Bacillus cecembensis sp. nov., isolated from the Pindari glacier of the Indian Himalayas

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Strain PN5^T is a Gram-positive, aerobic, motile, rod-shaped, peritrichously flagellated bacterium that was isolated from the Pindari glacier using nutrient agar medium. Cells of PN5^T are catalase-positive and oxidase-negative and contain lysine, glutamic acid and alanine in the peptidoglycan (peptidoglycan type A4 α). Further, the cells are characterized by the presence of iso-C_{15:0} and iso-C_{16:1} as the predominant fatty acids and MK-7 as the isoprenoid quinone. Based on the above characteristics, strain PN5^T was assigned to the genus *Bacillus*. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain PN5^T clustered with the type strain of *Bacillus silvestris* with a sequence similarity of 97.2%. DNA–DNA hybridization between PN5^T and *B. silvestris* DSM 12223^T resulted in a relatedness of only 15%, clearly indicating that strain PN5^T represents a novel species. Further, PN5^T was different from *B. silvestris* with respect to various phenotypic and chemotaxonomic characteristics. Therefore, strain PN5^T is identified as a representative of a novel species of the genus *Bacillus*, for which the name *Bacillus cecembensis* sp. nov. is proposed. *Bacillus cecembensis* is unique among psychrotolerant *Bacillus* species in containing L-Lys–D-Glu in the cell-wall peptidoglycan. The type strain is PN5^T (=LMG 23935^T =MTCC9127^T =JCM 15113^T).

The genus *Bacillus* was created by Cohn (1872), with *Bacillus subtilis* as the type species. Members of the genus *Bacillus* are distinguished from other endospore-forming bacteria on the basis of being strict or facultative aerobes, rod-shaped, (usually) catalase-positive and they contain meso-diaminopimelic acid (meso-Dap) in the peptidoglycan, MK-7 as the major menaquinone and iso-C_{15:0} and anteiso-C_{15:0} as major fatty acids and have DNA G+C contents of 32–69 mol%. Interestingly, *Bacillus* is the only genus that contains organisms with L-lysine (Lee *et al.*, 2006; Rheims *et al.*, 1999) or ornithine (Spanka & Fritze, 1993; Abd El-Rahman *et al.*, 2002) as the diamino acid in the peptidoglycan, in addition to meso-Dap, which is present in the majority of species. At the time of writing, 212 species and subspecies are recognized in the genus *Bacillus* (<http://www.dsmz.de/microorganisms>). The ubiquitous distribution of *Bacillus* species in nature is due to the resistance of their endospores to chemical and physical agents. Species of *Bacillus* have been shown to be resistant to radiation (Shivaji *et al.*, 2006; Venkateswaran *et al.*,

2003), extremely low temperatures (Brambilla *et al.*, 2001; Logan *et al.*, 2000; Forsyth & Logan, 2000; Rüger *et al.*, 2000; Nelson & Parkinson, 1978; Abd El-Rahman *et al.*, 2002; Lechner *et al.*, 1998), high temperatures (Baker *et al.*, 2001; Caccamo *et al.*, 2000; Combet-Blanc *et al.*, 1995), salinity (Lim *et al.*, 2006; Lee *et al.*, 2006; Gugliandolo *et al.*, 2003), alkalinity (Nogi *et al.*, 2005; Vargas *et al.*, 2005; Yumoto *et al.*, 1998), acidity (Albert *et al.*, 2005) and high pressure (Bae *et al.*, 2005). Although *Bacillus* species have been isolated from a wide range of habitats, only one *Bacillus* species with a validly published name, *Bacillus lehensis* (Ghosh *et al.*, 2007), has been isolated from the Himalayan region. In the present study, a unique psychrotolerant *Bacillus* strain (PN5^T) containing L-lysine as the diamino acid in the peptidoglycan was isolated from a soil sample of the Pindari glacier of the Indian Himalayas; based on polyphasic taxonomic characterization, it is identified as representing a novel species.

