Cryobacterium roopkundense sp. nov., a psychrophilic bacterium isolated from glacial soil

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Strain RuGI7^T was isolated from a soil sample collected at the periphery of the glacial Lake Roopkund in the Himalayan mountain range, India. Cells of RuGI7^T were Gram-positive, aerobic, rod-shaped, motile and grew optimally between 15 and 18 °C. Cells of RuGI7^T contained 2,4-diaminobutyric acid in the cell-wall peptidoglycan and the major menaquinones were MK-10, MK-11 and MK-12. The polar lipids present were diphosphatidylglycerol and phosphatidylglycerol and an unknown lipid and the major fatty acid was anteiso-C_{15:0}. Based on the above characteristics, strain RuGI7^T was assigned to the genus *Cryobacterium*. Strain RuGI7^T shared a 16S rRNA gene sequence similarity of 97.0 and 99.0 % with *Cryobacterium psychrotolerans* JCM 13925^T and *Cryobacterium psychrophilum* JCM 1463^T, respectively. However, DNA–DNA relatedness values between strain RuGI7^T and *C. psychrotolerans* and *C. psychrophilum* were 28 and 23 %, respectively. Furthermore, strain RuGI7^T exhibited several phenotypic and genotypic differences when compared with *C. psychrotolerans*, *C. psychrophilum* and *Cryobacterium mesophilum*. Based on these differentiating characteristics, strain RuGI7^T was identified as a novel species of the genus *Cryobacterium* for which the name *Cryobacterium roopkundense* sp. nov. is proposed. The type strain is RuGI7^T (=DSM 21065^T=JCM 15131^T).

The genus *Cryobacterium* was described by Suzuki *et al.* (1997) and emended by Dastager *et al.* (2008) to include Gram-positive aerobes that had a pleomorphic rod-shaped morphology. To date, the genus *Cryobacterium* comprises only three recognized species namely, *Cryobacterium psychrophilum* (Inoue & Komagata, 1976; Suzuki *et al.*, 1997), *Cryobacterium psychrotolerans* (Zhang *et al.*, 2007) and *Cryobacterium mesophilum* (Dastager *et al.*, 2008). In the present study, a new strain was isolated and was determined to represent a novel species of the genus *Cryobacterium*.

Strain RuGI7^T was isolated from a soil sample collected at the periphery of the glacial Lake Roopkund, India, as described earlier (Reddy *et al.*, 2008a, b). A soil sample of approximately 200 mg was suspended in 0.9 % (w/v) saline, subjected to shaking for 1 h, allowed to settle, plated (100 µl) on Antarctic bacterial medium (ABM) and incubated at 4 °C for 15 days. ABM consisted of 0.5 % peptone, 0.2 % yeast extract and 1.5 % agar at pH 7.0. Based on colony morphology, a pink-pigmented bacterium was clonally purified and maintained on Luria–Bertani agar plates (LB; 0.5 % yeast extract, 1.0 % tryptone, 1.0 % sodium chloride and 1.5 % agar, pH 7.0). Cell morphology was studied using light and transmission electron micro-

scopy as described previously (Reddy *et al.*, 2008a) and motility was assessed on 0.4 % nutrient agar plates. Growth and biochemical characteristics (Lányi, 1987; Smibert & Krieg, 1994), carbon assimilation and the sensitivity of the cultures to different antibiotics were determined according to previously described methods (Reddy *et al.*, 2004). All the tests were performed at 15 °C and the results were scored after 15–20 days. Biochemical characteristics were confirmed by double checking with a Hi25 *Enterobacteriaceae* identification kit (KB003; HiMedia) and HiCarbohydrate kit parts A, B and C (KB009; HiMedia) according to the manufacturer's protocol.

For quantitative analysis of whole-cell fatty acids, cells of RuGI7^T were grown on LB agar medium at 15 °C for 7 days and analysed by the Sherlock Microbial Identification System (MIDI) according to the protocol described by Agilent Technologies. For the analyses of fatty acids, lipids, pigments, cell-wall peptidoglycan and menaquinones, cells of RuGI7^T were grown in LB broth at 15 °C for 7 days. Lipids were extracted (Reddy *et al.*, 2003) and analysed by one-dimensional TLC using a pre-coated silica gel plate as described previously (Suresh *et al.*, 2004). Cells of RuGI7^T were grown on LB agar medium and the UV-visible spectrum of the pigment, *in vitro* and *in vivo*, was recorded as reported by Reddy *et al.* (2003). Peptidoglycan and cell-wall sugars were prepared and analysed according to the method of Komagata & Suzuki (1987). Isoprenoid

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain RuGI7^T is EF467640.

quinones were extracted according to the method of Collins *et al.* (1977) and separated by HPLC using an isocratic solvent system, methanol/isopropyl ether (3:1) (Tamaoka *et al.*, 1983; Tamaoka, 1986).

