

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 AHU1791^T 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 AHU1791^T and AHU1810 was 99.8%. The cell-wall peptidoglycan type of the two strains was B2 γ , containing 2,4-diaminobutyric acid as the diagnostic amino acid. The predominant menaquinones were MK-12 and MK-13 (strain AHU1791^T) and MK-11 and MK-12 (strain AHU1810). The major fatty acids of the two strains were 12-methyl tetradecanoic acid (anteiso-C_{15:0}), 14-methyl hexadecanoic acid (anteiso-C_{17:0}), 14-methyl pentadecanoic acid (iso-C_{16:0}) and 13-methyl tetradecanoic acid (iso-C_{15:0}). The DNA G+C contents of strains AHU1791^T 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 AHU1791^T and AHU1810. Although the predominant menaquinones found in strains AHU1791^T 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 AHU1791^T 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 AHU1791^T (=DSM 21135^T =NBRC 104264^T).

The GenBank/EMBL/DDBJ accession numbers of the 16S rRNA, *gyrB*, *rpoB* and *recA* gene sequences of strains AHU1791^T and AHU1810 are AB378301 and AB378302 (16S rRNA), AB436916 and AB436917 (*gyrB*), AB436918 and AB436919 (*rpoB*) and AB436920 and AB436921 (*recA*), respectively.

Details of the primers used for determination of housekeeping genes and the effect of growth temperature on the cellular fatty acid composition of strain AHU1791^T are available as supplementary material with the online version of this paper.

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^T and AHU1810, were distinct from genera within the family *Microbacteriaceae*. In the present paper, we describe strains AHU1791^T and AHU1810 further and suggest that they represent a novel species of a new genus in the family *Microbacteriaceae*.

Strains AHU1791^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^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 consisting of 0.1 % peptone, 0.1 % Casamino acids, 0.1 % K₂HPO₄, 0.05 % yeast extract and 0.03 % MgSO₄·7H₂O (pH 7.2) supplemented with 1 % D-glucose or 1 % (v/v) glycerol. Cell cultures used for all experiments were prepared in TSB at 20 °C unless indicated otherwise.

Chromosomal DNA was extracted and purified following the methods of Nakagawa *et al.* (2001). DNA G+C contents were determined by reversed-phase HPLC (Nakagawa *et al.*, 2001). 16S rRNA gene sequences (*Escherichia coli* positions 28–1509) were determined and were aligned with their relatives by using ARB software (Ludwig *et al.*, 2004) with reference to the SILVA database (Pruesse *et al.*, 2007). The sequences of the housekeeping genes coding for DNA gyrase subunit B (*gyrB*), RNA polymerase subunit B (*rpoB*) and recombinase A (*recA*) were determined by using the primers described in Supplementary Table S1 (see IJSEM Online). Phylogenetic trees were constructed according to the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods by using MEGA 4.0 (Tamura *et al.*, 2007) and PAUP* (Swofford, 2003). An evolutionary distance matrix for the neighbour-joining method was generated according to Jukes & Cantor (1969) by using complete deletion model analysis. Likelihood parameters were estimated via the hierarchical ratio tests implemented in MODELTEST 3.7 (Posada & Crandall, 1998). The robustness of tree topologies was assessed by bootstrap analyses (Felsenstein, 1985) based on 1000 replications.

Cell morphology was observed under a light microscope (Olympus BX50), with cells grown in TSB at 15 °C. Gram staining was performed as described by Hasegawa (1985). 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 swarming 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 % H₂O₂ 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 (JEOL). 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^T and AHU1810 were 65.3 and 65.4 mol%, respectively. 16S rRNA gene sequence analysis showed that strains AHU1791^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 fritillariae* (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^T and AHU1810. A 13-bp insertion in the same position was also observed in *Rhodoglobus vestalii* LV3^T. The presence of this insertion was mentioned as a distinctive characteristic when strain LV3^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^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^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

