Leifsonia pindariensis sp. nov., isolated from the Pindari glacier of the Indian Himalayas, and emended description of the genus *Leifsonia*

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Strain PON10^T is a yellow-pigmented, Gram-positive, aerobic, motile, rod-shaped bacterium isolated from the Pindari glacier of the Indian Himalayas. The cell-wall peptidoglycan contained DL-diaminobutyric acid as the diamino acid. The predominant fatty acids were anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} and the major isoprenoid quinones were MK-10 and MK-11. Based on the above characteristics, strain PON10^T was assigned to the genus *Leifsonia*. BLAST sequence similarity results indicated that *Leifsonia ginsengi* and *Leifsonia poae* were the nearest relatives, with 16S rRNA gene sequence similarity of 97.0 and 96.8% to the respective type strains. A difference of 3% in the 16S rRNA gene sequence indicated that PON10^T represents a novel species of the genus *Leifsonia*, and therefore DNA–DNA hybridization was not done. In addition, PON10^T showed a number of differences from *Leifsonia ginsengi* and *Leifsonia poae* with respect to phenotypic and chemotaxonomic characteristics. Thus, based on the differences it exhibited from *Leifsonia ginsengi* and *Leifsonia poae*, strain PON10^T was identified as representing a novel species named *Leifsonia pindariensis* sp. nov. The type strain is PON10^T (=LMG 24222^T =MTCC9128^T). An emended description of the genus *Leifsonia* is also presented.

Seventy per cent of the world's freshwater is frozen in glaciers, and the Himalayas have the largest concentration of glaciers outside the polar regions, covering about 33 000 km². The region has been referred to as the 'Water Tower of Asia', as it provides around 8.6 × 10⁶ m³ of water annually (Dyurgerov & Meier, 1997). Almost 70% of the glaciers in the Himalayas are retreating at a startling rate, and the major causal factor has been identified as climate change (Ageta & Kadota, 1992; Yamada, 1991; Yamada *et al.*, 1992; Fushimi *et al.*, 1985). The change in climate will affect the diversity of prokaryotic organisms and especially bacterial diversity. Due to the threat posed to the Himalayan glaciers from global warming and human intervention, there is an immediate need to explore and preserve their biodiversity. In an attempt to fingerprint the bacterial diversity of Himalayan glaciers, we have isolated several bacterial species from various habitats as part of an ongoing project (Chaturvedi & Shivaji, 2006; Chaturvedi *et al.*, 2005; Shivaji *et al.*, 2005). Besides our own study, there have been other reports on novel species of bacteria isolated from Himalayan glaciers (Ghosh *et al.*, 2007; Singla *et al.*, 2005; Mayilraj *et al.*, 2005, 2006a, b, c, d, e). In the

present study, another novel strain was isolated, subjected to polyphasic taxonomic characterization and identified as a member of a novel species of the genus *Leifsonia*.

The genus *Leifsonia* was described independently by Suzuki *et al.* (1999) and Evtushenko *et al.* (2000), but the description of *Leifsonia* by Evtushenko *et al.* (2000) predates the submission of the name in the paper by Suzuki *et al.* (1999) for inclusion in a Validation List. The genus *Leifsonia* Evtushenko *et al.* 2000 includes bacteria that are Gram-positive, non-spore-forming, irregular rods or filament-shaped, motile or non-motile and contain DL-2,4-diaminobutyric acid (DAB) in the peptidoglycan, MK-11 as the major menaquinone, phosphatidylglycerol and diphosphatidylglycerol as the major phospholipids and a high content of anteiso- and iso-branched fatty acids. The description contains many traits that are variable among the existing species. For instance, according to Evtushenko *et al.* (2000), the genus includes species that are white or yellow in colour, mesophilic and contain rhamnose as the major component in the cell wall, with glucose, galactose and mannose present in minor amounts. However, *Leifsonia rubra* is red pigmented, *L. rubra* and *Leifsonia aurea* are psychrophilic and *L. aurea* does not contain rhamnose in the cell wall (Reddy *et al.*, 2003). Therefore, the description of the genus given by Evtushenko *et al.* (2000) needs to be emended. Currently, eight *Leifsonia*

Abbreviation: DAB, DL-2,4-diaminobutyric acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PON10^T is AM900767.

