**Glaciibacter superstes** gen. nov., sp. nov., a novel member of the family *Microbacteriaceae* isolated from a permafrost ice wedge

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Gram-positive, aerobic, non-spore-forming, irregular rod-shaped bacteria, designated strains AHU1791T and AHU1810, were isolated from a permafrost ice wedge in Alaska. Cells were motile by means of a polar flagellum. The strains were psychrophilic, growing at −5 to 25 °C. Phylogenetic analysis of 16S rRNA gene sequences indicated that the ice-wedge isolates formed a clade distinct from other genera affiliated with the family *Microbacteriaceae*. The novel strains showed highest levels of 16S rRNA gene sequence similarity with members of the genera *Agreia* and *Subtercola* (95.6–95.9 %). The level of 16S rRNA gene sequence similarity between strains AHU1791T and AHU1810 was 99.8 %. The cell-wall peptidoglycan type of the two strains was B2c, containing 2,4-diaminobutyric acid as the diagnostic amino acid. The predominant menaquinones were MK-12 and MK-13 (strain AHU1791T) and MK-11 and MK-12 (strain AHU1810). The major fatty acids of the two strains were 12-methyl tetradecanoic acid (anteiso-C15:0), 14-methyl hexadecanoic acid (anteiso-C17:0), 14-methyl pentadecanoic acid (iso-C16:0) and 13-methyl tetradecanoic acid (iso-C15:0). The DNA G+C contents of strains AHU1791T and AHU1810 were approximately 65 mol%. These phenotypic characteristics differentiated the ice-wedge strains from their closest phylogenetic neighbours, namely *Subtercola boreus* and the two recognized species of the genus *Agreia*. The sequences of the housekeeping genes coding for DNA gyrase subunit B (*gyrB*), RNA polymerase subunit B (*rpoB*) and recombinase A (*recA*) were almost identical between strains AHU1791T and AHU1810. Although the predominant menaquinones found in strains AHU1791T and AHU1810 were different, no other distinct differences were found with regard to other phenotypic and genotypic characteristics, indicating that the two strains were members of the same species. Accordingly, strains AHU1791T and AHU1810 are considered to represent a single novel species of a new genus, for which the name *Glaciibacter superstes* gen. nov., sp. nov. is proposed. The type strain of *Glaciibacter superstes* is AHU1791T (=DSM 21135T =NBRC 104264T).

At the time of writing, the family *Microbacteriaceae* comprises 25 genera that possess a B-type cross-linked peptidoglycan and fully unsaturated respiratory menaquinones. Psychrophilic bacteria within the family, for example members of the genera *Cryobacterium* (Suzuki et al., 1997; Zhang et al., 2007), *Rhodoglobus* (Sheridan et al., 2003) and *Subtercola* (Männistö et al., 2000), have been isolated from cold environments including soils and lakes in Antarctica, glacier ice and groundwater. Previously, we
reported on the isolation of bacteria preserved within a permafrost ice wedge ice for 25,000 years (Katayama et al., 2007). Phylogenetic analysis based on 16S rRNA gene sequences indicated that two of the ice-wedge isolates, namely strains AHU1791\textsuperscript{T} and AHU1810, were distinct from genera within the family Microbacteriaceae. In the present paper, we describe strains AHU1791\textsuperscript{T} and AHU1810 further and suggest that they represent a novel species of a new genus in the family Microbacteriaceae.