Strain PN5^T was isolated from a soil sample collected close to the Pindari glacier at an altitude of approximately 3500 m. Approximately 200 mg of the soil sample was suspended in 0.9% saline, subjected to shaking for 1 h and allowed to settle and 100 μ l was plated on nutrient agar plates [0.3% beef extract, 0.5% peptone, 0.8% NaCl and 1.5% agar (all w/v)]. The viable bacterial cell count was approximately 2.1×10^5 c.f.u. and, based on colony morphology, 40 different morphotypes were observed. In the present study, one of the morphotypes in which the

Abbreviation: Dap, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PN5^T is AM773821.

A transmission electron micrograph of a cell of strain PN5^T and 16S rRNA gene sequence-based trees generated by UPGMA, minimum evolution and DNAPARS are available as supplementary material with the online version of this paper.

colony was opaque and white was picked up, purified, maintained on nutrient agar plates and studied in detail. Morphology was studied using light microscopy and transmission electron microscopy (Reddy *et al.*, 2006). Motility was assessed on 0.4 % nutrient agar plates and also by light microscopy. Spore staining was done using Schaeffer & Fulton's spore-staining kit (K006-1KT; HiMedia) according to the manufacturer's protocol. Growth and biochemical characteristics, carbon assimilation and sensitivity of cultures to different antibiotics were determined by previously described methods (Lányi, 1987; Reddy *et al.*, 2006; Smibert & Krieg, 1994). Biochemical characteristics were also checked with the Hi25 *Enterobacteriaceae* identification kit (KB003) and HiCarbohydrate kit parts A, B and C (KB009) (both from HiMedia) according to the manufacturer's protocol.

For quantitative analysis of whole-cell fatty acids, cells of PN5^T were grown on tryptic soy agar (TSA) at 28 °C for 2 days and extracted and analysed by using the Sherlock Microbial Identification System (MIDI) according to the protocol described by Agilent Technologies. Lipids were extracted and analysed by one-dimensional TLC using a pre-coated silica gel plate as described previously (Suresh *et al.*, 2004). Peptidoglycan was prepared and analysed according to the method of Komagata & Suzuki (1987). Isoprenoid quinones were extracted according to the method of Collins *et al.* (1977) and separated by HPLC using an isocratic solvent system of methanol/isopropyl ether (3:1) (Tamaoka *et al.*, 1983; Tamaoka, 1986).

DNA from strain PN5^T was isolated using the Microbial DNA isolation kit (Mo Bio Laboratories). The 16S rRNA gene was amplified by PCR and sequenced as described previously (Reddy *et al.*, 2000). The almost full-length sequence containing 1478 nucleotides (positions 27–1491, according to the *Escherichia coli* 16S rRNA gene sequence) was aligned with closely related sequences belonging to the genera *Bacillus*, *Caryophanon*, *Kurthia*, *Sporosarcina*, *Filibacter* and *Planococcus* using CLUSTAL W (Thompson *et al.*, 1994). Pairwise evolutionary distances were computed using the DNADIST program with Kimura's two-parameter model (Kimura, 1980). Phylogenetic trees were constructed using four different tree-making algorithms (neighbour-joining, maximum evolution, UPGMA and DNAPARS) using the MEGA 3 software package (Kumar *et al.*, 2001). Bootstrap analysis was performed with 1000 replicate datasets in order to assess stability among the clades recovered in the phylogenetic tree. DNA–DNA hybridization was performed by the membrane filter method as described previously (Pandey *et al.* 2002; Reddy *et al.*, 2002). *Bacillus silvestris* DSM 12223^T was used as a reference strain for comparing the peptidoglycan, lipids and menaquinones and for DNA–DNA hybridization.

Isolate PN5^T is a Gram-positive, motile, rod-shaped bacterium with peritrichous flagella and possesses terminally located spores. The rods are approximately 3.5 µm long and 1.25 µm wide (Supplementary Fig. S1, available in IJSEM Online). Cells are catalase-positive and oxidase-

