Paenibacillus glacialis sp. nov., isolated from the Kafni glacier of the Himalayas, India

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A novel strain of the genus *Paenibacillus*, KFC91^T, was isolated from the Kafni glacier of the Himalayas. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain KFC91^T clustered with *Paenibacillus antarcticus* LMG 22078^T (98.9%) and *Paenibacillus macquariensis* LMG 6935^T (98.7%). The cell wall peptidoglycan contained meso-diaminopimelic acid as the diamino acid, anteiso-C_{15:0} as the predominant fatty acid and MK-7 as the major isoprenoid quinone. Based on the phylogenetic analysis, chemotaxonomic characteristics and other phenotypic traits, strain KFC91^T was assigned to the genus *Paenibacillus*. DNA–DNA hybridization experiments of KFC91^T with *P. antarcticus* and *P. macquariensis* showed reassociation values of 39 and 52%, respectively. Thus, it is proposed that strain KFC91^T should be assigned the status of a novel species of the genus *Paenibacillus* and the name proposed is *Paenibacillus glacialis* sp. nov., with KFC91^T (=NCCB 100252^T =DSM 22343^T) as the type strain.

Ash *et al.* (1993), based on phylogenetic analysis of the 16S rRNA gene sequences of species known at the time, delineated the genus *Bacillus* into five phyletic lines (Groups 1 to 5) and this was in accordance with the earlier findings of Ash *et al.* (1991). Of particular interest was the observation that *Bacillus polymyxa* and its relatives belonging to Group 3 formed a coherent cluster compared with the other four *Bacillus* groups and possessed ‘signature’ bases unique to this group and a 22-base oligonucleotide specific to this group (Ash *et al.*, 1993). It was apparent that these Group 3 bacilli were phylogenetically distinct from the remaining four groups of the genus *Bacillus* and they were thus assigned to the genus *Paenibacillus*. The genus comprises species that are facultatively anaerobic or strictly aerobic, rod-shaped, motile and produce ellipsoidal endospores. The diamino acid in the peptidoglycan is meso-diaminopimelic acid and unsaturated MK-7 is the menaquinone. The G+C content of the DNA is 40–54 mol%. At the time of writing, 102 species of the genus and two subspecies have been described and all the species are either mesophilic (Enright *et al.*, 2003; Lim *et al.*, 2006; Saha *et al.*, 2005) or thermophilic (Chung *et al.*, 2000) except *Paenibacillus macquariensis* (Marshall & Ohye, 1966), which is psychrophilic. In this study, we describe a novel species of the genus *Paenibacillus* isolated from a soil sample from the Kafni glacier of the Himalayas, India.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences and *rpoB* gene sequence of strain KFC91^T are EU815294–EU815302 and GQ871757, respectively.

Southern blot analysis, DNA–DNA relatedness data and phylogenetic trees based on 16S rRNA gene and *rpoB* gene sequences are available with the online version of this paper.

Strain KFC91^T was isolated from a soil sample collected close to the Kafni glacier, at an altitude of 3500 m, located at 30° 13′ 5″ N 80° 3′ 20″ E in the Himalayan mountain ranges in India. Approximately 0.1 g soil sample was suspended in 1 ml sterile water, vortexed and plated on Luria–Bertani (LB) agar plates (Shivaji *et al.*, 2005). The bacterial count was 1×10^6 c.f.u. per gram soil. One of the colonies, KFC91^T, which was cream-coloured on tryptone soya agar [TSA (HiMedia M290), containing (l⁻¹) pancreatic digest of casein, 15 g; pancreatic digest of soybean meal, 5 g; agar, 15 g; pH 7.3] was studied in detail. Morphology was studied using light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as described previously (Reddy *et al.*, 2008) and the 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 by previously described methods (Reddy *et al.*, 2004). Biochemical characteristics were performed using the Hi25 *Enterobacteriaceae* identification kit (KB003) and HiCarbohydrate kit parts A, B and C (KB009, HiMedia) according to the manufacturer’s protocol. *Paenibacillus antarcticus* LMG 22078^T and *P. macquariensis* LMG 6935^T were used as reference strains for the study.

