Cryobacterium levicorallinum sp. nov., a psychrophilic bacterium isolated from glacier ice

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In this study, two psychrophilic bacterial strains were isolated from the China No. 1 glacier in Xinjiang, north-west China. Cells were Gram-positive rods. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the strains belonged to the genus Cryobacterium. Phylogenetic analysis showed that they clustered together and are most closely related to Cryobacterium luteum CGMCC 1.11210T, Cryobacterium flavum CGMCC 1.11215T, Cryobacterium psychrophilum CGMCC 1.4292T, Cryobacterium psychrotolerans CGMCC 1.5382T and Cryobacterium roopkundense CGMCC 1.10672T. The major cellular fatty acids of the novel strains were anteiso-C15:0, anteiso-C15:1, a-C16:0 and a-C15:0. Both strains contained diphosphatidylglycerol, phosphatidylglycerol and one unidentified glycolipid in the cell membrane. The results of DNA–DNA hybridization and physiological tests allowed the genotypic and phenotypic differentiation of strains Hh34T and Hh28 from related species. However, their high DNA–DNA relatedness showed that they belong to the same novel species. Strain Hh34T (=NBRC 107883T=CGMCC 1.11211T) was selected as the type strain to represent this novel species, for which the name Cryobacterium levicorallinum sp. nov. is proposed.

The genus Cryobacterium proposed by Suzuki et al. (1997) includes Gram-positive aerobes that have a pleomorphic rod-shaped morphology. At the time of writing, the genus Cryobacterium comprised seven recognized species, of which six are cold-adapted and one is mesophilic. They have been isolated from soil or ice: Cryobacterium psychrophilum, collected from an Antarctic soil sample by Inoue & Komagata (1976) (Suzuki et al., 1997), Cryobacterium psychrotolerans, isolated from frozen soil collected from the China No. 1 glacier by Zhang et al. (2007), Cryobacterium mesophilum, isolated from soil of Bigeum Island, Korea, by Dastager et al. (2008), Cryobacterium roopkundense, originated from Roopkund Glacier of the Himalayan mountain ranges (Reddy et al., 2010), Cryobacterium arcticum from mineral soils on Store Koldewey, north-east Greenland (Bajerski et al., 2011), and Cryobacterium flavum and Cryobacterium luteum, isolated from ice of the China No. 1 glacier (Liu et al., 2012).

As part of research on the bacterial biogeography of glaciers in the Xinjiang Uygur Autonomous Region, two psychrophilic bacterial strains, designated Hh34T and Hh28, were isolated from an ice sample collected from the China No. 1 glacier. In this study, the taxonomic status of the isolates was analysed using a polyphasic taxonomic approach.

Strains Hh34T and Hh28 were isolated using PYG medium [v/v: bacto peptone (Difco) 0.5 %, yeast extract 0.02 %, glucose 0.5 %, beef extract 0.3 %, NaCl 0.05 %, MgSO4.7H2O 0.15 %, pH adjusted to 7.0] at 14 °C and stored at −80 °C in 30 % (v/v) glycerol. C. psychrophilum CGMCC 1.4292T, C. psychrotolerans CGMCC 1.5382T, C. roopkundense CGMCC 1.10672T, C. flavum CGMCC 1.11215T and C. luteum CGMCC 1.11210T, obtained from the China General Microbiological Culture Collection Center, were used as reference strains for phenotypic tests and fatty acid analysis. These strains were routinely incubated at 14 °C in PYG medium.

Cell morphologies were examined using an Axioplan 2 microscope (Zeiss). Colony morphologies were observed on PYG agar plates after incubation at 14 °C. The temperature range (0–25 °C), pH range (4.0–13.0) and NaCl (0–6 %, w/v) tolerance for growth were determined on PYG medium. The strains were incubated in an anaerobic jar using the Oxoid atmosphere generation system to test their oxygen requirement. Utilization of various carbon sources was tested using a basal medium (containing 0.2 % (NH4)2SO4, 0.05 % NaH2PO4·H2O, 0.05 % KH2PO4, 0.02 % MgSO4·7H2O and 0.01 % CaCl2·2H2O). Catalase activity was determined by bubble production in 3 % (v/v) H2O2; cytochrome oxidase activity

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of Cryobacterium levicorallinum Hh34T and Hh28 are JF267312 and JF267310, respectively.

