Cryobacterium arcticum sp. nov., a psychrotolerant bacterium from an Arctic soil

Felizitas Bajerski,¹ Lars Ganzert,¹ Kai Mangelsdorf,² André Lipski³ and Dirk Wagner¹

Correspondence

Felizitas Bajerski
felizitas.bajerski@awi.de

¹Alfred Wegener Institute for Polar and Marine Research, Research Department Potsdam, Telegrafenberg A45, 14473 Potsdam, Germany

²Helmholtz Centre Potsdam, GFZ German Research Centre for Geosciences, Sektion 4.3, Organic Geochemistry, Telegrafenberg, B 423, 14473 Potsdam, Germany

³Rheinische Friedrich-Wilhelms-Universität Bonn, Institute of Nutrition and Food Sciences, Department of Food Microbiology and Hygiene, Meckenheimer Allee 168, 53115 Bonn, Germany

A psychrotolerant, Gram-stain-positive, yellow-pigmented, aerobic rod, designated SK1^T, was isolated from a soil sample collected from Store Koldewey, north-east Greenland. Cells were catalase- and methyl red-positive, produced H₂S and produced acid from glucose, mannitol and salicin. Strain SK1^T was able to grow between –6 and 28 °C, with an optimum at 20 °C. The isolate contained 2,4-diaminobutyrate, glycine, alanine and glutamic acid in the cell wall and the major menaquinones were MK-10 and MK-11. Identified polar lipids were phosphatidylglycerol and diphosphatidylglycerol. The major fatty acids were anteiso-C_{15:0} (53.5 %), anteiso-C_{17:0} (17.0 %) and C_{18:0} (12.1 %). The genomic DNA G+C content was 67.8 mol%. Strain SK1^T showed the highest 16S rRNA gene sequence similarity with *Cryobacterium psychrotolerans* 0549^T (97.6 %) and *Cryobacterium roopkundense* RuGI7^T (96.8 %). Considering morphological, physiological, biochemical and chemotaxonomic characters and phylogenetic analysis, strain SK1^T represents a novel species in the genus *Cryobacterium*, for which the name *Cryobacterium arcticum* sp. nov. is proposed. The type strain is SK1^T (=DSM 22823^T =NCCB 100316^T).

The genus *Cryobacterium* was first described by Suzuki *et al.* (1997) to accommodate psychrophilic Gram-positive irregular rods. The type species, *Cryobacterium psychrophilum*, had been isolated from an Antarctic soil sample by Inoue & Komagata (1976) and was originally named '*Curtobacterium psychrophilum*'. Currently, the genus comprises only three other known species: *Cryobacterium psychrotolerans*, isolated from the China No. 1 glacier by Zhang *et al.* (2007), *Cryobacterium mesophilum*, collected from Bigeum Island by Dastager *et al.* (2008), and *Cryobacterium roopkundense*, originating from Roopkund Glacier of the Himalayan mountain ranges (Reddy *et al.*, 2010). An emended description of the genus *Cryobacterium* includes Gram-positive, aerobic pleomorphic rods with optimal growth between 9 and 28 °C (Dastager *et al.*, 2008).

Within the scope of ecological studies of microbial communities in extreme polar environments, several cold-adapted aerobic bacteria were isolated from mineral soils on Store Koldewey, north-east Greenland. Strain SK1^T was isolated from a soil sample from the bottom of a slope

on the shore of Duck Lake, Store Koldewey (76° 25' N 18° 44' W). Nutrient agar (0.5 % peptone, 0.3 % meat extract, 0.1 % yeast extract, 0.5 % glucose, 1.5 % agar, all w/v, in deionized water; pH 7.2) was used for isolation. The isolate was maintained at 16 °C on half-diluted LB medium (0.5 % tryptone, 0.25 % yeast extract, 0.5 % NaCl, 1.5 % agar for solid medium, all w/v, in deionized water; pH 7.2). The same conditions and media were used for all experiments, unless otherwise noted.