Cellular fatty acids were determined as described previously (Reddy *et al.*, 2008) using cells of KFC91^T grown on TSA medium for 4 days at 22 °C. Fatty acids were analysed by using the Sherlock Microbial Identification System (MIDI), according to the protocol described by Agilent Technologies. The predominant fatty acids of KFC91^T were anteiso-C_{15:0} (56.8%), iso-C_{15:0} (9.9%) and C_{16:1}ω11c

(6.0%). These fatty acids are typical of the genus *Paenibacillus*.

Menaquinones and polar lipids were determined in freeze-dried cells. Menaquinones were extracted as described by Collins *et al.* (1977) and were analysed by HPLC (Groth *et al.*, 1997). DNA was isolated according to the procedure of Marmur (1961) and G+C content (mol%) was determined from the melting point (T_m) curves (Sly *et al.*, 1986) obtained by using a Lambda 2 UV-Vis spectrophotometer (Perkin Elmer) equipped with the Templab 2.0 software package (Perkin Elmer). Cell walls were prepared (Komagata & Suzuki, 1987) from 500 mg bacterial cell pellet and amino acids and peptides in the acid hydrolysates were analysed by two-dimensional TLC on cellulose plates (Schleifer & Kandler, 1972).

Direct sequencing of the 16S rRNA gene of strain KFC91^T, performed as described previously (Reddy *et al.*, 2000, 2008), gave ambiguous results, suggesting multiple copies of the rRNA gene. Presence of multiple copies of the 16S rRNA gene is a characteristic feature of the genus *Paenibacillus* (Bosshard *et al.*, 2002; Hamasaki *et al.*, 2005; Nubel *et al.*, 1996). Therefore, to overcome this problem, the 16S rRNA gene of KFC91^T was amplified with primers 16S1 (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S2 (5'-AAGGAGGTGATCCAGCCGCA-3') corresponding to positions 8 to 28 and 1542 to 1522, respectively, of the *Escherichia coli* 16S rRNA gene. The PCR product was then purified using a Quiaquick PCR purification kit (Qiagen), ligated into the pGEM-T plasmid vector (Kiran *et al.*, 2005; Prabakaran *et al.*, 2007) according to the manufacturer's protocol (Promega) and cloned in *E. coli* DH5 α cells. Transformants were selected on LB plates containing 100 μ g ampicillin ml⁻¹, 100 mM IPTG and 50 mg X-Gal ml⁻¹. A total of nine transformants were sequenced.

The copy number of the 16S rRNA gene was determined by Southern blotting using ³²P-labelled 16S rRNA as a probe to hybridize the genomic DNA of strain KFC91^T digested with *Bam*HI, *Pst*I or both *Bam*HI/*Pst*I (restriction sites for both *Bam*HI and *Pst*I are absent in the KFC91^T 16S rRNA gene) as described by Sambrook *et al.* (1989). Genomic DNA was purified according to the protocol of Marmur (1961). Results indicated that KFC91^T contains multiple rRNA operons (Supplementary Fig. S1 available in IJSEM

Online), which is in agreement with the cloning experiment, where nine different sequences were found with a sequence difference of 0.1 to 0.9%. In bacteria, the number of rRNA operons present varies in number from one to 15 (Klappenbach *et al.*, 2001). At least eight 16S rRNA gene operons were found in *Paenibacillus macerans* (Hamasaki *et al.*, 2005) and heterogeneity in the sequence among the multiple 16S rRNA operons may range from 0 to 5% (Bricker, 2000; Cilia *et al.*, 1996; Mylvaganam & Dennis, 1992; Ninet *et al.*, 1996; Nubel *et al.*, 1996; Pettersson *et al.*, 1998). In *Paenibacillus polymyxa*, pairwise comparison of six sequences showed a difference of 2.5% in 12 rRNA operons (Nubel *et al.*, 1996). In another strain, *Paenibacillus turicensis*, six to eight different rRNA operons have been reported with seven different sequences with a sequence difference of 0.15 to 0.77% (Bosshard *et al.*, 2002).

