Glaciimonas frigoris sp. nov., a psychrophilic bacterium isolated from ancient Siberian permafrost sediment, and emended description of the genus *Glaciimonas*

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The bacterial strain N1-38^T was isolated from ancient Siberian permafrost sediment. The strain was Gram-reaction-negative, motile by gliding, rod-shaped and psychrophilic, and showed good growth over a temperature range of −5 to 25 °C. Phylogenetic analysis of 16S rRNA gene sequences revealed that strain N1-38^T was most closely related to members of the genus *Glaciimonas* and shared the highest 16S rRNA gene sequence similarities with the type strains of *Glaciimonas alpina* (99.3 %), *Glaciimonas immobilis* (98.9 %) and *Glaciimonas singularis* (96.5 %). The predominant cellular fatty acids of strain N1-38^T were summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{16:0}$ and $C_{18:1}\omega7c$. The major respiratory quinone was ubiquinone 8 and the major polar lipids were phosphatidylethanolamine and diphosphatidylglycerol. The genomic DNA G+C content was 53.0 mol%. Combined data of phenotypic, phylogenetic and DNA–DNA relatedness studies demonstrated that strain N1-38^T represents a novel species of the genus *Glaciimonas*, for which the name *Glaciimonas frigoris* sp. nov. is proposed. The type strain is N1-38^T (=LMG 28868^T=CCOS 838^T). An emended description of the genus *Glaciimonas* is also provided.

The genus *Glaciimonas* belongs to the family *Oxalobacteraceae* of the class *Betaproteobacteria* and, at the time of writing, included three species with validly published names: *Glaciimonas immobilis* (Zhang *et al.*, 2011), *Glaciimonas singularis* (Chung *et al.*, 2013) and *Glaciimonas alpina* (Frasson *et al.*, 2015). While the type strain of the species *Glaciimonas singularis* was isolated from a water sample collected in a uranium mine (Chung *et al.*, 2013), representatives of *Glaciimonas immobilis* and *Glaciimonas alpina* described so far are psychrophilic isolates from alpine glaciers (Zhang *et al.*, 2011; Frasson *et al.*, 2015). In this study, we report the characterization of a psychrophilic representative of a novel species of the genus *Glaciimonas* isolated from ancient Siberian permafrost sediment.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain N1-38^T is JX545209.

Four supplementary figures and a supplementary table are available with the online Supplementary Material.

Strain N1-38^T was isolated as previously described from ancient (Neogene) permafrost sediment collected from Mammoth Mountain in the Aldan river valley in Central Yakutia, Eastern Siberia (Zhang *et al.*, 2013). The strain was routinely cultured on R2A agar at 10–15 °C and stored as a suspension in skimmed milk (10 %, w/v) at −80 °C. The type strains *Glaciimonas singularis* LMG 27070^T (Chung *et al.*, 2013), *Glaciimonas alpina* Cr9-12^T (Frasson *et al.*, 2015), and *Glaciimonas immobilis* Cr9-30^T (Zhang *et al.*, 2011) were routinely grown on R2A agar at 10–15 °C and used as reference strains in this study.

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. On the basis of pairwise comparisons of the 16S rRNA gene sequences using the recent version of the EzTaxon program (www.ezbiocloud.net/eztaxon; Kim *et al.*, 2012), strain N1-38^T shared the highest sequence similarities with the type

strains of *G. alpina* (99.3 % 16S rRNA gene sequence similarity), *G. immobilis* (98.9 %) and *G. singularis* (96.5 %). Multiple sequence alignments were performed using the CLUSTAL W program integrated in MEGA software package version 6 (Tamura *et al.*, 2013). Phylogenetic trees were reconstructed by using the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms using MEGA version 6.0. The resultant tree topologies generated from all three methods were evaluated by bootstrap analysis based on 1000 replicates. The reconstructed phylogenetic tree based on the neighbour-joining algorithm (Saitou & Nei, 1987) revealed that strain N1-38^T formed a distinct cluster together with *G. alpina* Cr9-12^T and *G. immobilis* Cr9-30^T as supported by high bootstrap values (Fig. 1). This phylogenetic position was confirmed in the trees generated by using the maximum-likelihood and maximum-parsimony algorithms (Figs S1 and S2, available in the online Supplementary Material).