Colony characteristics were determined visually after incubation for 7 and 14 days. Cell morphology was examined by light microscopy (Axioskop 2 Plus; Zeiss) after incubation for 2 days. Gram staining was carried out by the classical procedure as described by Gram (Schröder, 1991). The presence of flagella was determined in the way of Leifson (Schröder, 1991). Spores were detected according to Wirtz (Schröder, 1991). Conditions for growth were determined between –6 and 32 °C, with 0–10 % (w/v) NaCl and at pH 4–10. The medium was buffered with glycine (pH 4.0–5.0 and pH 10.0), MES (pH 5.0–6.5), HEPES (pH 7.0–8.0) or Bis Tris propane (pH 8.5–9.5) (Hoaki *et al.*, 1994). Growth was measured by monitoring turbidity at 600 nm twice a day over a period of 2 weeks. Anaerobic growth was tested on PYG agar (0.1 % peptone,

Abbreviation: Dab, 2,4-diaminobutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SK1^T is GQ406814.

0.1 % yeast extract, 0.2 % glucose, 1.5 % agar, all w/v, in deionized water; pH 7.2) incubated under a N_2/CO_2 (80:20, v/v) atmosphere. Susceptibility to nine antibiotics and lysozyme (all 1 mg ml⁻¹) was examined by using filter discs and measuring the inhibition zones. Acid production from carbohydrates was tested with peptone water (1 % peptone, 0.5 % NaCl, both w/v, in deionized water) containing 1 % (w/v) sugar solutions and bromothymol blue as an indicator, according to Hugh & Leifson (1953). The methyl-red test was performed as described by Schröder (1991). Catalase activity was determined by bubble production in a 10 % hydrogen peroxide solution. Oxidase activity was analysed with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine as a redox indicator, as described by Kovács (1956). Hydrolysis of starch and casein and the production of urease, hydrogen sulfide and indole from tryptophan were determined as described by Schröder (1991). Hydrolysis of gelatin was tested by flooding gelatin agar plates with saturated ammonium sulfate solution after incubation. To check the utilization of carbon compounds as sole carbon

sources, a minimal medium (0.1 % NH_4Cl , 0.1 % K_2HPO_4 , 0.05 % KH_2PO_4 , 0.02 % $MgSO_4 \cdot 7H_2O$, 0.005 % $CaCl_2 \cdot 2H_2O$, all w/v, 0.2 %, v/v, trace metal mixture, 1.5 % agar, in deionized water) with 0.25 % (w/v) of each carbon compound was used. The trace metal mixture contained (w/v) 0.5 % H_3BO_3 , 0.04 % $CuSO_4 \cdot 5H_2O$, 0.2 % $FeCl_3 \cdot 6H_2O$, 0.4 % $MnCl_2 \cdot 4H_2O$, 0.2 % NH_4MoO_4 and 0.4 % $ZnSO_4 \cdot 7H_2O$ dissolved separately in deionized water.

Genomic DNA was extracted by using the Microbial DNA isolation kit (MoBio Laboratories), following the manufacturer's instructions. 16S rRNA gene was amplified by PCR using the universal bacterial primers 8F (Ravenschlag *et al.*, 1999) and 1492R (Dojka *et al.*, 1998). Sequencing of the purified PCR products (MinElute PCR Purification kit; Qiagen) was performed by GATC (Konstanz, Germany) using the ABI 3730XL system (Applied Biosystems). The 16S rRNA gene sequences of strain SK1^T and closely related reference organisms were obtained from GenBank and aligned using the integrated SINA alignment tool from the

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain SK1^T within the family Microbacteriaceae. Bootstrap values ($\geq 50\%$) based on 1000 replications are shown at branch nodes. Asterisks indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony method. Plus signs indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood method. Bar, 0.01 substitutions per nucleotide position.