Strains AHU1791\textsuperscript{T} and AHU1810 were isolated from an ice wedge in the Fox permafrost tunnel, Alaska, USA (64.952' N 147.617' W), which is preserved at about −3 °C by the US Army's Cold Regions Research and Engineering Laboratory. Sample collection and isolation methods were as described by Katayama et al. (2007). Strains AHU1791\textsuperscript{T} and AHU1810 were originally isolated from agar plates containing Luria broth and minimal medium plus 0.5 % glucose (Stanier et al., 1986), respectively. The strains showed good aerobic growth at 20 °C in trypticase soy broth (TSB) or R2A revised medium (R2AR), the latter supplemented with 1 % glycerol and 0.5 % NaCl. Colony pigmentation and morphology were observed for colonies grown on trypticase soy agar (TSA) at 15 °C for 10 days. Motility was determined based on the swimming test on R2AR agar (0.25 % agar) at 15 °C. Flagella were stained according to the method of Nishizawa and Sugawara (see Hasegawa, 1985). Cell growth under anaerobic conditions was examined on TSA supplemented with 1 % D-glucose or 1 % l-arabinose. Catalase and oxidase activities were determined by bubble formation in 3 % H\textsubscript{2}O\textsubscript{2} solution and by use of an oxidase test kit (Wako), respectively. Acid production and assimilation of carbohydrates were determined by using API 50 CH (bioMérieux) test strips. Growth at −5, 0, 5, 10, 15, 18, 20, 23 and 25 °C on liquid R2AR supplemented with 1 % glycerol was determined based on increases in optical density at 600 nm. The upper temperature limit for growth was determined from colony formation on R2AR agar supplemented with 1 % glycerol at 25, 27 and 30 °C.

Menaquinones were extracted as described by Collins et al. (1977) and were analysed by reversed-phase HPLC (Tamaoka et al., 1983). Cell walls were prepared by using the protocol of Komagata & Suzuki (1987). Amino acids in acid hydrolysates of the cell walls were identified and quantified by TLC (Schleifer et al., 1967) and by using a JLC-500/S amino acid analyser (JOEL). Peptidoglycan acyl types were determined according to the method of Uchida et al. (1999). Fatty acid methyl esters were prepared as described by Suzuki & Kudo (2001) and were analysed by using GC-MS (QP2010; Shimadzu).

The DNA G+C contents of strains AHU1791\textsuperscript{T} and AHU1810 were 65.3 and 65.4 mol%, respectively. 16S rRNA gene sequence analysis showed that strains AHU1791\textsuperscript{T} and AHU1810 were related most closely to the type strains of Agreia pratensis (95.9 % similarity), Agreia bicolorata (95.6 %), Subtercola boreus (95.7–95.8 %), Microterricola viridarii (95.5 %) and Okibacterium frigilatii (95.5 %). Lower levels of 16S rRNA gene sequence similarity (91.6–94.7 %) were found with the type strains of all other type species within the family Microbacteriaceae. The neighbour-joining phylogenetic tree indicated that the ice-wedge strains formed a branch distinct from those of their related species (Fig. 1). This relationship was supported by a high level of bootstrap support. During alignments of the 16S rRNA gene sequences, 12-bp insertions (E. coli position 457) were found in strains AHU1791\textsuperscript{T} and AHU1810. A 13-bp insertion in the same position was also observed in Rhodoglobus vestalii LV3\textsuperscript{T}. The presence of this insertion was mentioned as a distinctive characteristic when strain LV3\textsuperscript{T} was proposed as a member of a new genus (Sheridan et al., 2003). The insertions were not observed in related taxa, such as A. pratensis, A. bicolorata and S. boreus. Phylogenetic relationships plus the presence of the insertions discussed above supported the designation of strains AHU1791\textsuperscript{T} and AHU1810 as representatives of a new genus. As shown in Fig. 1, S. boreus, the type species of the genus Subtercola, and Subtercola frigoramans were not closely related to each other. Thus, in the present study, the type strains of S. boreus, A. bicolorata and A. pratensis were used for further comparisons. The 16S rRNA gene sequence of strain AHU1791\textsuperscript{T} showed 99.8 % similarity to that of strain AHU1810.

