Glaciimonas immobilis gen. nov., sp. nov., a member of the family *Oxalobacteraceae* isolated from alpine glacier cryoconite

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Strains Cr9-30^T and Cr9-12 were isolated from alpine glacier cryoconite. Both strains were Gram-negative-staining, non-motile, rod-shaped and psychrophilic, showing good growth over the temperature range 1–20 °C. Phylogenetic analysis of 16S rRNA gene sequences revealed that the two strains formed a distinct branch within the family *Oxalobacteraceae* and were most closely related to members of the genus *Collimonas*. The 16S rRNA gene sequence similarity between strains Cr9-30^T and Cr9-12 was 99.0 %. The two strains showed highest 16S rRNA gene sequence pairwise similarity with *Collimonas pratensis* LMG 23965^T (96.6 and 96.1 % for strains Cr9-30^T and Cr9-12, respectively), *Collimonas arenae* LMG 23964^T (96.5 and 96.3 %, respectively) and *Collimonas fungivorans* LMG 21973^T (96.4 and 96.2 %, respectively). The predominant cellular fatty acids were summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{16:0} and C_{18:1}ω7c. The DNA G + C content of strain Cr9-30^T was 51.0 mol%. On the basis of phenotypic characteristics and phylogenetic analysis, strains Cr9-30^T and Cr9-12 represent a novel species in a new genus of the family *Oxalobacteraceae*, for which the name *Glaciimonas immobilis* gen. nov., sp. nov. is proposed. The type strain of *Glaciimonas immobilis* is Cr9-30^T (=DSM 23240^T=LMG 25547^T).

The bacterial family *Oxalobacteraceae* belongs to the class *Betaproteobacteria* and, at the time of writing, included 11 genera (Garrity *et al.*, 2005; Kämpfer *et al.*, 2007). The family *Oxalobacteraceae* represents a phylogenetically diverse family of typically aquatic and soil micro-organisms within the class *Betaproteobacteria* (Eder *et al.*, 2011) and the majority of the organisms in this family form yellowish-pigmented colonies on nutrient-rich media such as nutrient agar (Kämpfer *et al.*, 2007). Cold-adapted members within this family have been described recently, for example *Herminiimonas glaciei* was isolated from a Greenland glacier ice core (Loveland-Curtze *et al.*, 2009) and *Massilia brevitalea* was detected in lysimeter soil (Zul *et al.*, 2008). In the present paper, the characterization of two novel psychrophilic strains, designated Cr9-30^T and Cr9-12, is reported. The term psychrophile is used in this report as a general term to describe a micro-organism that grows in a cold environment (Margesin *et al.*, 2008). The use of growth rates to define the optimum growth temperature as described by Morita (1975) has been shown to be ambiguous and inappropriate (Feller & Gerday, 2003; Cavicchioli, 2006; Margesin, 2009).

Strains Cr9-30^T and Cr9-12 were isolated from alpine glacier cryoconite collected under sterile conditions in

spring 2006 from the Tiefenbachferner glacier in the Oetztaler Alps, Tyrol, Austria, at an altitude of 2900 m above sea level. A sample (1 g dry mass) was shaken with 9 ml sterile 1 % sodium pyrophosphate for 20 min at 150 r.p.m. Appropriate dilutions, prepared with sterile saline solution (0.9 % NaCl), were plated on R₂A agar (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) and incubated at 10 °C. Two pure white cultures were identified and designated Cr9-30^T and Cr9-12. *Collimonas fungivorans* LMG 21973^T (type species of the genus), *Collimonas arenae* LMG 23964^T and *Collimonas pratensis* LMG 23965^T were routinely grown on R₂A agar at 20 °C and used as reference strains.

DNA was extracted and purified as described by Sambrook *et al.* (1989). The 16S rRNA gene was amplified by PCR with two universal primers (Zhang *et al.*, 2010). PCR products were cloned in pGEM-T vectors (Promega) according to the manufacturer's instructions. Sequencing reactions were performed as described by Zhang *et al.* (2006). Phylogenetic analysis using the program MEGA version 3.0 (Kumar *et al.*, 2004) revealed that strains Cr9-30^T and Cr9-12 formed a distinct phylogenetic lineage with a high bootstrap value (100 %) within the family *Oxalobacteraceae* in the neighbour-joining tree (Fig. 1). Strains Cr9-30^T and Cr9-12 had almost identical 16S rRNA

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Cr9-30^T and Cr9-12 are GU441679 and HM756647, respectively.