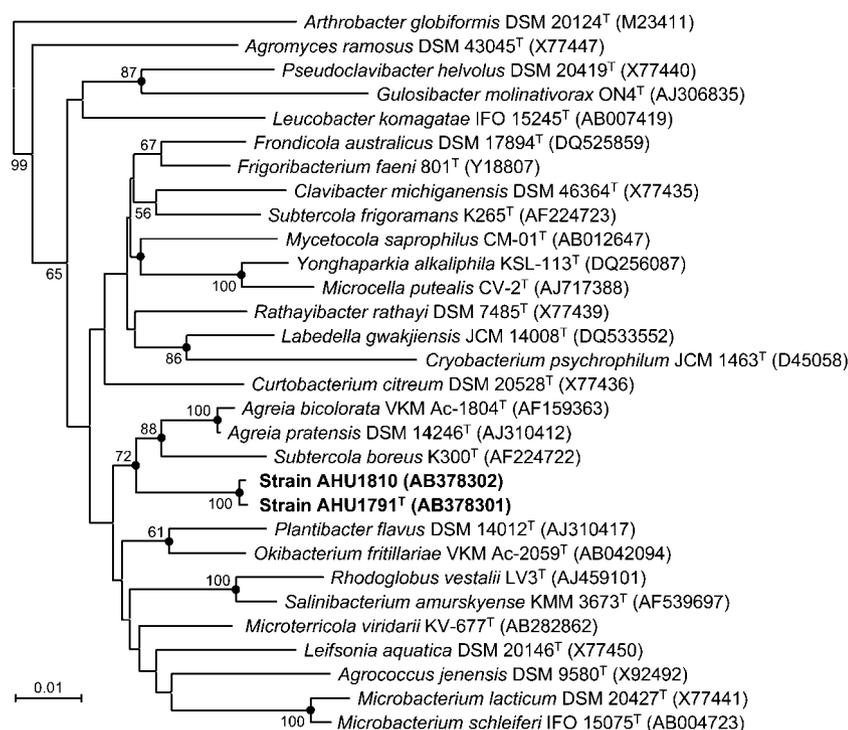


Fig. 1. Neighbour-joining tree showing the relationship between strains AHU1791^T and AHU1810 and the type strains of related taxa within the family *Microbacteriaceae*. Bootstrap values of >50% are shown at nodes. Dots indicate branches that were also found in the maximum-likelihood tree. *Brevibacterium linens* DSM 20425^T (GenBank accession no. X77451) served as an outgroup (not shown). Bar, 0.01 substitutions per nucleotide position.

and species descriptions below. Physiological differences found between strains AHU1791^T and AHU1810 were as follows. The maximum growth rate of strain AHU1791^T was observed at 20 °C, whereas that of strain AHU1810 was observed at 18–20 °C. Strain AHU1810 produced acid from D-arabinose whereas strain AHU1791^T did not.

The predominant menaquinones of strain AHU1791^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^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 *Agreia* and *Subtercola* in place of glutamic acid, was not detected in strains AHU1791^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 *S. boreus* DSM 13056^T, *A. pratensis* DSM 14246^T and the ice-wedge strains under identical conditions.

Table 1. Differential characteristics between strains AHU1791^T and AHU1810 and *S. boreus*, *A. pratensis* and *A. bicolorata*

Data for reference species were taken from Männistö *et al.* (2000), Evtushenko *et al.* (2001) and Behrendt *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_{15:0} DMA, 1,1-dimethoxy anteiso-pentadecane; i-C_{16:0} DMA, 1,1-dimethoxy iso-hexadecane.

Characteristic	Strains AHU1791 ^T /AHU1810	<i>S. boreus</i>	<i>A. bicolorata</i> / <i>A. pratensis</i>
Colony colour	White	Yellow	Yellow or orange/yellow
Growth temperature range (°C)	–5 to 25	2–28	≤37/≥–2
Optimum growth temperature (°C)	18–20	15–17	24–26
Motility	+	–	+/–
Peptidoglycan amino acid(s)	DAB	DAB, Hyg	DAB, Orn, Hyg
Predominant menaquinones (MK)	12, 13/11, 12	9, 10	10/10, 11
Major fatty acids (>5% of the total)*	a-C _{15:0} , i-C _{16:0} , a-C _{17:0} , i-C _{15:0}	a-C _{15:0}	a-C _{15:0} , i-C _{16:0} , a-C _{17:0} /C _{16:0} †
Major 1,1-dimethoxy alkanes	None detected	a-C _{15:0} DMA, i-C _{16:0} DMA	a-C _{15:0} DMA
DNA G + C content (mol%)	65	64	67/65

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

†Hexadecanoic acid (C_{16:0}) comprised >5% of the total fatty acids in *A. pratensis*.