Those physiological and morphological characteristics common to the two novel strains are given in the genus **Glaciibacter superstes** gen. nov., sp. nov.
and species descriptions below. Physiological differences found between strains AHU1791\textsuperscript{T} and AHU1810 were as follows. The maximum growth rate of strain AHU1791\textsuperscript{T} was observed at 20\textdegree{}C, whereas that of strain AHU1810 was observed at 18–20\textdegree{}C. Strain AHU1810 produced acid from D-arabinose whereas strain AHU1791\textsuperscript{T} did not. The predominant menaquinones of strain AHU1791\textsuperscript{T} were MK-12 (50\% of the total quinones) and MK-13 (32\%), whereas those of strain AHU1810 were MK-11 (32\%) and MK-12 (45\%). Purified peptidoglycans of strains AHU1791\textsuperscript{T} and AHU1810 contained 2,4-diaminobutyric acid, alanine, glycine and glutamic acid at a molar ratio of 2.0 : 0.9 : 1.3 : 1.0. Threo-3-hydroxyglutamic acid, found in the peptidoglycans of members of the genera \textit{Agreia} and \textit{Subtercola} in place of glutamic acid, was not detected in strains AHU1791\textsuperscript{T} or AHU1810. Schleifer & Kandler (1972) reported that the hydroxylation of glutamic acid depends on oxygen supply during culturing. We confirmed the presence or absence of threo-3-hydroxyglutamic acid by culturing \textit{S. boreus} DSM 13056\textsuperscript{T}, \textit{A. pratensis} DSM 14246\textsuperscript{T} and the ice-wedge strains under identical conditions.

The predominant menaquinones of strain AHU1791\textsuperscript{T} were MK-12 (50\% of the total quinones) and MK-13 (32\%), whereas those of strain AHU1810 were MK-11 (32\%) and MK-12 (45\%). Purified peptidoglycans of strains AHU1791\textsuperscript{T} and AHU1810 contained 2,4-diaminobutyric acid, alanine, glycine and glutamic acid at a molar ratio of 2.0 : 0.9 : 1.3 : 1.0. Threo-3-hydroxyglutamic acid, found in the peptidoglycans of members of the genera \textit{Agreia} and \textit{Subtercola} in place of glutamic acid, was not detected in strains AHU1791\textsuperscript{T} or AHU1810. Schleifer & Kandler (1972) reported that the hydroxylation of glutamic acid depends on oxygen supply during culturing. We confirmed the presence or absence of threo-3-hydroxyglutamic acid by culturing \textit{S. boreus} DSM 13056\textsuperscript{T}, \textit{A. pratensis} DSM 14246\textsuperscript{T} and the ice-wedge strains under identical conditions.

**Table 1.** Differential characteristics between strains AHU1791\textsuperscript{T} and AHU1810 and \textit{S. boreus}, \textit{A. pratensis} and \textit{A. bicolorata}