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequence data, showing the phylogenetic position of strains Cr9-30^T, Cr9-12 and representatives of other related taxa. Bootstrap values (%) are based on 1000 replicates and are shown for branches with >50% support. Asterisks indicate nodes that were also found in the maximum-likelihood tree. GenBank accession numbers of 16S rRNA gene sequences are given in parentheses. Bar, 0.5% sequence divergence.

gene sequences (99.0% similarity) and showed the highest 16S rRNA gene sequence pairwise similarity with *C. pratensis* LMG 23965^T (96.6 and 96.1% for Cr9-30^T and Cr9-12, respectively), *C. arenae* LMG 23964^T (96.5 and 96.3%, respectively) and *C. fungivorans* LMG 21973^T (96.4 and 96.2%, respectively). 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).

Strains Cr9-30^T and Cr9-12 were routinely cultured in R₂A medium at 20 °C and maintained as a suspension in skimmed milk (10%, w/v) at –80 °C. Cell morphology was studied by phase-contrast microscopy (×1000 magnification) and by transmission electron microscopy (Libra 120 EFTEM; Zeiss). Motility was examined at 10 and 20 °C by microscopy (×1000), by the API M system (bioMérieux) and additionally by the DSMZ (Braunschweig, Germany) on soft agar. Colonial morphology was observed on R₂A agar plates incubated at 20 °C.

All tests described in this paragraph were done with strains Cr9-30^T and Cr9-12 and with the reference strains *C. fungivorans* LMG 21973^T, *C. arenae* LMG 23964^T and *C. pratensis* LMG 23965^T. Gram-reaction was tested by standard Gram-staining. Catalase activity was determined by bubble production in 3% (v/v) H₂O₂; oxidase activity was determined using 1% (w/v) *N*, *N*,*N*',*N*'-tetramethyl-*p*-phenylenediamine. API strips (API 20E, API 20NE, API ZYM; bioMérieux) incubated at 20 °C were used according to the manufacturer's instructions to determine physiological and biochemical characteristics as well as enzyme activities of strains Cr9-30^T and Cr9-12. β-Galactosidase, lipase esterase, amylase and protease activities were also tested by using R₂A agar plates supplemented with the appropriate substrates (Margesin *et al.*, 2003). Chitinase activity was assayed on chitin-yeast agar as described by de Boer *et al.* (2004). The temperature range for growth was determined aerobically between 1 and 42 °C using R₂A medium. Growth at various pH values (pH 5–9) and salt tolerance [0–10% (w/v) NaCl] was determined on R₂A agar plates. Susceptibility to antibiotics was determined at

20 °C by using antibiotic discs (bioMérieux). Growth under anaerobic conditions was determined after 5 days of incubation at 20 °C in an anaerobic jar [containing Anaerocult A (Merck) to produce anaerobic conditions] on R₂A agar plates supplemented with 10 mM KNO₃. Growth under microaerophilic conditions was assessed after 5 days at 20 °C on R₂A agar plates that were incubated in a microaerophilic atmosphere containing 8–10 % (v/v) carbon dioxide and 5–7 % (v/v) oxygen; this atmosphere was generated in sealed jars containing Anaerocult C (Merck). Features that serve to differentiate strains Cr9-30^T and Cr9-12 from the phylogenetically most closely related members of the genus *Collimonas* are given in Table 1. Morphological, physiological and biochemical characteristics of strains Cr9-30^T and Cr9-12 are given in the species description (see below) or are shown in Table 1.

Table 1. Differential phenotypic characteristics of strains Cr9-30^T and Cr9-12 and members of the genus *Collimonas*

Strains: 1, Cr9-30^T; 2, Cr9-12; 3, *Collimonas fungivorans* LMG 21973^T; 4, *Collimonas arenae* LMG 23964^T; 5, *Collimonas pratensis* LMG 23965^T. All data are from this study. +, Positive; –, negative; w, weak. All strains are positive for acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase activities and for assimilation of D-glucose and L-arabinose. All strains are negative for β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase, lysine dihydrolase and ornithine dihydrolase activities, for assimilation of maltose and phenylacetic acid, and for acid production from D-glucose, D-mannitol, sucrose, inositol, sorbitol, L-rhamnose, melibiose, amygdalin and L-arabinose.