Pairwise alignment of the KFC91^T 16S rRNA gene sequence (clone EU815300) performed using the ExPASy bioinformatics tool (<http://www.expasy.org>) showed similarities of 98.9, 98.7 and 93.2% with *P. antarcticus* LMG 22078^T (AJ605292), *P. macquariensis* LMG 6935^T (X60625) and *Paenibacillus nematophilus* DSM 13559^T (AF480935), respectively. To determine the phylogenetic affiliation of KFC91^T, the 16S rRNA gene sequence of the strain was aligned with all species of the genus *Paenibacillus* using CLUSTAL W (Thompson *et al.*, 1997) and phylogenetic analysis was carried out as described previously (Bhadra *et al.*, 2008). Phylogenetic trees were reconstructed using three tree-making algorithms, namely the maximum-likelihood method according to Guindon & Gascuel (2003) and the neighbour-joining and maximum-parsimony methods according to Kimura's two-parameter model (Kimura, 1980) using MEGA3.1 (Kumar *et al.*, 2004). Bootstrap analysis, based on 1000 replicate datasets, was performed to assess stability among the clades recovered in the phylogenetic tree. Phylogenetic analyses based on the above three methods, with the closely related species, indicated that strain KFC91^T clustered with *P. antarcticus* and *P. macquariensis* (Fig. 1). All cloned sequences of strain KFC91^T clustered with *P. antarcticus* and *P. macquariensis* (Supplementary Figs S2–S4).

In the DNA–DNA relatedness study performed according to Reddy *et al.* (2000), strain KFC91^T showed 39 and 52%

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationship between strain KFC91^T and related species of the genus *Paenibacillus*. *Escherichia coli* ATCC 11775^T (X80725) was used as an outgroup. Bootstrap values (expressed as percentages of 1000 replications) >50% are given at branch nodes. Bar, 2 substitutions per 100 nucleotides.

Table 1. Distinguishing characteristics of strain KFC91^T, *Paenibacillus antarcticus* and *Paenibacillus macquariensis*

Strains: 1, *Paenibacillus glacialis* sp. nov. KFC91^T; 2, *Paenibacillus antarcticus* LMG 22078^T; 3, *Paenibacillus macquariensis* LMG 6935^T. All data from this study. For fatty acid analysis, strains were cultured in TSA at 22 °C. All isolates of strain KFC91^T were motile and positive for aesculin hydrolysis and acid production from maltose and D-glucose, but could not tolerate 5 % NaCl and were negative for H₂S production, gelatin liquefaction and acid production from glycerol, sorbitol, inulin and xylitol. +, Positive; –, negative; ND, not detected.

Characteristics	1	2	3
Habitat	Soil	Sediment	Soil
Growth range (°C)	4–30	4–31	0–25
Oxidase	+	+	–
Voges–Proskauer test	–	–	–
Nitrate reduction	+	–	–
Arginine dihydrolase	+	–	–
Casein hydrolysis	+	–	–
Starch hydrolysis	–	+	+
Urease	–	+	–
Acid production from:			
L-Arabinose	–	+	+
D-Xylose	–	+	+
N-Acetylglucosamine	–	+	+
Lactose	–	+	+
D-Mannose	–	+	+
Mannitol	–	–	+
Melezitose	–	–	+
Methyl α-D-glucoside	–	+	+
Sodium gluconate	–	–	+
Galactose	–	+	+
Sucrose	–	+	+
Methyl α-D-mannoside	–	–	+
Fatty acids			
C _{10:0}	1.1	0.7	ND
C _{12:0}	0.5	0.5	0.5
anteiso-C _{13:0}	0.5	0.5	0.5
iso-C _{14:0}	5.0	6.0	1.9
C _{14:0}	1.7	1.0	1.3
iso-C _{15:0}	9.9	7.7	7.5
anteiso-C _{15:0}	56.8	65.3	55.3
C _{16:1ω7c} alcohol	4.0	5.0	0.9
iso-C _{16:0}	2.1	2.0	6.9
C _{16:1ω11c}	6.0	5.1	2.1
C _{16:0}	2.7	1.2	7.8
iso-C _{17:0}	0.9	0.5	2.1
anteiso-C _{17:0}	1.5	1.0	3.9
iso-C _{17:1ω10c}	0.7	0.5	0.5
C _{17:1ω9c}	0.6	0.6	ND
iso-C _{18:0}	ND	ND	0.7
Polar lipids*	PE, PG, DPG	PE, PG, DPG	PE, PG, DPG
DNA G + C content (mol%)	42.0	40.7	39

*PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol.