One supplementary table and three supplementary figures are available with the online version of this paper.
was determined using 1 % (w/v) \( N,N,N',N'-\text{tetramethyl-p-phenylenediamine} \). Hydrolysis of casein was assessed as described by Smibert & Krieg (1994). Hydrolysis of starch and lipase (Tweens 20, 40 and 80) were determined using PYG agar medium as the modified basal medium (Gordon et al., 1974). In addition, the strains were characterized biochemically using API 20E, API 20NE and API50CH strips (bioMérieux), according to the manufacturer’s instructions. Table 1 summarizes the data obtained from these comparative studies.

Two universal primers (27f, \( 5'-\text{AGAGTTTGATCCTGG-CTCAG-3'} \); 1492r, \( 5'-\text{GGTTACCTTGTTACGACTT-3'} \)) were used to amplify the 16S rRNA genes from strains Hh34\(^T\) and Hh28. The CLUSTAL W program (Thompson et al., 1994) was used to perform a multiple sequence alignment of the novel sequences and related sequences. The phylogenetic tree was reconstructed according to the neighbour-joining (Fig. 1) (Saitou & Nei, 1987), maximum-likelihood (Fig. S1 available in IJSEM Online) (Felsenstein, 1981) and maximum-parsimony (Fig. S2) (Fitch, 1971) algorithms using the software package MEGA 5.0 (Tamura et al., 2011). The genetic distances for the neighbour-joining analysis were calculated using Kimura’s two-parameter model. The tree topologies were evaluated using bootstrap analyses based on 1000 resamplings.

The 16S rRNA gene sequences of strains Hh34\(^T\) and Hh28 were identical to each other. Comparison of these sequences with those available in the public databases indicated that the two strains belonged to the genus *Cryobacterium* (Fig. 1) and were related most closely to *C. luteum* CGMCC 1.11210\(^T\) and *C. flavum* CGMCC 1.11215\(^T\) with 99.7 and 99.3 % sequence similarity, respectively; the next most closely related strains to the new isolates were *C. roopkundense* CGMCC 1.10672\(^T\) (98.7 %), *C. psychrophilum* CGMCC 1.4292\(^T\) (98.3 %) and *C. psychrotolerans* CGMCC 1.5382\(^T\) (97.3 %).

The initial DNA–DNA liquid reassociation rate was used for determination of DNA–DNA hybridization as described by De Ley et al. (1970). The tests were performed on a model Lambda 35 UV/VIS spectrometer equipped with a temperature programme controller (Perkin Elmer). Levels of DNA–DNA hybridization were determined between the isolates and the type strains of the closely related species of the genus *Cryobacterium*. DNA–DNA hybridization analysis showed that strain Hh34\(^T\) shared a mean (±SD) DNA–DNA relatedness of 36 ± 10 % with *C. flavum* CGMCC 1.11215\(^T\), 22 ± 6 % with *C. luteum* CGMCC 1.11210\(^T\), 31 ± 10 % with *C. psychrophilum* CGMCC 1.4292\(^T\), 23 ± 6 % with *C. roopkundense* CGMCC 1.10672\(^T\) and 20 ± 5 % with *C. psychrotolerans* CGMCC 1.5382\(^T\). These values are all below the 70 % threshold recommended by Wayne et al. (1987) for the recognition of genomospecies. The level of DNA–DNA relatedness between strains Hh34\(^T\) and Hh28 was 100 %, indicating that they belong to the same genomospecies (Wayne et al., 1987). The result of DNA–DNA relatedness was obtained from three independent determinations. The

### Table 1. Phenotypic characteristics that differentiate strains Hh34\(^T\) and Hh28 from the type strains of related species of the genus *Cryobacterium*

<table>
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<th>Characteristic</th>
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</table>
G+C content of the DNA was tested by the thermal denaturation method (Marmur & Doty, 1962) with *Escherichia coli* K-12 as the reference strain. The DNA G+C content of strains Hh34T and Hh28 was 63.7 mol%.

Polar lipids were extracted, separated by two-dimensional TLC and identified as described by Minnikin *et al.* (1977). Menaquinones were extracted according to the method of Collins (1985) and further analysed by HPLC (Wu *et al.*, 1989). For analysis of cellular fatty acid composition, cell mass of strains Hh34T and Hh28 and *C. psychrophilum* CGMCC 1.10672T, *C. psychrotolerans* CGMCC 1.11215T and *C. luteum* CGMCC 1.5382T, *C. levicorallinum* was harvested on PYG plates after incubation at 14 °C. Analysis was performed according to the standard protocol of the MIDI (Microbial Identification) system (Sasser, 1990). The samples were analysed on an Agilent Technologies 6890N gas chromatograph. The database used was TSBA (version 6.0). The amino acid of the cell-wall peptidoglycan was determined as described by Komagata & Suzuki (1987).