Data for reference species were taken from Männisto \textit{et al.} (2000), Evtushenko \textit{et al.} (2001) and Behrendt \textit{et al.} (2002). DAB, 2,4-Diaminobutyric acid; Hyg, threo-3-hydroxyglutamic acid; Orn, ornithine; a, anteiso-branched fatty acids; i, iso-branched fatty acids; a-C\textsubscript{15} : 0 DMA, 1,1-dimethoxy anteiso-pentadecane; i-C\textsubscript{16} : 0 DMA, 1,1-dimethoxy iso-hexadecane.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strains AHU1791\textsuperscript{T}/AHU1810</th>
<th>\textit{S. boreus}</th>
<th>\textit{A. pratensis}/\textit{A. bicolorata}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony colour</td>
<td>White</td>
<td>Yellow</td>
<td>Yellow or orange/yellow</td>
</tr>
<tr>
<td>Growth temperature range (\textdegree{}C)</td>
<td>–5 to 25</td>
<td>2–28</td>
<td>(\leq 37)/(\geq 2)</td>
</tr>
<tr>
<td>Optimum growth temperature (\textdegree{}C)</td>
<td>18–20</td>
<td>15–17</td>
<td>24–26</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>–</td>
<td>+/-</td>
</tr>
<tr>
<td>Peptidoglycan amino acid(s)</td>
<td>DAB</td>
<td>DAB, Hyg</td>
<td>DAB, Orn, Hyg</td>
</tr>
<tr>
<td>Predominant menaquinones (MK)</td>
<td>12, 13/11, 12</td>
<td>a-C\textsubscript{15} : 0</td>
<td>a-C\textsubscript{15} : 0</td>
</tr>
<tr>
<td>Major fatty acids (&gt;5% of the total)</td>
<td>a-C\textsubscript{15} : 0, i-C\textsubscript{16} : 0, a-C\textsubscript{17} : 0, i-C\textsubscript{15} : 0</td>
<td>a-C\textsubscript{17} : 0, a-C\textsubscript{16} : 0, a-C\textsubscript{17} : 0/i-C\textsubscript{16} : 0</td>
<td>a-C\textsubscript{15} : 0, i-C\textsubscript{16} : 0, a-C\textsubscript{17} : 0/D\textsubscript{16} : 0f</td>
</tr>
<tr>
<td>Major 1,1-dimethoxy alkanes</td>
<td>None detected</td>
<td>a-C\textsubscript{15} : 0 DMA, i-C\textsubscript{16} : 0 DMA</td>
<td>a-C\textsubscript{15} : 0 DMA</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>65</td>
<td>64</td>
<td>67/65</td>
</tr>
</tbody>
</table>

\(f\)Fatty acid profiles are for cells cultured at the respective optimal growth temperature, i.e. 20 (ice-wedge strains), 15 (\textit{S. boreus}) and 25 (\textit{Agreia} species) \textdegree{}C.

\(f\)Hexadecanoic acid (C\textsubscript{16} : 0) comprised >5\% of the total fatty acids in \textit{A. pratensis}. 

**Fig. 1.** Neighbour-joining tree showing the relationship between strains AHU1791\textsuperscript{T} and AHU1810 and the type strains of related taxa within the family \textit{Microbacteriaceae}. Bootstrap values of >50\% are shown at nodes. Dots indicate branches that were also found in the maximum-likelihood tree. \textit{Brevibacterium linens} DSM 20425\textsuperscript{T} (GenBank accession no. X77451) served as an outgroup (not shown). Bar, 0.01 substitutions per nucleotide position.
Muramic acid residues in the peptidoglycans of strains AHU1791T and AHU1810 were acetylated, as were those of S. boreus DSM 13056T. Fatty acids found in cells of strain AHU1791T cultured at 20 °C were anteiso-C15:0 (64.3% of the total), anteiso-C17:0 (11.8%), iso-C16:0 (11.0%), iso-C15:0 (7.5%) and anteiso-C15:1 (4.4%). This profile was almost identical to that of strain AHU1810. The profile of major fatty acids distinguished the ice-wedge strains from S. boreus and the two recognized species of the genus Agreia (Table 1). In addition, 1,1-dimethoxy alkanes, the unique methanolsyates observed in members of the genera Agreia, Subtercola and Frigoribacterium (Schumann et al., 2003; Kämpfer et al., 2000), were not detected in the ice-wedge strains. The proportion of anteiso-C15:1 in strain AHU1791T increased to 29% of the total fatty acids with a decrease in cultivation temperature to −5 °C, while the proportions of anteiso-C15:0 and anteiso-C17:0 decreased (see Supplementary Table S2). It is notable that, when strain AHU1791T was cultured at −5 °C, the proportion of iso-C15:0 increased (21%) and that of iso-C16:0 decreased to a greater extent compared with that at 0 °C, indicating that strain AHU1791T altered its fatty acid composition by increasing the ratio of short-chain components as well as that of unsaturated components to maintain membrane fluidity at subzero temperatures.

Phenotypic characteristics indicated that the ice-wedge strains were clearly distinguished from their phylogenetic neighbours, S. boreus, A. bicolorata and A. pratensis (Table 1).