DNA–DNA relatedness with *P. antarcticus* LMG 22078^T and *P. macquariensis* LMG 6935^T, respectively. Reverse DNA–DNA hybridizations also yielded similar results (Supplementary Table S1). In addition, when the *rpoB* gene encoding the β subunit of RNA polymerase of strain KFC91^T was amplified using primers *rpoB*1698f (5'-AACATCGGTTTGATCAAC-3'; corresponding to *E. coli* position 1643) and *rpoB*2041r (5'-CGTTGCATGTTGGTACCCAT-3'; corresponding to *E. coli* position 2041) (Dahllöf *et al.*, 2000) and sequenced it showed 87.0 and 65.0 % similarity with *P. antarcticus* LMG 22078^T and *P. macquariensis* LMG 6935^T, respectively. Phylogenetic analyses of the *rpoB* gene sequence based on a neighbour-joining tree with the closely related species indicated that strain KFC91^T clustered with *P. antarcticus* with a sequence similarity of 87.0 % (Supplementary Fig S5). Considering 97.7 % sequence similarity of the *rpoB* gene as a cut-off for species delineation correlated with <70 % DNA–DNA relatedness (Adekambi *et al.*, 2008), and based on differences observed in the phenotypic characteristics and chemotaxonomic properties (Table 1), strain KFC91^T is regarded as a new species of the genus *Paenibacillus*, for which the name *Paenibacillus glacialis* sp. nov. is proposed. The type strain is KFC91^T (NCCB 100252^T = DSM 22343^T).

Description of *Paenibacillus glacialis* sp. nov.

Paenibacillus glacialis (gl.a.cia'lis. L. fem. adj. *glacialis* icy, referring to the cold, icy environment from which the strain was isolated).

Cells are Gram-positive, motile, aerobic and rod-shaped (0.5 μm $\times$ 2.2 μm). Ellipsoidal endospores are visible in swollen sporangia in the subterminal region of the cell. Colonies grown on TSA are circular, convex with entire margins, cream-coloured and non-pigmented. Growth occurs between 4 and 30 °C and at pH 6–7.5. Optimum growth occurs at 22 °C and pH 7.0. Tolerates 2 % NaCl but not 5 % NaCl. Positive for catalase but negative for lysine decarboxylase, ornithine decarboxylase, phenylalanine deamination, citrate utilization, indole production and the methyl red test. Cells utilize D-fructose, cellobiose, D-glucose, inositol, ribose, trehalose, maltose, erythritol, sucrose, inulin and salicin as the sole carbon source but do not utilize raffinose, adonitol, D-arabinose, dulcitol, D-xylose, D-mannose or glycerol. Acid is produced from D-fructose, trehalose, salicin and cellobiose. Acid is not produced from raffinose, melibiose, dulcitol, inositol, adonitol, D-rhamnose, D-arabinose or sorbose. The cell-wall diamino acid is meso-diaminopimelic acid. The major fatty acids are anteiso-C_{15:0} (56.8 %), iso-C_{15:0} (9.9 %) and C_{16:1} ω 11c (6.0 %). The major menaquinone is MK-7. The DNA G+C content of the type strain is 42.0 mol%. The major cellular phospholipids are phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine. Sensitive to ($\mu\text{g disc}^{-1}$) kanamycin (30), novobiocin (30), lomefloxacin (30) and chloramphenicol (30) but resistant to streptomycin (10).

The type strain is KFC91^T (=NCCB 100252^T =DSM 22343^T), isolated from a soil sample from the Kafni glacier of the Himalayas, India.

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