Characteristic	1	2	3	4	5
Motility	–	–	+	+	+
Growth at/in presence of:					
30 °C	–	–	+	+	+
pH 5	–	–	+	+	+
2 % (w/v) NaCl	–	w	w	+	+
Enzyme activity:					
Nitrate reduction	–	–	+	+	+
Chitinase	–	–	+	+	+
Lipase (C14)	–	w	+	+	+
α-Galactosidase	–	–	–	–	+
β-Galactosidase	–	–	+	–	+
Trypsin	–	–	w	+	+
α-Chymotrypsin	–	–	+	+	+
Gelatinase	–	–	w	+	–
Urease	–	–	–	+	–
Assimilation of (API 20NE):					
D-Mannose	–	–	+	w	+
D-Mannitol	–	+	+	–	+
N-Acetylglucosamine	–	–	+	–	+
Gluconate	+	+	–	w	w
Capric acid	–	–	+	+	w
Malic acid	–	+	+	+	+

For fatty acid methyl ester analysis, cell masses of strains Cr9-30^T, Cr9-12 and *C. fungivorans* LMG 21973^T (the type strain of the type species of the genus) were harvested from R₂A plates after incubation at 10 °C for 4 days. Fatty acid methyl esters were prepared and extracted according to the standard protocol of the MIDI (Microbial Identification) system (Sasser, 1990). The fatty acid profiles of strains Cr9-30^T, Cr9-12 and *C. fungivorans* LMG 21973^T are shown in Table 2. The predominant cellular fatty acids were summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0}2-OH), C_{16:0} and C_{18:1}ω7c.

The DNA G + C content of strain Cr9-30^T, as determined by using standard HPLC analysis (Mesbah *et al.*, 1989), was 51.0 mol%. Cellular fatty acids and DNA G + C content were both analysed by the Identification Service of the DSMZ.

Strains Cr9-30^T and Cr9-12 were isolated from the same site. No distinct differences were found between the two strains with regard to their morphological, physiological and biochemical characteristics, and their fatty acid profiles were almost identical. These data suggest that the two cryoconite strains belong to the same species. Similar cases have been described for representatives of the species *Glaciibacter superstes* (Katayama *et al.*, 2009) and *Undibacterium pigrum* (Kämpfer *et al.*, 2007). Within the family *Oxalobacteraceae*, strains Cr9-30^T and Cr9-12 formed a distinct subclade with three species of the genus *Collimonas*, which are the nearest phylogenetic neighbours of strain Cr9-30^T. However, strain Cr9-30^T cannot be assigned to the genus *Collimonas* for the following reasons. (i) One unique continuous stretch of 18 bp (V6 region), which is absent in all other recognized type strains of the family *Oxalobacteraceae*, but shared by members of the genus *Collimonas* and highly predictive of the genus *Collimonas* (de Boer *et al.*, 2004; Höppener-Ogawa *et al.*,

Table 2. Cellular fatty acid compositions (%) of strains Cr9-30^T, Cr9-12 and *C. fungivorans* LMG 21973^T

Strains: 1, Cr9-30^T; 2, Cr9-12; 3, *C. fungivorans* LMG 21973^T. Values are percentages of total fatty acids. ND, Not detected.

Fatty acid	1	2	3
C _{10:0}	0.2	0.2	ND
C _{10:0} 3-OH	1.6	1.2	1.1
C _{12:0}	3.5	3.2	3.6
C _{12:0} 2-OH	2.7	2.4	2.8
C _{12:0} 3-OH	3.3	2.8	3.4
Summed feature 3*	54.7	47.7	43.9
C _{16:0}	17.5	22.5	23.4
C _{16:1} 2-OH	1.6	0.8	2.7
C _{17:0} cyclo	1.0	6.9	3.4
C _{18:1} ω7c	13.6	10.6	15.2
C _{18:0}	0.5	0.7	0.5
11-methyl C _{18:1} ω7c	ND	0.8	ND

*Summed feature 3 comprises C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