The lengths of the housekeeping gene sequences for strains AHU1791T and AHU1810 were 1862 bp for gyrB [corresponding to gyrB positions 100–1973 of Leifsonia xyli subsp. xyli CTCB07 (GenBank accession no. NC_006087); 93.5% of the total length], 2840 bp for rpoB [corresponding to rpoB positions 436–3276 of L. xyli subsp. xyli CTCB07 (NC_006087); 81.2% of the total] and 819 bp for recA [corresponding to recA positions 79–897 of L. xyli subsp. xyli CTCB07 (NC_006087); 72.8% of the total]. The gyrB, rpoB and recA gene sequences of strains AHU1791T and AHU1810 were almost identical, with 1 nt difference in those of the gyrB and rpoB genes.

Levels of sequence similarity of the gyrB, rpoB and recA genes within the same region between Clavibacter michiganensis subsp. michiganensis NCPPB 382 (GenBank accession no. NC_009480) and C. michiganensis subsp. sepedonicus ATCC 33113T (NC_010407), which were compared as one of the criteria for subspecies discrimination, were 95.4, 98.5 and 95.0%, respectively. By comparison, levels of sequence similarity for these three genes between the ice-wedge strains were much higher. Accordingly, although the predominant menaquinones were different between strains AHU1791T and AHU1810, no further distinct differences were found with regard to other phenotypic and genotypic characteristics, suggesting that the two ice-wedge strains were affiliated with the same species.

Thus, strains AHU1791T and AHU1810 are considered to represent a single novel species of a new genus, for which the name Glaciibacter superstes gen. nov., sp. nov. is proposed.

Description of Glaciibacter gen. nov.

Glaciibacter (Gla.ci.i.bacter.ter. L. n. glacies ice; N.L. masc. n. bacter rod; N.L. masc. n. Glaciibacter a rod of the ice).

Cells are aerobic, Gram-positive, non-spore-forming, irregular rods. The cell-wall peptidoglycan is of B2 type, containing 2,4-diaminobutyric acid as the diagnostic amino acid. The muramic acid is of acetyl type. The predominant menaquinones are MK-12 and MK-13 or MK-11 and MK-12. The major fatty acids are 12-methyl tetradecanoic acid (anteiso-C15:0), 14-methyl hexadecanoic acid (anteiso-C17:0), 14-methyl pentadecanoic acid (iso-C16:0) and 13-methyl tetradecanoic acid (iso-C15:0). The G+C content of the DNA is about 65 mol%. The type species is Glaciibacter superstes.

Description of Glaciibacter superstes sp. nov.

Glaciibacter superstes (su.per’stes. L. masc. adj. superstes surviving).

Has the following characteristics in addition to those given for the genus. Cells are 1.1–1.8×0.3–0.5 μm in size, motile by means of a single polar flagellum. Mycelium formation does not occur. V-shaped forms are found at the early stages of growth in liquid culture. Cells exhibit a rod/coccus life cycle in TSB. Colonies are round, smooth, slightly shiny, convex and white on TSA. Cell aggregations are observed when cells are grown at above 15 °C in TSB. Growth occurs at −5 to 25 °C. The optimal temperature for growth is approximately 20 °C. Catalase-positive and oxidase-negative. Acid is produced from L-arabinose, D-fructose, D-galactose, D-glucose, D-mannose, L-rhamnose, D-xylose, D-ribose, cellobiose, maltose, sucrose, turanose, D-mannitol, xylitol and glycerol, but not from DL-fucose, D-xylose, trehalose, raffinose, D-sorbitol, starch or inulin. Acid production from D-arabinose is strain-dependent. The G+C content of the DNA is 65.3–65.4 mol%. The 16S rRNA gene sequence contains a 12-nt insertion (E. coli position 457).

The type strain, AHU1791T (＝DSM 21135T＝NBRC 104264T), was isolated from an ice wedge in the Fox permafrost tunnel, Alaska, USA. Strain AHU1810, isolated from the same source, is a second strain of the species.

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References