negative and contain lysine, glutamic acid and alanine in the peptidoglycan (peptidoglycan type A4α). The cells are characterized by the presence of iso-C_{15:0} and iso-C_{16:1} as the predominant fatty acids and MK-7 as the lone isoprenoid quinone. Based on the above characteristics, strain PN5^T was assigned to the genus *Bacillus*. The placement of the strain within the genus *Bacillus* was further supported by phylogenetic analysis based on the 16S rRNA gene sequence, wherein it clustered with the type strain of *Bacillus silvestris* (Rheims *et al.*, 1999) with bootstrap resampling values of 83, 94, 87 and 54 %, respectively, with the neighbour-joining (Fig. 1), UPGMA, maximum evolution and DNAPARS methods (Supplementary Fig. S2). In addition, BLAST analysis based on the 16S rRNA gene sequence further supported its close relationship with *Bacillus silvestris* by exhibiting a sequence similarity of 97.2 % to the type strain (a difference of 39 nucleotides with 2 gaps out of 1478 nucleotides). The other closely related type strains that shared more than 95 % 16S rRNA gene sequence similarity with PN5^T are those of *Bacillus sphaericus* (96.8 %), *Bacillus fusiformis* (95.6 %), *Caryophanon tenue* (95.9 %) and *Caryophanon latum* (95.8 %). The above similarity suggests that strain PN5^T may represent a distinct species. However, DNA–DNA hybridization between strain PN5^T and *B. silvestris* DSM 12223^T resulted in a relatedness of only 15 %, clearly suggesting that strain PN5^T represents a novel species (Wayne *et al.*, 1987). In addition to these important characteristics, PN5^T exhibited number of differences from *B. silvestris*, the phylogenetically closest relative, with respect to growth characteristics, biochemical parameters and lipid and fatty acid composition (Tables 1 and 2).

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences (1478 bases), showing the phylogenetic relationships between *Bacillus cecembensis* sp. nov. PN5^T and related taxa. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are given at nodes. *Sulfolobus thermosulfidooxidans* DSM 9293^T was used as the outgroup. Bar, 2 substitutions per 100 nucleotides.

Table 1. Characteristics that differentiate strain PN5^T from *B. silvestris* DSM 12223^T

Data for *B. silvestris* DSM 12223^T were taken from Rheims *et al.* (1999).

Characteristic	PN5 ^T	<i>B. silvestris</i> DSM 12223 ^T
Growth temperature (°C)		
Range	4–30	10–40
Optimum	25	20–30
Tolerance to NaCl (%)	0–3	0–5
pH range for growth	6–8	6–7
Lipase	+	–
Utilization of:		
D-Ribose, glycerol	–	+
Trehalose, glucosamine, citrate	+	–
Lipids*	PG, DPG, (PC)	PG, DPG, PE, (PS), (PL)

*DPG, Diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PL, unknown phospholipid. Minor components are listed in parentheses.

Based on these differences, it is proposed to assign PN5^T to a novel species of the genus *Bacillus*, and the name suggested is *Bacillus cecembensis* sp. nov.

It is interesting to note that strain PN5^T and *B. silvestris* cluster with the clade representing species of the genus *Caryophanon*, implying that they are phylogenetically closely related to the genus *Caryophanon* (Fig. 1). However, *B. cecembensis* sp. nov. and *B. silvestris* can be differentiated from the species of *Caryophanon* since they contain MK-7 as the isoprenoid quinone, whereas *Caryophanon* species contain MK-6. Thus, the affiliation of *B. cecembensis* and *B. silvestris* to the genus *Bacillus* is unambiguous.

B. cecembensis is one of the very few psychrotolerant species of the genus *Bacillus*. Only six species have so far been reported. Five of these species exhibit growth temperature ranges of 0–30 °C [*Bacillus psychrotolerans* and *B. psychrodurans* (Abd El-Rahman *et al.*, 2002), *B. psychrosaccharolyticus* (Priest *et al.*, 1988), *B. insolitus* (Larkin & Stokes, 1967) and *B. marinus* (Rüger *et al.*, 2000)], whereas *Bacillus weihenstephanensis* is able to grow at 4–43 °C (Lechner *et al.*, 1998). Rüger *et al.* (2000) and Abd El-Rahman *et al.* (2002) compared the morphological and physiological characteristics of psychrotolerant *Bacillus* species, but *Bacillus globisporus* and *Bacillus psychrophilus* have since been transferred to the genus *Sporosarcina* (Yoon *et al.*, 2001). With the addition of *B. cecembensis* sp. nov., an attempt was made to re-evaluate the morphological, biochemical, chemotaxonomic and phylogenetic differences between psychrotolerant strains of *Bacillus*.