species have been described, from various habitats such as nematode galls on *Poa annua* roots (Evtushenko *et al.*, 2000), distilled water (Leifson, 1962), diseased plants (a *Saccharum* interspecific hybrid and *Cynodon dactylon*) (Davis *et al.*, 1984), soil samples (Suzuki *et al.*, 1999), cyanobacterial mat samples (Reddy *et al.*, 2003) and roots of ginseng (Qiu *et al.*, 2007). The present isolate, PON10^T, resembles *L. rubra* and *L. aurea* in that it was isolated from an extremely cold habitat. However, based on a polyphasic taxonomic study, strain PON10^T was identified as representing a novel species.

Strain PON10^T was isolated from a soil sample collected close to the Pindari glacier at an altitude of 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 µl of the suspension was plated on an oligotrophic nutrient agar plate (one-tenth-strength nutrient agar, containing 0.03 % beef extract, 0.05 % peptone, 0.08 % NaCl and 1.5 % agar; Shivaji *et al.*, 2005). Based on colony morphology, a yellow-pigmented bacterium was purified and maintained on nutrient agar plates (0.3 % beef extract, 0.5 % peptone, 0.8 % NaCl and 1.5 % agar). Morphology was studied using light microscopy and transmission electron microscopy as described previously (Reddy *et al.*, 2006) 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 cultures to different antibiotics were determined by previously described methods (Reddy *et al.*, 2004). Biochemical characteristics were confirmed by using the Hi25 *Enterobacteriaceae* identification kit and HiCarbohydrate kits parts A, B and C (HiMedia) according to the manufacturer's protocol.

For quantitative analysis of whole-cell fatty acids, cells of PON10^T were grown on tryptic soy agar (TSA) at 28 °C for 2 days and fatty acids were analysed by 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 plate as described previously (Suresh *et al.*, 2004). Cells of PON10^T were grown on nutrient agar medium, and *in vitro* and *in vivo* UV-visible spectra of the pigments were recorded as described by Reddy *et al.* (2006). Peptidoglycan and cell-wall sugars were 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 PON10^T was isolated using the Microbial DNA isolation kit (Mo Bio Laboratories) according to the manufacturer's protocol. The 16S rRNA gene was amplified by PCR and sequenced as described previously (Reddy *et al.*, 2000). The almost full-length sequence containing 1492 nucleotides was aligned with closely

related sequences of the genus *Leifsonia* 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, minimum-evolution, UPGMA and maximum-parsimony) using the MEGA 3 software package (Kumar *et al.*, 2004). Bootstrap analysis was performed employing 1000 replicate datasets in order to assess stability among the clades recovered in the phylogenetic tree.

Strain PON10^T possessed all the characteristics described for the genus *Leifsonia*: cells were Gram-positive, motile, catalase-positive, curved rods that contained DAB in the peptidoglycan, MK-10 and MK-11 as the major menaquinones and phosphatidylglycerol and diphosphatidylglycerol as polar lipids and had a high content of anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} fatty acids. Besides the above characteristics, BLAST sequence similarity results based on the 16S rRNA gene sequence also supported the affiliation of PON10^T to the genus *Leifsonia*, as it was closely related to members of the genus, with similarity of 95.8–97.0 %. Of *Leifsonia* species, *Leifsonia ginsengi* (Qiu *et al.*, 2007) and *Leifsonia poae* (Evtushenko *et al.*, 2000) were the most closely related, with 16S rRNA gene sequence similarity of 97.0 % (44 nucleotide differences including 4 gaps out of 1451 nt, excluding primer sequences) and 96.8 % (46 nucleotide differences including 10 gaps out of 1451 nt, excluding the primer sequences) to the respective type strains. Phylogenetic analyses based on the neighbour-joining, minimum evolution, UPGMA and maximum-parsimony algorithms supported the association of PON10^T with the genus *Leifsonia* (Fig. 1). In all the phylogenetic methods tested in the present study, the species of *Leifsonia* formed two major clades. Clade I

Fig. 1. Neighbour-joining tree, based on 16S rRNA (1451 nt) gene sequences, showing the phylogenetic relationship between *Leifsonia pindariensis* sp. nov. strain PON10^T and related taxa. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are given at nodes. Bar, 0.005 substitutions per nucleotide position.