Cell morphology was examined by phase-contrast microscopy (× 1000; Leitz Diaplan) and transmission electron microscopy (Libra 120 EFTEM; Zeiss) of cells grown on R2A agar at 15 °C. Motility was examined by microscopy (× 1000) and on R2A soft agar (0.3 % agar) plates. Gram-staining was tested by using the bioMérieux Gram-stain kit and by the KOH (3 %, w/v) method. Catalase activity was determined by bubble production in 3 % (v/v) H_2O_2 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 20NE, API 20E and API ZYM systems (bioMérieux) at 10 °C. Oxidative/fermentative metabolism of glucose was determined as described by Süßmuth *et al.* (1987) on Hugh and Leifson's OF basal medium (1 % glucose, 0.2 % peptone, 0.1 % yeast extract, 0.5 % NaCl, 0.02 % K_2HPO_4, 0.008 % bromothymol blue and 0.3 % agar). Aerobic growth on different media was assessed at 10 °C on R2A agar, nutrient agar (NA) and trypticase soy agar (TSA). Growth under anaerobic conditions was examined after incubation on R_2A agar supplemented with 10 mM KNO_3 for 7 days at 10 °C in an anaerobic jar [containing Anaerocult A (Merck) to produce anaerobic conditions]. Growth under microaerophilic conditions was investigated at 10 °C on R_2A agar after incubation 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). Degradation of starch (amylase), skimmed milk (protease), carboxymethyl-cellulose (cellulase), Tween 80 and lignosulfonic acid was tested on agar plates supplemented with the appropriate substrates as described previously (Zhang *et al.*, 2013). The utilization of hydrocarbons (n-hexadecane, diesel oil, phenol, naphthalene, phenanthrene and anthracene) as sole carbon sources was determined as described previously (Zhang *et al.*, 2013). Growth at 1, 5, 10, 15, 20, 25, 30 and 35 °C was assessed on R2A agar and in R2A broth at 150 r.p.m. Growth at −5 °C was assessed in R2A broth (unshaken). Growth at pH 5, 6, 7, 8, 9 and 10 (using medium buffered with citrate buffer for pH 5, phosphate buffer for pH 6–7 or Tris buffer for pH 8–10), and with 0, 1, 2, 3, 5, 7 and 10 % (w/v) NaCl was determined on R2A agar. All tests were carried out simultaneously with strain N1-38^T and the reference strains *G. singularis* LMG 27070^T, *G. alpina* Cr9-12^T and *G. immobilis* Cr9-30^T. The morphological, physiological and biochemical characteristics of strain N1-38^T are given in the species description, and the features that differentiate this strain from the reference strains are given in Table 1.

For fatty acid methyl ester analysis, strain N1-38^T and the reference strains *G. singularis* LMG 27070^T, *G. alpina* Cr9-12^T and *G. immobilis* Cr9-30^T were grown on R2A agar at 10 °C for 5 days. All four strains were in the stationary growth phase and shared similar growing behaviour; after cultivation under the applied conditions, a sufficient amount of cells of comparable physiological age could be harvested from the third streak-quadrant of the plates. The fatty acid methyl esters were extracted and prepared using minor modifications of the methods of Miller (1982) and Kuykendall *et al.* (1988). The fatty acid methyl esters mixtures were separated using Sherlock Microbial Identification System (MIS) (MIDI; version 6.1) (Sasser, 1990), and the database TSBA6 was used for calculation. Fatty acid analyses were carried out by the Identification Service of the Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). The predominant cellular fatty acids of strain N1-38^T were summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{16:0}$ and $C_{18:1}\omega7c$ (Table S1).