Table 2. Fatty acid methyl ester profiles of strain PN5^T and *B. silvestris* DSM 12223^T

Data for *B. silvestris* DSM 12223^T were taken from Rheims *et al.* (1999) (only components present at 0.9% or above were reported). Values are percentages of total fatty acids. –, Not detected (PN5^T) or not reported (*B. silvestris* DSM 12223^T).

Fatty acid	PN5 ^T	<i>B. silvestris</i> DSM 12223 ^T
C _{12:0}	0.1	–
iso-C _{13:0}	0.6	–
C _{13:0}	0.1	–
C _{14:0}	1.1	–
iso-C _{14:0}	8.8	2.9
iso-C _{15:0}	36.5	44.2
anteiso-C _{15:0}	8.9	5.6
C _{16:1} ω7c alcohol	14.5	–
iso-C _{16:0}	10.6	6.2
C _{16:1} ω11c	5.4	3.1
iso-C _{16:1}	–	18.7
C _{16:1} ω5c	0.1	–
C _{16:0}	2.6	–
C _{16:0} 3-OH	0.1	–
iso-C _{17:1} ω10c	1.8	7.8
anteiso-C _{17:1} A	0.1	2.8
iso-C _{17:0}	1.9	5.1
anteiso-C _{17:0}	2.0	3.0
C _{17:1} ω9c	0.8	–
C _{17:1} ω5c	0.3	–
C _{17:0}	0.2	1.0
iso-C _{18:0}	0.2	–
C _{18:1} ω9c	0.4	–
C _{18:1} ω5c	0.1	–
C _{18:0}	0.1	–
C _{18:1} 2-OH	0.1	–
C _{20:4} ω6,9,12,15c	0.1	–
Summed features*		
3	0.3	–
4	1.1	–
5	0.5	–
8	0.1	–

*Summed feature 3 contains C_{16:1}ω7c and/or C_{16:1}ω6c; summed feature 4 contains iso-C_{17:1} I and/or anteiso-C_{17:1} B; summed feature 5 contains ante-C_{18:0} and/or C_{18:2}ω6,9c; summed feature 8 contains C_{18:1}ω7c and/or C_{18:1}ω6c.

Comparative analyses indicate that all the species are distinct, as they all differ with respect to biochemical properties (Table 3). Interestingly, most of the psychrotolerant strains cluster together (supported by a significant bootstrap value of 96%) with the exception of *B. psychrosaccharolyticus* and *B. marinus* (very low bootstrap values, of less than 50%) (Fig. 2), so it is tempting to suggest that the 16S rRNA gene sequence contains adaptive signatures for psychrotolerance. *B. cecembensis* PN5^T also is unique among psychrotolerant strains in possessing urease activity and L-Lys–D-Glu in its cell wall (Table 3); the other

Table 3. Characteristics that distinguish the psychrotolerant species of the genus *Bacillus*

Taxa: 1, *B. cecembensis* sp. nov. PN5^T (data from the present study); 2, *B. psychrotolerans* (Abd El-Rahman *et al.*, 2002); 3, *B. psychrodurans* (Abd El-Rahman *et al.*, 2002); 4, *B. psychrosaccharolyticus* (Larkin & Stokes, 1967; Priest *et al.*, 1988); 5, *B. marinus* (Claus & Berkeley, 1986; Rüger *et al.*, 2000); 6, *B. insolitus* (Larkin & Stokes, 1967; Claus & Berkeley, 1986); 7, *B. weihenstephanensis* (Lechner *et al.*, 1998). +, Positive; –, negative; v, variable; NA, data not available.

Property	1	2	3	4	5	6	7
Spore shape	Round	Round	Round	Ellipsoid	Round or ellipsoid	Round or cylindrical	Not round
Sporangium shape	Swollen	Swollen	Swollen	Swollen	Not swollen	Not swollen	Not swollen
Growth temperature (°C)							
Minimum	4	–2	–2	0–2	<1–4	<0	4
Maximum	30	30	30	30	30	25	43
Requirement for NaCl	–	–	–	–	+	–	–
Maximum NaCl concentration (%)	3	3	5	3	2.4	2	10
Anaerobic growth	–	–	–	+	–	–	–
Oxidase	–	+	+	–	+	+	–
Hydrolysis of:							
Starch	–	+	+	–	–	–	+
Aesculin	–	–	–	–	–	NA	+
Gelatin	–	–	+	–	NA	–	+
Urea	+	–	–	–	–	–	–
Lipase	+	+	+	–	–	NA	+
Nitrate reduction	+	–	+	+	v	–	+
Cell-wall type	L-Lys–D-Glu	L-Orn–D-Glu	L-Orn–D-Glu	meso-Dap	L-Lys (direct)	L-Orn–D-Glu	meso-Dap
rRNA group	2	2	2	1	2	2	2