2008; Leveau *et al.*, 2010), was absent in strain Cr9-30^T. (ii) The DNA G+C content of strain Cr9-30^T (51.0 mol%) was considerably lower than values reported for members of the genus *Collimonas* (57–62 mol%). (iii) With regard to cellular fatty acids, strain Cr9-30^T contained higher amounts of summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0}2-OH) and lower amounts of C_{16:0} compared with the type strain of the type species of the genus *Collimonas* (see Table 2). (iv) Strain Cr9-30^T can be differentiated from all three recognized species of the genus *Collimonas* by a number of phenotypic properties (Table 1), e.g. strain Cr9-30^T is non-motile and negative for the following: growth at 30 °C and pH 5; nitrate reduction; lipase (C14) and α-chymotrypsin activities; and assimilation of capric acid, malic acid and trisodium citrate. In addition, strain Cr9-30^T is not able to hydrolyse colloidal chitin or milk proteins, which are typical characteristics of members of the genus *Collimonas*.

Thus, strains Cr9-30^T and Cr9-12 represent a novel species in a new genus, for which the name *Glaciimonas immobilis* gen. nov., sp. nov. is proposed.

Description of *Glaciimonas* gen. nov.

Glaciimonas (Gla.ci.i.mo'nas. L. fem. n. *glacies* ice; L. n. *monas* a unit, monad; N.L. fem. n. *Glaciimonas* a cell from the glacier).

Cells are Gram-negative-staining, rod-shaped and non-motile. Able to grow under aerobic and microaerophilic conditions; unable to grow under anaerobic conditions. Oxidase- and catalase-positive. Negative for nitrate reduction. The predominant cellular fatty acids are summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0}2-OH), C_{16:0} and C_{18:1}ω7c. The most closely related genus phylogenetically is *Collimonas*. The genus is affiliated to the family *Oxalobacteraceae* of the order *Burkholderiales* (class *Betaproteobacteria*). The type species is *Glaciimonas immobilis*.

Description of *Glaciimonas immobilis* sp. nov.

Glaciimonas immobilis (im.mo'bi.lis. L. fem. adj. *immobilis* motionless).

In addition to the characteristics listed above for the genus, the following properties are observed. Cells are 0.9–1.0 × 1.1–1.5 µm in size after 7 days at 20 °C on R₂A agar plates. Flagella or pili are absent. Colonies on R₂A agar are white, round, convex and smooth with entire margins; slime is produced. Good growth occurs in liquid R₂A medium and on R₂A agar plates at 1–20 °C; growth at 25 °C is absent on agar plates and weak in liquid culture. Growth is weak on nutrient agar plates and almost absent on tryptic soy agar plates at 20 °C. On R₂A agar plates, grows in the presence of 0–1 % (w/v) NaCl and at pH 6–8. Negative for indole and H₂S production, hydrolysis of urease, aesculin, gelatin, milk proteins (skimmed milk) and starch. Negative for arginine dihydrolase, lysine dihydrolase and ornithine dihydrolase. In

the API ZYM system, positive for acid phosphatase, alkaline phosphatase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, esterase (C4) and esterase lipase (C8) activities. Valine arylamidase activity is weak. Negative for lipase (C14), trypsin, α-chymotrypsin, N-acetyl-β-glucosaminidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase activities. In the API 20NE system, positive for assimilation of D-glucose, L-arabinose and potassium gluconate. D-Mannose, D-mannitol, maltose, N-acetylglucosamine, trisodium citrate, capric acid, adipic acid, malic acid and phenylacetic acid are not assimilated. Negative for acid production from D-glucose, D-mannitol, sucrose, inositol, D-sorbitol, L-rhamnose, D-melibiose, amygdalin and L-arabinose. Resistant to ampicillin (10 µg), fusidic acid (10 µg) and erythromycin (15 µg); sensitive to streptomycin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg), kanamycin (30 µg), gentamicin (10 µg), amikacin (30 µg), rifampicin (30 µg) and nalidixic acid (30 µg).

The type strain is Cr9-30^T (=DSM 23240^T=LMG 25547^T), isolated from alpine glacier cryoconite collected from the Tiefenbachferner glacier in the Oetztaler Alps, Tyrol, Austria. The DNA G+C content of strain Cr9-30^T is 51.0 mol%. Strain Cr9-12 (=DSM 22814), isolated from the same source, is a second strain of the species.

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