Table 1. Physiological and biochemical characteristics of strain SK1^T and related *Cryobacterium* species

Strains: 1, *Cryobacterium arcticum* sp. nov. SK1^T; 2, *C. roopkundense* DSM 21065^T (data from Reddy *et al.*, 2010); 3, *C. psychrotolerans* JCM 13925^T (Zhang *et al.*, 2007); 4, *C. psychrophilum* JCM 1463^T (Suzuki *et al.*, 1997); 5, *C. mesophilum* DSM 19267^T (Dastager *et al.*, 2008). +, Positive; ±, weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5
Colony colour*	LY	P	Y	OP	LY
Cell dimensions (µm)					
Length	0.4–2.0	~2.5	1.8–3.6	1.0–1.8	1.0–1.4
Width	0.2–0.4	~0.8	0.5–0.8	0.5–0.7	0.3–0.6
Motility	–	+	+	–	+
Temperature for growth (°C)					
Range	–6 to 28	0–18	4–27	<18	20–28
Optimum	20	15–18	20–22	9–12	28
pH for growth					
Range	5.0–9.5	ND	5.0–10.0	ND	6.0–11.0
Optimum	6.5–7.5	7.0	~6.0–7.0	ND	7.0–8.0
Acid production from:					
Glucose	+	–	+	+	+
Galactose	+	–	–	+	+
Maltose	+	–	+	–	–
Mannitol	+	ND	–	–	+
Arabinose	–	–	+	–	–
Inositol	–	–	–	–	+
Rhamnose	–	ND	–	–	+
Xylose	±	–	+	–	ND
Use of sole carbon sources					
D-Fructose	+	–	+	ND	–
D-Glucose	+	–	+	ND	+
<i>myo</i> -Inositol	+	–	–	ND	+
Lactose	+	–	+	ND	+
Maltose	+	–	+	ND	–
D-Mannose	+	–	+	ND	+
Raffinose	+	–	–	ND	+
D-Ribose	+	–	+	ND	–
Pyruvic acid	+	–	–	+	+
Lactate	–	–	+	+	ND
Hydrolysis of:					
Starch	–	–	+	±	+
Gelatin	–	+	–	–	–
Casein	–	–	+	–	+
DNA G + C content (mol%)	67.8	64.7	67.0	65.0	70.0
Menaquinones	10, 11	11, 10, 12	11, 12, 10	10, 11, 8, 9	11, 12
Lipids†	PG, DPG	PG, DPG, UL	PG, DPG, UL	PG, DPG, UL	PG, DPG

*LY, Light yellow; OP, opaque pink; P, pink; Y, yellow.

†DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; UL, unidentified lipids.

ARB-SILVA website (Pruesse *et al.*, 2007). The ARB program (Ludwig *et al.*, 2004) was utilized for phylogenetic analysis. Evolutionary distance matrices were calculated using the correction of Jukes & Cantor (1969) and a phylogenetic tree (Fig. 1) was constructed using the neighbour-joining method (Saitou & Nei, 1987). Base-to-base comparisons of strain SK1^T with its nearest neighbours revealed the highest 16S rRNA gene sequence similarity with *C. psychrotolerans* 0549^T (97.6%), followed by *C. roopkundense* RuG17^T

(96.8%), *C. psychrophilum* DSM 4894^T (96.4%) and *C. mesophilum* MSL-15^T (96.1%). As the 16S rRNA gene sequence similarity between strain SK1^T and its nearest phylogenetic relative was well below the value of 98.5% (Stackebrandt & Ebers, 2006), DNA–DNA hybridization was not conducted. Determination of G + C content of DNA was done by HPLC according to the method of Mesbah *et al.* (1989). The G + C content of strain SK1^T was 67.8 mol%.