Respiratory quinones were extracted from cells grown in R2A broth at 10 °C for 3 days, purified according to Collins (1985) and analysed by HPLC (Wu *et al.*, 1989), using ubiquinone 8 (UQ-8) from *G. singularis* LMG 27070^T as a reference. The respiratory quinone of strain N1-38^T was UQ-8, which is in agreement with the description of respiratory quinones of *G. singularis* LMG 27070^T (Chung *et al.*, 2013), as well as with *G. alpina* Cr9-12^T and three other representatives of this species (Frasson *et al.*, 2015).

Polar lipid profiles were analysed according to Tindall (1990a, b). The polar lipid profile of strain N1-38^T contained phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, three unidentified phospholipids, one unidentified aminolipid, two unidentified aminophospholipids and one unidentified polar lipid (Fig. S3).

The DNA G+C content was determined by the initial renaturation method (Marmur & Doty, 1962) using *Escherichia coli* K-12 as calibration standard. The genomic DNA G+C content of of strain N1-38^T was 53.0 mol%. The degree of DNA–DNA hybridization was determined by the liquid renaturation method (De Ley *et al.*, 1970) as modified by Huss *et al.* (1983). DNA–DNA hybridizations were carried out in 2× SSC at 73 °C and each

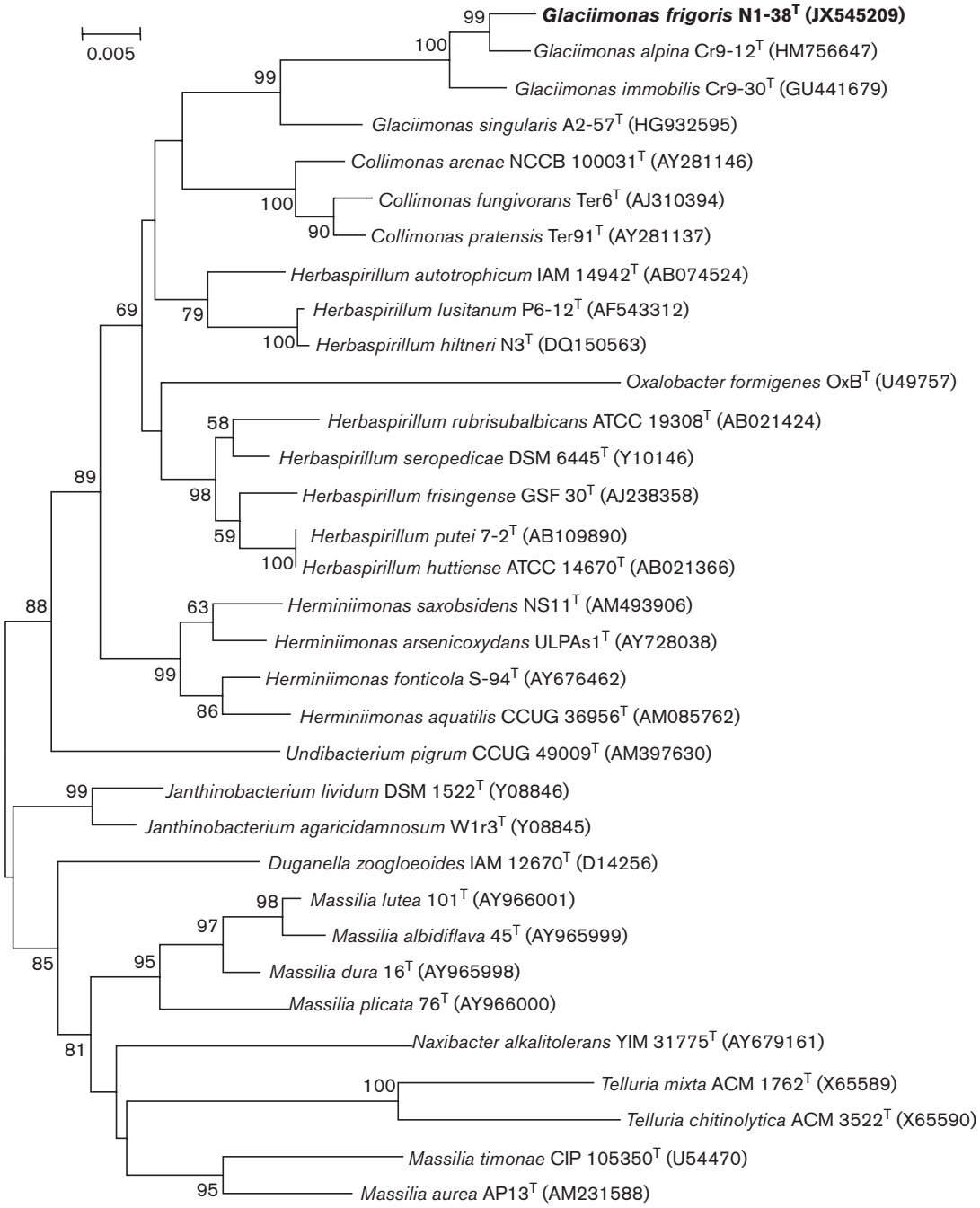

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequence data, showing the phylogenetic position of strain N1-38^T, recognized members of the genus *Glaciimonas*, and representatives of some related taxa. Bootstrap values (%) are based on 1000 replicates and are shown for branches with >50 % support. GenBank accession numbers of 16S rRNA gene sequences are given in parentheses. Bar, 0.05 % sequence divergence.

determination was done in triplicate. Both experiments were performed at 260 nm with a model Lambda 35 UV/VIS spectrometer equipped with a Peltier System (PTP 1+1) (Perkin–Elmer). The DNA–DNA hybridization experiments revealed that strain N1-38^T shared 42 % DNA–DNA relatedness with *G. alpina* Cr9-12^T and 35 % with *G. immobilis* Cr9-30^T. These values were well below the 70 % cut-off point recommended for the assignment of strains to the same genospecies (Wayne *et al.*, 1987).

