Cryobacterium psychrotolerans sp. nov., a novel psychrotolerant bacterium isolated from the China No. 1 glacier

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A novel psychrotolerant, Gram-positive, yellow-pigmented, aerobic bacterium, strain 0549^T, was isolated from the China No. 1 glacier. The cells of the isolate were catalase-positive, motile, irregular rods. The diamino acid content of the cell-wall peptidoglycan was determined to be 2,4-diaminobutyric acid. Strain 0549^T was able to grow at 4–27 °C, with optimum growth occurring at 20–22 °C. The major fatty acids were anteiso-C_{15:0}, anteiso-C_{15:1}, iso-C_{16:0} and anteiso-C_{17:0}. The genomic DNA G + C content was 67 mol%. A phylogenetic analysis based on 16S rRNA gene sequences indicated that strain 0549^T belonged to the genus *Cryobacterium* and exhibited 96.5% similarity to *Cryobacterium psychrophilum* JCM 1463^T. On the basis of the phenotypic, chemotaxonomic and phylogenetic properties and the DNA–DNA relatedness data, strain 0549^T represents a novel species of the genus *Cryobacterium*, for which the name *Cryobacterium psychrotolerans* is proposed. The type strain is 0549^T (= CGMCC 1.5382^T = JCM 13925^T).

The genus *Cryobacterium* was described by Suzuki *et al.* (1997) as including Gram-positive, aerobic, obligately psychrophilic bacteria that had a pleomorphic rod-shaped morphology. To date, the genus *Cryobacterium* comprises only one species, *Cryobacterium psychrophilum*, which was isolated from Antarctic soil (Inoue, 1976) and named '*Curtobacterium psychrophilum*' (Inoue *et al.*, 1976) until amended and renamed as *Cryobacterium psychrophilum* by Suzuki *et al.* (1997).

The China No.1 glacier, located in Xinjiang Uygur Autonomous Region, north-west China, is a relatively simple and closed ecosystem. Three psychrophiles were isolated from the area during our survey of cold-adapted micro-organisms (Zhu *et al.*, 2003; Zhang *et al.*, 2006). In this study, we report the isolation and identification of strain 0549^T. Physiological, biochemical, chemotaxonomic characterization and a phylogenetic analysis (based on 16S rRNA gene sequences) indicated that the new isolate could represent a novel species of the genus *Cryobacterium*.

Strain 0549^T was isolated from frozen soil collected from the China No. 1 glacier using previously described media and methods (Zhu *et al.*, 2003). The strain was obtained in pure culture after three successive transfers to fresh agar medium and was stored at –80 °C in 30% (v/v) glycerol. Strain 0549^T was routinely grown aerobically at 20 °C in PYG medium, which comprised (l⁻¹): 5 g Bacto peptone (Difco), 0.2 g yeast extract (Oxoid), 5 g glucose, 3 g beef extract (Oxoid), 0.5 g NaCl and 1.5 g MgSO₄·7H₂O (pH adjusted to 7.0). *Cryobacterium psychrophilum* JCM 1463^T was obtained from the Japan Collection of Microorganisms (Saitama, Japan) and was used as a reference strain. This culture was grown using medium containing (l⁻¹): 10 g Bacto peptone (Difco), 5 g yeast extract, 5 g Casamino acids (Difco), 2 g meat extract (Difco), 5 g malt extract (Difco), 2 g glycerol, 1 g MgSO₄·7H₂O and 0.05 g Tween 80 (pH adjusted to 7.2).

DNA was extracted and purified as described by Sambrook *et al.* (1989). The 16S rRNA gene was amplified by a PCR with the forward primer 5'-AGAGTTTGATCCTGGCTC-AG-3' and the reverse primer 5'-AAGGAGGTGATCCA-GCCGCA-3' (Liu *et al.*, 2000). The PCR product was sequenced by using the ABI BigDye 3.1 sequencing kit (Applied Biosystems) and an automated DNA sequencer (model ABI3730; Applied Biosystems). The 16S rRNA gene sequence of strain 0549^T (1399 bp) was submitted to

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 0549^T is DQ515963.

The fatty acid profiles of strain 0549^T and *Cryobacterium psychrophilum* JCM 1463^T are available in a supplementary table in IJSEM Online.