For quantitative analysis of the cellular fatty acid composition, cells were harvested after 3 days of incubation. Extraction and analysis of polar lipids and fatty acid methyl esters was conducted according to the protocol of Zink & Mangelsdorf (2004). Isoprenoid quinones were extracted using the small-scale integrated procedure of Minnikin *et al.* (1984). Menaquinones were analysed by HPLC using a Hewlett Packard model 1050 equipped with an ODS Hypersil column (Agilent Technologies) and a diode-array detector. Methanol/isopropyl ether (9:2, v/v) was used as the mobile phase with a flow rate of 1.0 ml min⁻¹ and a column temperature of 30 °C (Hu *et al.*, 1999). To determine the peptidoglycan structure, purified peptidoglycans were prepared according to the method of Schleifer & Kandler (1972). After derivatization (MacKenzie, 1987), the molar ratio of the amino acids was determined by GC (Groth *et al.*, 1996). The fatty acids identified in strain SK1^T were anteiso-C_{15:0} (53.5%), anteiso-C_{17:0} (17.0%), C_{18:0} (12.1%), iso-C_{16:0} (8.6%), C_{16:0} (6.5%), C_{18:1ω9c} (1.7%) and iso-C_{15:0} (0.6%). The identified polar lipids were phosphatidylglycerol and diphosphatidylglycerol. The identified menaquinones were MK-10 (56%) and MK-11 (44%). Amino acid analysis of the cell walls showed the presence of 2,4-diaminobutyrate (Dab), glycine, alanine and glutamic acid in the ratio 1.6:0.5:0.4:1.0. Peptidoglycan structure analysis revealed type B2γ with a D-Glu-D-Dab interpeptide bridge (type B7 according to DSMZ, 2001).

On the basis of morphological, physiological and biochemical characteristics, strain SK1^T could be clearly differentiated from its closest neighbours within the genus *Cryobacterium* (Table 1). This finding was supported by 16S rRNA gene sequence analysis. Therefore, strain SK1^T is a member of a novel species, for which the name *Cryobacterium arcticum* sp. nov. is proposed.

Description of *Cryobacterium arcticum* sp. nov.

Cryobacterium arcticum (arc'ti.cum. L. neut. adj. *arcticum* northern, arctic, from the Arctic, referring to the place from where the type strain was isolated).

Colonies are small, round, slightly convex with entire margins and viscous in consistency. Colour is matt light yellow and not translucent. Cells are Gram-stain-positive, non-spore-forming, non-flagellated short, irregular rods, approximately 0.4–2.0 µm long and 0.2–0.4 µm wide after culture for 3 days. Grows at between –6 and 28 °C (optimum 20 °C), at pH 5.0–9.5 (optimum pH 6.5–7.5) and with 0–3% (w/v) NaCl (optimum 0% NaCl). Acid is produced from (+)-D-galactose, glucose, maltose, D-mannitol, sucrose, (–)-D-salicin and (+)-D-xylose, but not from adonitol, L-arabinose, dulcitol, lactose, *myo*-inositol, (+)-L-rhamnose, (–)-D-sorbitol or trehalose. As sole carbon sources, utilizes L-arabitol, cellobiose, D-fructose, D-glucose, *myo*-inositol, inulin, lactose, maltose, D-mannose, melezitose (weakly), melibiose, raffinose, L-rhamnose, D-ribose, sucrose, D-salicin, sorbitol, trehalose, D-xylose, glycerol, glycogen, acetate and pyruvate, but not

adonitol, dulcitol, i-erythritol, L-fucose, L-asparagine, glycine, formate, lactate or succinate. Does not hydrolyse starch, casein or gelatin. Positive for H₂S production, catalase and methyl-red test, but negative for indole production from tryptophan and urease and oxidase. The major fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and C_{18:0}. 2,4-Diaminobutyrate, glycine, alanine and glutamic acid are present in the cell wall. The major menaquinones are MK-10 and MK-11. The polar lipids were phosphatidylglycerol and diphosphatidylglycerol. Sensitive to neomycin, streptomycin, oxytetracycline, novobiocin and rifampicin. Resistant to penicillin, ampicillin, kanamycin sulfate and erythromycin. Inhibited by lysozyme. The genomic DNA G+C content of the type strain is 67.8 mol%.

The type strain is SK1^T (=DSM 22823^T =NCCB 100316^T), isolated from a soil sample from Store Koldewey, north-east Greenland.

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