The data presented in this study demonstrate that strain N1-38^T is a psychrophilic member of the genus

Table 1. Phenotypic characteristics that differentiate strain N1-38^T from other species of the genus *Glaciimonas*

Strains: 1, N1-38^T; 2, *Glaciimonas singularis* LMG 27070^T; 3, *Glaciimonas alpina* Cr9-12^T; 4, *Glaciimonas immobilis* Cr9-30^T. All data are from this study. All strains are positive for aerobic and microaerophilic growth, catalase, cytochrome *c* oxidase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase in API ZYM, API 20NE and API 20E tests, and for assimilation of D-glucose. All strains are negative for anaerobic growth; H_2S production; hydrolysis of aesculin and gelatin; citrate utilization; activities of urease, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, mannosidase, α-fucosidase, tryptophan deaminase, arginine dihydrolase, lysine dihydrolase and ornithine dihydrolase in API ZYM, API 20NE and API 20E tests; assimilation of D-mannose, maltose, capric acid, adipic acid and phenylacetic acid; and for fermentation of glucose. +, Positive; −, negative; W, weakly positive.

Characteristic	1	2	3	4
Isolation source	Siberian permaforst sediment	Wastewater treatment plant	Alpine Glacier cryoconite	Alpine Glacier cryoconite
Motility	gliding	−	−	−
Growth on R2A agar at/with:				
−5 °C	+	−	+	+
25 °C	+	+	−	−
30 °C	−	+	−	−
2 % (w/v) NaCl	−	W	+	W
Growth on NA and TSA	−	+	−	−
Nitrate reduction to nitrite or nitrogen (manual test)	+ (to nitrite)	−	+ (to nitrogen)	+ (to nitrogen)
Valine arylamidase (API ZYM)	−	−	−	W
Assimilation of (API 20NE):				
L-Arabinose	−	+	+	+
D-Mannitol	+	−	+	−
N-Acetylglucosamine	−	+	−	−
Potassium gluconate	−	−	+	+
Malic acid	W (delayed)	−	+	−
Trisodium citrate	−	−	+	−