GenBank and EMBL to search for similar sequences by using the BLAST algorithm. Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony algorithms with Kimura's two-parameter model (Kimura, 1980) implemented in MEGA, version 3.0 (Kumar *et al.*, 2004). The resulting tree topologies were evaluated with a bootstrap analysis based on 1000 replicates. A phylogenetic analysis (Fig. 1) based on a consensus 1166 bp length of 16S rRNA gene sequence showed that strain 0549^T grouped as a member of the genus *Cryobacterium*, forming a distinct cluster with *Cryobacterium psychrophilum* JCM 1463^T (96.5 %) with strong support (100 %).

Cell morphology was examined under a light microscope (BH-2; Olympus). The growth temperature was determined with a TN3F temperature-gradient incubator (Advantec). The pH range for growth was determined for the culture in PYG at various pH values obtained by adjustment with HCl or NaOH (1 mol l⁻¹). The following tests were performed according to Dong & Cai (2001): catalase and oxidase activities, Voges–Proskauer reaction, Simmons' citrate test, hydrolysis of DNA, starch, gelatin and casein, activities of lipase, arginine dihydrolase, lysine decarboxylase, ornithine

decarboxylase, lecithinase and tryptophan deaminase, and the utilization of various substrates as sole carbon and energy sources. Acid production from carbohydrates was determined as described by Leifson (1963). The cells of strain 0549^T were irregularly rod-shaped, Gram-positive and motile. Colonies on PYG agar were yellow, smooth and convex with entire margins.

Menaquinones were extracted and purified according to Collins (1985) and were analysed by using HPLC (Wu *et al.*, 1989). The cellular polar lipids were extracted and analysed on silica-gel plates (Kieselgel 60 F; Merck) by using TLC (Kates, 1986). Cell-wall peptidoglycan was prepared by using a method described previously (Komagata & Suzuki, 1987). Amino acid compositions were determined using an automatic amino acid analyser (model S 433D; Sykam) equipped with a separation column (LCA K06/Na). TLC determination of cell-wall sugar was performed as described previously (Lechevalier & Lechevalier, 1970). Cellular fatty acids were determined from a culture grown in PYG at 20 °C for 3 days and were extracted, methylated and analysed using the standard MIDI (Microbial Identification) system (Sasser, 1990). The major menaquinones found in strain 0549^T included MK-12 (38 %), MK-11 (39 %) and MK-10

Fig. 1. Neighbour-joining phylogenetic dendrogram, based on 16S rRNA gene sequence similarity, of strain 0549^T and species of the family Microbacteriaceae. *Brevibacterium linens* DSM 20425^T (X77451) served as an outgroup. Numbers at the nodes represent bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets. GenBank accession numbers are given in parentheses. Bar, 1 % sequence divergence.

(16 %), followed by small amounts of MK-8 and MK-9. The main polar lipids present included diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid. Cell-wall hydrolysates of strain 0549^T contained 2,4-diaminobutyric acid as the diagnostic diamino acid; the molar ratio for glutamic acid/glycine/alanine/2,4-diaminobutyric acid was estimated to be 1.0:0.9:0.5:1.1. The main components of the whole-cell sugars were glucose and ribose. The predominant cellular fatty acids of strain 0549^T were anteiso-C_{15:0} (39.7 %), anteiso-C_{15:1} (17.2 %), iso-C_{15:0} (11.8 %), iso-C_{16:0} (14.6 %) and anteiso-C_{17:0} (11.2 %). The chemotaxonomic characteristics, such as the cell-wall peptidoglycan, the polar lipid profile and the fatty acid profile were in good agreement with those determined for *Cryobacterium psychrophilum* JCM 1463^T. In particular, both strains possessed very similar amounts of monounsaturated fatty acid (anteiso-C_{15:1}), which contributes to the maintenance of membrane fluidity at low temperatures (see the fatty acid profiles in Supplementary Table S1, available in IJSEM Online).

The G + C content of the genomic DNA was determined by using the thermal denaturation method (Sly *et al.*, 1986) with *Escherichia coli* K-12 as the reference. DNA–DNA hybridization experiments were carried out by using the liquid renaturation method (De Ley *et al.*, 1970) as modified by Huß *et al.* (1983). Both experiments were carried out using a DU800 spectrophotometer (Beckman). The DNA G + C content of strain 0549^T was 67 mol%. The DNA–DNA relatedness between strain 0549^T and *Cryobacterium psychrophilum* JCM 1463^T was 34 %.

