6) and putrescine (0.5). 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; Xie & Yokota, 2006; Kämpfer *et al.*, 2007; Huang *et al.*, 2009; Roh *et al.*, 2009; Nigam *et al.*, 2010; Zhang *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.

The DNA G + C content was determined using the thermal denaturation method with *E. coli* K-12 as reference.

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic positions of strain C16y^T, some members of the genus *Sphingomonas* and representatives of other related taxa. Bootstrap values (50%) based on 1000 replicates are shown at branch nodes. Bar, 2% sequence divergence.

DNA–DNA hybridization was done using the liquid renaturation method (De Ley *et al.*, 1970) as modified by Huß *et al.* (1983). Both experiments were carried out using a Lambda 35 UV/VIS spectrometer equipped with a temperature program controller (PerkinElmer). The DNA G + C content of strain C16y^T was 67.9 mol%. DNA–DNA relatedness between strain C16y^T and *Sphingomonas oligophenolica* JCM 12082^T and *Sphingomonas echinoides* DSM 1805^T were 23.0% and 19.5%, respectively, which were much lower than the 70% DNA–DNA relatedness threshold that is recommended for species demarcation (Wayne *et al.*, 1987), thus verifying that the isolate represented a species different to *Sphingomonas oligophenolica* and *Sphingomonas echinoides*.

The data presented above demonstrate that strain C16y^T is a psychrophilic member of the genus *Sphingomonas*. Strain C16y^T could be easily differentiated from its closest phylogenetic neighbours, *Sphingomonas oligophenolica* JCM 12082^T and *Sphingomonas echinoides* DSM 1805^T, by its inability to produce *N*-acetyl- β -glucosaminidase, its weak growth at 30 °C and the relative amounts of C_{14:0} 2-OH and summed feature 4. In addition, strain C16y^T could be easily differentiated from *Sphingomonas oligophenolica* JCM 12082^T by its inability to reduce nitrate and hydrolyse

gelatin, the absence of α -fucosidase and its ability to assimilate D-glucose and maltose, to grow with 1% (w/v) NaCl and to tolerate penicillin G (30–100 μ g ml⁻¹) and ampicillin (30 μ g ml⁻¹). Strain C16y^T could be distinguished from *Sphingomonas echinoides* DSM 1805^T on the basis of absence and presence of certain lipids, its ability to hydrolyse Tween 80, its inability to assimilate gluconate, its lower resistance to ampicillin and its immotility. Therefore, on the basis of phenotypic, phylogenetic and genomic evidence, strain C16y^T is a representative of a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas glacialis* sp. nov. is proposed.

Description of *Sphingomonas glacialis* sp. nov.

Sphingomonas glacialis (gla.cia'lis. L. fem. adj. *glacialis* referring to the frozen, icy environment from which the type strain was isolated).

Cells are aerobic, Gram-negative, non-motile, short rods (0.5 $\times$ 0.8 μ m after 6 days at 20 °C on R2A agar). Colonies on R2A agar are yellow (orange after approx. 14 days), convex and round with entire margins (2 mm in diameter after 6 days at 20 °C). Grows well at 1–25 °C and weakly at 30 °C (in R2A broth, biomass at 30 °C is about one-third of that produced at 1–25 °C) and does not grow at 37 °C.

Table 1. Phenotypic characteristics that differentiate strain C16y^T from its closest phylogenetic neighbours

Strains: 1, *Sphingomonas glacialis* sp. nov. C16y^T; 2, *S. oligophenolica* JCM 12082^T; 3, *S. echinoides* DSM 1805^T. Data were taken from this study. All strains are positive for aesculin hydrolysis, oxidase, alkaline phosphatase, acid phosphatase, cystine arylamidase, leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase and α -glucosidase. All strains are negative for indole production, glucose fermentation, lipase (C14), arginine dihydrolase, amylase, urease, α -mannosidase, assimilation of D-mannose, D-mannitol, trisodium citrate, adipic acid, capric acid and phenylacetic acid and resistance to ($\mu\text{g ml}^{-1}$) chloramphenicol (100), kanamycin (30), rifampicin (30) and tetracycline (30). +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3
Motility	–	–	+
Growth at 30 °C	w	+	+
Growth with 1 % (w/v) NaCl	+	–	+
Nitrate reduction	–	+	–
Hydrolysis of:			
Gelatin	–	+	–
Tween 80	+	+	–
Enzymes (API ZYM)			
<i>N</i> -Acetyl- β -glucosaminidase	–	+	+
α -Fucosidase	–	+	–
Assimilation of (API 20 NE):			
D-Glucose	+	–	+
Maltose	+	–	+
Malic acid	w	–	w
Gluconate	–	w	+
Resistance to ($\mu\text{g ml}^{-1}$):			
Ampicillin (30)	+	–	+
Ampicillin (100)	–	–	+
Penicillin G (30–100)	+	–	+
Streptomycin (100)	w	–	+

Grows at pH 7–8 and with 0–1 % (w/v) NaCl. Positive for catalase and cytochrome *c* oxidase. Negative for nitrate reduction, indole production, H₂S production and urease. Positive for aesculin hydrolysis, alkaline phosphatase, acid phosphatase, esterase (C4; weak) esterase lipase (C8), cystine arylamidase, leucine arylamidase, valine arylamidase, trypsin, α -chymotrypsin (weak), naphthol-AS-BI-phosphohydrolase, α - and β -glucosidase, β -galactosidase, β -glucuronidase. Negative for lipase (C14), gelatinase, arginine dihydrolase, lysine dihydrolase, ornithine dihydrolase, tryptophan deaminase, *N*-acetyl- β -glucosaminidase, α -fucosidase and α -mannosidase. Assimilates L-arabinose, D-glucose, maltose and malic acid (weakly) but does not assimilate D-mannose, D-mannitol, citrate, potassium gluconate, adipic acid, capric acid or phenylacetic acid. Does not ferment L-arabinose, D-glucose, melibiose, L-rhamnose, sucrose, inositol, D-mannitol, sorbitol or amygdalin. Resistant to ($\mu\text{g ml}^{-1}$) ampicillin (30), penicillin G (100) and streptomycin (30) and sensitive to ampicillin (100), chloramphenicol (30), kanamycin (30), rifampicin (30) and tetracycline (30).

The predominant cellular fatty acids (>5 %) are summed feature 7 (comprising one or more of C_{18:1} ω 7*c*, C_{18:1} ω 9*t* and C_{18:1} ω 12*t*), C_{16:0}, summed feature 4 (comprising C_{16:1} ω 7*c* and/or iso-C_{15:0} 2-OH) and C_{14:0} 2-OH. The predominant ubiquinone is Q-10. The polar lipid profile contains phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, sphingoglycolipid, five unidentified glycolipids, two unidentified aminophospholipids and two unidentified lipids. The polyamine pattern contains major amounts of *sym*-homospermidine and relatively high amounts of spermidine. The DNA G+C content of the type strain is 67.9 mol%.

The type strain, C16y^T (=DSM 22294^T =CGMCC 1.8957^T =CIP 11013^T), was isolated from a glacier cryoconite collected from the Stubai Glacier, Austria.

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