Glaciimonas, able to grow at sub-zero temperatures (−5 °C). 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 (Feller & Gerday, 2003; Margesin, 2009). Strain N1-38^T could be easily differentiated from the three reference strains of the genus *Glaciimonas* by its gliding motility and its inability to grow in the presence of 2 % (w/v) NaCl and to assimilate L-arabinose. Strain N1-38^T could be further distinguished from *G. singularis* LMG 27070^T by its ability to grow at −5 °C and to reduce nitrate to nitrite, and its inability to grow on R2A at 30 °C and to grow on nutrient-rich media (NA and TSA). These features point out the oligotrophic character of this strain due to its natural environmental conditions. On the basis of these combined data, strain N1-38^T represents a novel species of the genus *Glaciimonas*, for which the name *Glaciimonas frigoris* sp. nov. is proposed. An emended description of the genus *Glaciimonas* is also provided.

EMENDED DESCRIPTION OF THE GENUS *GLACIIMONAS*

In addition to the properties described for the genus *Glaciimonas* by Zhang *et al.* (2011), cells are non-motile or motile by gliding and are able or unable to reduce nitrate to nitrite or nitrogen.

Description of *Glaciimonas frigoris* sp. nov.

Glaciimonas frigoris (fri′go.ris. L. gen. neut. n. *frigoris* from frost).

Cells are Gram-stain-negative, short rods, 0.6–0.8 × 1.3–2.3 μm in size after 4 days at 15 °C on R2A. Flagella are absent (Fig. S4), and cells are motile by gliding. Colonies on R2A are creamy white, glossy, convex and circular with entire margin. Flexirubin and carotenoid pigments are absent. Grows under aerobic and microaerophilic conditions, but unable to grow under anaerobic conditions. Grows well at 1–25 °C in R2A broth (with highest biomass production at 1–5 °C) and on R2A agar; no growth occurs at 30 °C. Positive for growth at −5 °C in R2A broth. Grows at pH 6–8 and with 0–1 % (w/v) NaCl. Growth is

absent on NA and TSA. Positive for cytochrome *c* oxidase, catalase, nitrate reduction to nitrite, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase (weak) and naphthol-AS-BI-phosphohydrolase (weak). Negative for H_2S production, indole production from tryptophan, hydrolysis of aesculin and gelatin, citrate utilization, urease, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, mannosidase, α-fucosidase, tryptophan deaminase, arginine dihydrolase, lysine dihydrolase and ornithine dihydrolase activities in API ZYM, API 20NE and API 20E tests. Negative for degradation of starch, skimmed mild (protease), Tween 80, carboxymethylcellulose and lignosulfonic acid on agar plates supplemented with appropriate substrates. Negative for utilization a range of hydrocarbons (n-hexadecane, diesel oil, phenol, naphthalene, phenanthrene and anthracene) as sole carbon source. Positive for assimilation of glucose and D-mannitol, assimilation of malic acid is weak and delayed. Negative for assimilation of L-arabinose, D-mannose, *N*-acetyl-β-D-glucosamine, maltose, potassium gluconate, capric acid, adipic acid, trisodium citrate and phenylacetic acid, and for glucose fermentation. The predominant cellular fatty acids are summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{16:0}$ and $C_{18:1}\omega7c$. The polar lipid profile contains phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, three unidentified phospholipids, one unidentified aminolipid, two unidentified aminophospholipids and one unidentified polar lipid. The major respiratory quinone is ubiquinone 8 (UQ-8).

The type strain is N1-38^T (=LMG 28868^T=CCOS 838^T) and was isolated from sediment collected from ancient permafrost in Siberia, Russia. The genomic DNA G+C content of the type strain is 53.0 mol%.

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