Strain 0549^T was distinguished from *Cryobacterium psychrophilum* by the following phenotypic and chemotaxonomic characteristics. Strain 0549^T was psychrotolerant and grew at 4–27 °C, whereas *Cryobacterium psychrophilum* was obligately psychrophilic and grew optimally at 9–12 °C, but not at 18 °C. The whole-cell sugars of *Cryobacterium psychrophilum* contained rhamnose and fucose, which were lacking in the cell wall of strain 0549^T. Cells of strain 0549^T contained mainly MK-12, MK-11 and MK-10, whereas MK-10 was the major menaquinone in *Cryobacterium psychrophilum*. Other phenotypic features that can be used to differentiate strain 0549^T from *Cryobacterium psychrophilum* are shown in the species description and Table 1. On the basis of these results, it is concluded that strain 0549^T represents a novel species of the genus *Cryobacterium*, for which the name *Cryobacterium psychrotolerans* sp. nov. is proposed.

Description of *Cryobacterium psychrotolerans* sp. nov.

Cryobacterium psychrotolerans (psy.chro.tol'er.ans. Gr. adj. *psychros* cold; L. pres. part. *tolerans* tolerating; N.L. part. adj. *psychrotolerans* cold-tolerating).

Cells are Gram-positive, irregular rods, aerobic, psychrotolerant, motile and 0.5–0.8 × 1.8–3.6 µm in size. Colonies are

Table 1. Characteristics that differentiate strain 0549^T from *Cryobacterium psychrophilum*

Taxa: 1, strain 0549^T; 2, *Cryobacterium psychrophilum* (Suzuki *et al.*, 1997). +, Positive; –, negative; W, weakly positive.

Characteristic	1	2
Colony colour	Yellow	Pink
Motility	+	–
Growth temperature (°C):		
Range	4–27	4–17
Optimum	20–22	9–12
Hydrolysis of:		
Casein	+	–
DNA	–	+
Starch	+	W
Acid from:		
Arabinose	+	–
Fructose	–	+
Galactose	–	+
Maltose	+	–
Mannose	–	+
Sucrose	–	+
Assimilation of:		
Fumarate	–	+
Glutamate	+	–
Hippurate	–	+
Malate	–	+
Pyruvate	–	+
Nitrate reduction	+	–
DNA G + C content (mol%)	67	65

yellow, smooth, circular and convex with entire margins. Catalase-positive and oxidase-negative. Growth occurs at 4–27 °C and pH 5.0–10.0, with optimum growth at 20–22 °C and approximately pH 6.0–7.0. Growth occurs in the presence of 0–4 % (w/v) NaCl. Nitrate is reduced. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, lecithinase, urease, lipase and tryptophan deaminase activities are absent. The Simmons' citrate and Voges–Proskauer tests are negative. Hydrolyses casein, starch and aesculin, but not gelatin or DNA. The following substrates are utilized as sole carbon sources: glucose, maltose, sucrose, D-mannose, fructose, D-cellobiose, D-galactose, lactose, L-arabinose, xylose, glycerol, melezitose, ribose, salicin, inulin, glutamate, lactate, succinate and acetate. Acids are produced from glucose, arabinose, maltose and xylose. The following substrates are not utilized as sole carbon sources: mannitol, raffinose, L-rhamnose, D-sorbitol, sorbinose, galactitol, inositol, erythritol, gluconate, pyruvate, citrate, fumarate, hippurate, malate and tartrate. The major menaquinones are MK-11, MK-12 and MK-10. The main polar lipids are diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid. The amino acids of the cell-wall peptidoglycan are 2,4-diaminobutyric acid, alanine, glycine and glutamic acid. The main components of the whole-cell sugars are glucose and ribose.

The predominant cellular fatty acids are anteiso- $C_{15:0}$ (39.7 %), anteiso- $C_{15:1}$ (17.2 %), iso- $C_{15:0}$ (11.8 %), iso- $C_{16:0}$ (14.6 %), anteiso- $C_{17:0}$ (11.2 %), iso- $C_{14:0}$ (1.7 %), iso- $C_{17:0}$ (1.4 %) and $C_{16:0}$ (0.9 %). The genomic DNA G + C content is 67 mol%.

The type strain, 0549^T (=CGMCC 1.5382^T=JCM 13925^T), was isolated from the China No. 1 glacier (Xinjiang Uygur Autonomous Region).

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