Muramic acid residues in the peptidoglycans of strains AHU1791^T and AHU1810 were acetylated, as were those of *S. boreus* DSM 13056^T. Fatty acids found in cells of strain AHU1791^T cultured at 20 °C were anteiso-C_{15:0} (64.3 % of the total), anteiso-C_{17:0} (11.8 %), iso-C_{16:0} (11.0 %), iso-C_{15:0} (7.5 %) and anteiso-C_{15:1} (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 methanololates 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-C_{15:1} in strain AHU1791^T increased to 29 % of the total fatty acids with a decrease in cultivation temperature to -5 °C, while the proportions of anteiso-C_{15:0} and anteiso-C_{17:0} decreased (see Supplementary Table S2). It is notable that, when strain AHU1791^T was cultured at -5 °C, the proportion of iso-C_{15:0} increased (21 %) and that of iso-C_{16:0} decreased to a greater extent compared with that at 0 °C, indicating that strain AHU1791^T 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 AHU1791^T 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 AHU1791^T 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 33113^T (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 AHU1791^T 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 AHU1791^T 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.bac'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 γ 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-C_{15:0}), 14-methyl hexadecanoic acid (anteiso-C_{17:0}), 14-methyl pentadecanoic acid (iso-C_{16:0}) and 13-methyl tetradecanoic acid (iso-C_{15:0}). 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 \times 0.3–0.5 μ m 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, L-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, AHU1791^T (=DSM 21135^T =NBRC 104264^T), 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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Supplementary Table S1. Primers used for determination of housekeeping gene sequences

Positions are given relative to the corresponding genes of *Leifsonia xyli* subsp. *xyli* CTCB07 (GenBank accession no. NC_006087). N, Any; R, A or G; S, C or G; Y, C or T; M, A or C; W, A or T; K, G or T; B, C, G or T; V, A, C or G.

Gene	Primer	Sequence (5'-3')	Position
<i>gyrB</i>	gyrB-1F	AAG CGY CCS GGY ATG TAC ATC GG	88-110
	gyrB-2R	GAT GTC RAG GAA SCK SAC GTC CTT	1966-1989
	gyrB-AF	CAG ATG GCG TTC CTG AAC AAG GG	598-620
	gyrB-BR	TCG CCC GAG AGG TTG TCA TCC TT	985-1007
<i>rpoB</i>	rpoB-1F	ACS GTS TTY ATG GGY GAY TTC C	418-439
	rpoB-2R	CGA TGA ANC CGA ASG VGT TGA T	1486-1507
	rpoB-3F	CAY TAC GGY CGY ATG TGC CC	1405-1424
	rpoB-4R	CCT TSA CGA TSG CYT CGT ASA CCT T	3265-3289
	rpoB-AF	AAG CTS CGY CCV GGC GAG CA	805-824
	rpoB-BR	TCG CGV ACV ACG CGC TCC AT	1156-1175
	rpoB-CF	GCG CCA ACC AGG GCA CCA GCT A	2000-2021
	rpoB-DR	TTG TGG CCY TCC CAM GGC ATG A	2138-2159
	rpoB-ER	AGC GGC GCT GTG CGC TTC CTC A	2847-2868
	<i>recA</i>	recA-1F	AAR GGY WCN GTS ATG CG
recA-2R		GTT CTC YTT RCC CTG NCC	256-275
recA-AF		GAR TCS TCS GGW AAG ACB AC	796-812
recA-BR		GGN GGC ATC TTG TTC TT	949-966

Supplementary Table S2. Effect of growth temperature on the cellular fatty acid composition of strain AHU1791^T

Values are percentages of total fatty acids.

Fatty acid	Growth temperature (°C)					
	25	20	15	10	0	-5
iso-C _{14:0}	0.4	0.3	0.3	0.5	0.4	0.3
anteiso-C _{15:1}	0.0	4.4	7.4	11.8	22.3	29.2
iso-C _{15:0}	4.1	7.5	8.7	8.7	9.0	21.2
anteiso-C _{15:0}	70.1	64.3	55.5	54.7	49.9	36.3
iso-C _{16:0}	11.7	11.0	11.8	12.7	11.5	4.7
C _{16:0}	1.1	0.2	0.5	0.5	0.6	0.4
iso-C _{17:0}	0.2	0.6	1.1	0.6	1.3	1.9
anteiso-C _{17:0}	12.3	11.8	14.7	10.4	4.9	6.0