DNA from cells of strain RuG17^T was isolated using a Microbial DNA isolation kit (MoBio Laboratories) according to the manufacturer's protocol. The 16S rRNA gene was PCR-amplified and sequenced as described previously (Reddy *et al.*, 2000). The almost full-length sequence containing 1459 nucleotides was aligned with closely related bacterial 16S rRNA gene sequences using CLUSTAL W (Thompson *et al.*, 1994). Pairwise evolutionary distances were computed using the DNADIST program with the Kimura two-parameter model as developed by Kimura (1980). Phylogenetic trees were constructed using three different tree-making algorithms (neighbour-joining, minimum evolution and maximum-parsimony) using the MEGA4 software package (Kumar *et al.*, 2004). Bootstrap analysis was performed with 1000 replicate datasets in

order to assess stability among the clades recovered in the phylogenetic tree.

The morphological, biochemical and chemotaxonomic characteristics of the strain RuG17^T are given in the species description and also summarized in Table 1. Colonies of strain RuG17^T on LB agar plates incubated at 15 °C were convex, shiny, 2–3 mm in diameter and pink. Cells were Gram-positive, aerobic, rod-shaped, motile and occurred singly or in pairs. The cell size was approximately 0.8 µm in width and 2.5 µm in length (Fig. 1). The novel strain grew optimally between 15 and 18 °C. The cell-wall peptidoglycan contained 2,4-diaminobutyric acid as the diagnostic diamino acid. The cellular fatty acid composition (relative percentage) of strain RuG17^T was anteiso-C_{15:1} (14.1), iso-C_{15:0} (8.0), anteiso-C_{15:0} (20.3), iso-C_{16:0} (3.6), C_{16:1}ω9c (1.9), C_{16:0} (6.5), anteiso-C_{17:1}ω9c (2.9), iso-C_{17:0} (5.3), anteiso-C_{17:0} (7.5), C_{17:1}ω9c (1.0), C_{17:1}ω8c (1.1), C_{17:1}ω7c (1.4), C_{17:1}ω6c (1.1), iso H-C_{18:1} (1.1), iso-C_{18:0} (1.7), C_{18:1}ω9c (7.9), iso-C_{17:0}

Table 1. Characteristics that differentiate strain RuG17^T from recognized species of the genus *Cryobacterium*

Taxa: 1, strain RuG17^T; 2, *C. psychrophilum* JCM 1463^T (data from Suzuki *et al.*, 1997); 3, *C. psychrotolerans* JCM 13925^T (Zhang *et al.*, 2007); 4, *C. mesophilum* DSM 19267^T (Dastager *et al.*, 2008). +, Positive; –, negative; w, weakly positive; ND, not determined; PG, phosphatidylglycerol; DPG, diposphatidylglycerol; UGL, unknown glycolipid; UL, unknown lipid.

Characteristic	1	2	3	4
Morphology				
Motility	+	–	+	+
Colour	Pink	Pink	Yellow	Cream to pale yellow
Growth temperature (°C):				
Range	0–18	4–17	4–27	20–28
Optimum	15	9–12	20–22	25–28
Biochemical characteristics:				
Aesculin hydrolysis	–	–	+	–
DNase	–	+	–	–
Gelatinase	+	–	–	–
Nitrate reduction	+	–	+	–
Starch hydrolysis	–	w	+	+
Acid production from:				
L-Arabinose	–	–	+	+
D-Fructose	–	+	–	–
Galactose	–	+	–	+
D-Glucose	–	+	ND	ND
Maltose	–	–	+	–
D-Mannose	–	+	–	–
Sucrose	–	+	–	–
Assimilation of carbon compounds:				
Lactic acid	–	+	+	ND
Malate	–	+	–	+
Pyruvic acid	–	+	–	+
Major menaquinones (%)	MK-10 (26.0), MK-11 (53.0), MK-12 (21.0)	MK-8 (14.2), MK-9 (11.2), MK-10 (50.4), MK-11 (17.3)	MK-10 (16), MK-11 (39.0), MK-12 (38.0)	MK-11 (48.0), MK-12 (47.0)
Lipids	PG, DPG, UL	PG, DPG, UGL	PG, DPG, UGL	PG, DPG

Fig. 1. A transmission electron micrograph of a cell of strain RuGI7^T. Cells were grown in LB agar at 15 °C for 7 days and negatively stained with uranyl acetate. Bar, 0.5 μm.