psychrotolerant strains contained either L-Orn–D-Glu or meso-Dap or L-Lys (direct) peptidoglycan types. The phylogenetic analyses also indicated that strain PN5^T is different from *B. lehensis* from the Indian Himalayas (Ghosh *et al.*, 2007) and *Bacillus fumarioli* from Antarctica (Logan *et al.*, 2000), raising the possibility that these species are epidemic in their distribution. *B. cecembensis* sp. nov. PN5^T is an interesting organism as it can grow on very few carbon sources (only three of almost 40 tested) and is sensitive to all but two of the tested antibiotics.

Fig. 2. Neighbour-joining tree, based on 16S rRNA gene sequences (1478 bases), showing the phylogenetic relationships between psychrotolerant species of *Bacillus*. See Fig. 1 for further details.

Description of *Bacillus cecembensis* sp. nov.

Bacillus cecembensis (ce.cem.ben'sis. N.L. masc. adj. *cecembensis* arbitrary name derived from the acronym CCMB for the Centre for Cellular and Molecular Biology, where the type strain was characterized).

Colonies are irregular, opaque and flat and cells are Gram-positive, motile, rod-shaped (3.5 µm long and 1.25 µm wide), peritrichously flagellated and possess round, terminally located spores. Grows at 4–30 °C with optimum growth at 25 °C. Positive for catalase, lipase, phosphatase, urease, ornithine decarboxylase, nitrate reduction and β-galactosidase (nitrate reduction and β-galactosidase negative on HiMedia kits) but negative for oxidase, lysine decarboxylase, phenylalanine deamination, indole production, methyl red and Voges–Proskauer tests and hydrolysis of aesculin, gelatin and starch. Utilizes trehalose, glucosamine and citrate but not D-glucose, erythritol, D- or L-arabinose, D- or L-xylose, adonitol, methyl α-D-glucoside, methyl α-D-mannoside, galactose, mannose, D-mannitol, D-fructose, L-sorbose, D-rhamnose, dulcitol, myo-inositol, sorbitol, N-acetylglucosamine, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, inulin, melezitose, raffinose, xylitol, D-glucuronic acid, sucrose, dextran, sodium gluconate, malonate, L-isoleucine, L-glutamine, L-alanine, L-threonine, L-phenylalanine, L-proline, L-lysine, L-serine, L-tyrosine, L-histidine, L-leucine or L-aspartic acid. Cells are resistant to (µg per disc unless indicated) nalidixic acid (30) and streptomycin (10) but sensitive to

amikacin (30), ampicillin (10), bacitracin (10), carbenicillin (100), cefazolin (30), cefuroxime (30), cefoperazone (75), cefotaxime (30), chloramphenicol (30), ciprofloxacin (5), colistin (10), co-trimoxazole (25), doxycycline (10), erythromycin (15), gentamicin G (30), kanamycin (30), leucomycin (30), lomefloxacin (30), nitrofurantoin (300), norfloxacin (10), novobiocin (30), oleandomycin (15), penicillin G (10), polymyxin B (50 U), rifampicin (30), roxithromycin (30), spectinomycin (100), tetracycline (30), tobramycin (10) and vancomycin (30). The peptidoglycan contains lysine, glutamic acid and alanine, representing peptidoglycan type A4 α . Polar lipids comprise phosphatidylglycerol, diphosphatidylglycerol and a compound that co-migrates with phosphatidylcholine; the predominant isoprenoid quinone is MK-7. The fatty acid composition is detailed in Table 2. Phylogenetically, a member of *Bacillus* RNA group 2.

The type strain is PN5^T (=LMG 23935^T =MTCC9127^T =JCM 15113^T), which was isolated from soil samples collected at an altitude of approximately 3500 m near the Pindari glacier of the Indian Himalayas.

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