The polar lipids detected were diphosphatidylglycerol, phosphatidylglycerol and one unidentified glycolipid (Fig. S3). The predominant menaquinones of strain Hh34T were MK-9 (9.0 %), MK-10 (74.6 %) and MK-11 (16.4 %). The cellular fatty acids (relative percentage) detected in strain Hh34T were iso-C14:0 (1.7 %), iso-C15:0 (11.4 %), anteiso-C15:0 (41.7 %), iso-C15:1 G (1.4 %), anteiso-C15:1 A (20.1 %), C16:0 (1.3 %), iso-C16:0 (14.3 %) and anteiso-C17:0 (6.4 %) (Table S1). The cell-wall peptidoglycan contained 2,4-diaminobutyric acid as the diagnostic diamino acid.

The two new isolates exhibited identical biochemical characteristics. A detailed description of the morphological, physiological and biochemical characteristics of the isolates is given in the species description. Growth of strain Hh34T occurred at 0–18 °C and pH 6.5–11.0, and growth of strain Hh28 occurred at 0–20 °C and pH 6.0–12.0.

The results of the present study show that the new isolates belong to the genus *Cryobacterium* (Fig. 1). Based on phenotypic differences and genotypic criteria it is clear that the new isolates merit classification as representatives of a novel species within the genus *Cryobacterium*, for which the name *Cryobacterium levicorallinum* sp. nov. is proposed.

**Description of *Cryobacterium levicorallinum* sp. nov.**


Cells are Gram-positive, aerobic, rod-shaped and 0.93 × 0.43 μm in size. Colonies are light-coral, convex, round and 2.0 mm in diameter after 8 days of incubation on PYG plates at 14 °C. Growth occurs at 0–18 °C, at pH 6.0–12.0 and in the presence of 0–3.0 % (w/v) NaCl. Optimum growth temperature is 8–14 °C. Positive for catalase, but negative for oxidase. Does not hydrolyse Tween 20, 60 or 80, gelatin, aesculin, casein or starch. Indole and H2S are not formed. Does not utilize citrate. Reduces nitrate to nitrite. Negative for arginine dihydro- lase, lysine decarboxylase, ornithine decarboxylase, urease and tryptophan deaminase, but positive for β-galactosidase and Voges–Proskauer test. Uses the following substances as carbon sources: fructose, ribose, cellobiose, maltose, l-arabinose, mannose, glucose, sucrose, trehalose, inulin, xylose, galactose, glycerol, mannitol, sorbitol, sodium acetate, sodium gluconate and sodium malate. Does not use the following substances as carbon sources: melezitose,
raffinose, melibiose, rhamnose, sorbose, lactose, amygdalin, lin, sodium propionate or erythrose. Acids are produced from glycerol, L-arabinose, D-ribose, D-xylene, D-fructose, D-mannitol, D-sorbitol, aesculin, sucrose, xylitol and D-arabitol, but not from erythritol, D-arabinose, L-xylene, D-aldonitol, methyl β-D-xylpyranoside, D-galactose, D-glucose, D-mannose, l-sorbose, l-rhamnose, dulcitol, inositol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, trehalose, inulin, melezitose, raffinose, starch, glycogen, gentiobiose, turanose, D-lxysose, D-tagatose, D-fucose, L-fucose, L-arabitol, potassium gluconate, potassium 2-ketogluconate and sodium 5-ketogluconate. The predominant fatty acids are anteiso-C_{15:0}, iso-C_{16:0}, iso-C_{15:0} and anteiso-C_{15:1} A. The predominant menaquinone is MK-10. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol and one unidentified glycolipid. 2,4-Diaminobutyric acid is the major cell-wall diamino acid. The DNA G+C content is 63.7 mol%.

The type strain is Hh34^{T} (=NBRC 107883^{T}=CGMCC 1.11211^{T}), which was isolated from ice collected at the China No. 1 glacier in Xingjiang Uygur Autonomous Region, north-west China. Hh28, collected from the same source, is a second strain of the species.

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**References**