3-OH (1.4) and cyclo-C_{19:0}ω8c (1.0). The major menaquinones were MK-10, MK-11 and MK-12 and the polar lipids were diphosphatidylglycerol and phosphatidylglycerol and one unidentified glycolipid. Based on the above characteristics, strain RuGI7^T was assigned to the genus *Cryobacterium*.

The BLAST sequence similarity search, based on the 16S rRNA gene sequence of strain RuGI7^T, also indicated that it was closely affiliated to the genus *Cryobacterium*. Base to base comparisons of the 16S rRNA gene sequence of strain RuGI7^T with species of the genus *Cryobacterium* resulted in the identification of *C. psychrotolerans* and *C. psychrophilum* as the nearest phylogenetic neighbours with 16S rRNA gene sequence similarities of 97.0 % and 99.0 %, respectively. The similarity of strain RuGI7^T with the other recognized species of the genus, *Cryobacterium mesophilum* (Dastager *et al.*, 2008) was 95.2 %. However, strain RuGI7^T shared DNA–DNA relatedness values of 28.0 % and 23.0 %, respectively, with *C. psychrophilum* and *C. psychrotolerans*. This clearly suggests that the strain RuGI7^T belongs to a novel species of the genus *Cryobacterium* (Stackebrandt &

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship between strain RuGI7^T and other related reference micro-organisms. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are indicated at the nodes. Bar, 0.01 substitutions per site.

Goebel, 1994). In addition, phylogenetic analyses based on the neighbour-joining (Fig. 2), minimum-evolution and maximum-parsimony (DNAPARS) methods indicated that the strain was closely related to the genus *Cryobacterium* and formed a robust clade with *C. psychrophilum* JCM 1463^T (GenBank accession no. D45058). In addition, strain RuGI7^T showed several phenotypic and genotypic differences when compared with the three recognized species of the genus *Cryobacterium* (Table 1). On the basis of the results obtained in this study, strain RuGI7^T represents a novel species of the genus *Cryobacterium* for which the name *Cryobacterium roopkundense* sp. nov. is proposed.

Description of *Cryobacterium roopkundense* sp. nov.

Cryobacterium roopkundense (ro.op.kun.den'se. N.L. neut. adj. *roopkundense* pertaining to Lake Roopkund, the area from where the type strain was isolated).

Colonies of RuGI7^T on LB agar plates are convex, shiny, 2–3 mm in diameter and are pink in colour. Cells are Gram-positive, aerobic, rod-shaped, motile and occur singly or in pairs. The cells are approximately 0.8 µm in width and 2.5 µm in length (Fig. 1). The optimum growth temperature is between 15 and 18 °C and the optimum pH is 7.0. Cells of strain RuGI7^T are positive for catalase and lipase activities, but negative for hydrolysis of casein and starch, for H₂S production, and for activities of lysine, arginine and ornithine decarboxylase, oxidase, urease, phosphatase, β-galactosidase and DNase. Gives a negative result in the methyl red test, the Voges–Proskauer test and in tests for indole production and phenylalanine deamination. Does not produce acid from erythritol, *myo*-inositol, inulin, D-melibiose, ribose, D-sorbitol, sorbose, trehalose or D-xylose. Cells of strain RuGI7^T can utilize citrate and malonate, but not acetate, adonitol, D-arabinose, L-arabinose, cellobiose, dulcitol, D-fructose, galactose, gluconate, D-glucose, glucosamine, glycerol, *myo*-inositol, inulin, lactose, maltose, D-mannitol, D-mannose, melibiose, melezitose, methyl α-D-glucoside, methyl α-D-mannoside, raffinose, D-rhamnose, D-ribose, salicin, sorbitol, L-sorbose, sucrose, trehalose, xylitol or D-xylose. Cells are sensitive to the following antibiotics (µg per disc): bacitracin (10), carbenicillin (100), cefotaxime (30), chloramphenicol (30), erythromycin (15), gentamicin-G (30), oleandomycin (15), penicillin-G (10), rifampicin (30), spectinomycin (100), streptomycin (10), tetracycline (30) and colistin (10). Additional characteristics of the species are listed in Table 1. The major fatty acid is anteiso-C_{15:0}. The absorption maxima (nm) of the pink pigment are 360, 470 and 490. The cell-wall peptidoglycan of strain RuGI7^T contains 2,4-diaminobutyric acid as the diagnostic diamino acid. The major menaquinones are MK-10, MK-11 and MK-12 and the polar lipids are diphosphatidylglycerol and phosphatidylglycerol and one unidentified glycolipid.

The type strain, RuGI7^T (=DSM 21065^T=JCM 15131^T), was isolated from a soil sample from the banks of Lake

Roopkund, Himalayan region, India. The G + C content of the genomic DNA of strain RuGI7^T is 64.7 mol%.

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