contains *Leifsonia naganoensis* (Suzuki *et al.*, 1999), *Leifsonia aquatica* (Evtushenko *et al.*, 2000), *Leifsonia xyli* subsp. *xyli* (Davis *et al.*, 1984; Evtushenko *et al.*, 2000), *L. xyli* subsp. *cynodontis* (Davis *et al.*, 1984; Evtushenko *et al.*, 2000), *Leifsonia shinshuensis* (Davis *et al.*, 1984; Evtushenko *et al.*, 2000) and *L. poae* (Davis *et al.*, 1984; Evtushenko *et al.*, 2000) and clade 2 contains *L. aurea* (Reddy *et al.*, 2003), *L. rubra* (Reddy *et al.*, 2003), *L. ginsengi* (Qiu *et al.*, 2007), *Subtercola boreus* (Männistö *et al.*, 2000) and *Agreia bicolorata* (Evtushenko *et al.*, 2001). Interestingly, strain PON10^T (like *L. ginsengi*) clustered with clade 2, despite showing the closest 16S rRNA gene sequence similarity to *L. poae* (Evtushenko *et al.*, 2000), albeit with insignificant bootstrap values. Clade 2 includes members of three different genera, *Leifsonia*, *Subtercola* and *Agreia*. The branching presented in the neighbour-joining tree (Fig. 1) was highly conserved and consistent in other trees generated by using minimum-evolution, UPGMA and maximum-parsimony (not shown). These results highlight the existing ambiguity with respect to the phylogenetic position of strain PON10^T. However, polyphasic characteristics of PON10^T undoubtedly assigned it to the genus *Leifsonia* (Evtushenko *et al.*, 2000) and it could thus be differentiated from species of the genera *Subtercola* and *Agreia* (Table 1). Furthermore, it showed several differences from its closest neighbours *L. ginsengi* and *L. poae* with respect to biochemical and chemotaxonomic characteristics (Table 2). For instance, PON10^T is motile and possessed an extra unknown lipid and MK-9 in minor amounts (Table 2). Furthermore, PON10^T had significantly larger amounts of the fatty acids iso-C_{15:0} and anteiso-C_{15:0} and smaller amounts of iso-C_{16:0} and anteiso-C_{17:0}. Strain PON10^T also showed differences from *L. ginsengi* and *L. poae* with respect to production of acid from sugars, utilization of carbon compounds and sensitivity to antibiotics (Table 2). Thus, based on these cumulative differences, it is proposed to assign strain PON10^T to a novel species, *Leifsonia pindariensis* sp. nov.

It is interesting to observe that strain PON10^T was unable to utilize most of the carbon compounds tested (it utilized

only 19 out of 59 carbon sources, which included 17 amino acids) and is sensitive to most of the antibiotics tested (resistant to 5/32 antibiotics), implying that the strain is adapted to the oligotrophic conditions and pristine environment of the Himalayas.

Emended description of the genus *Leifsonia* Evtushenko *et al.* 2000

Leifsonia (Leif.so'ni.a. N.L. fem. n. *Leifsonia* named after Einar Leifson, who isolated and described the first organism of this genus).

Cells are Gram-positive, non-spore-forming, rod-shaped or filamentous, obligately aerobic and catalase-positive. Cell-wall peptidoglycan is of B-type and contains DAB as the diamino acid and the major menaquinone is MK-11. Principal phospholipids are phosphatidylglycerol and diphosphatidylglycerol and the major fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The G+C content of the DNA ranges from 66 to 73 mol%. The genus is a member of the family *Microbacteriaceae* of the order *Actinomycetales*. *Leifsonia aquatica* is the type species.

Description of *Leifsonia pindariensis* sp. nov.

Leifsonia pindariensis (pin.da.ri.en'sis. N.L. fem. adj. *pindariensis* pertaining to the Pindari glacier of the Himalayan region of India, from where the type strain was isolated).

Displays the following properties in addition to those given in the emended genus description. Colonies are convex, yellow-pigmented and opaque. Cells are motile, short rods. Positive for β -galactosidase and urease but negative for oxidase, phosphatase, ornithine decarboxylase, lysine decarboxylase, nitrate reduction, phenylalanine deamination, indole production and the Voges-Proskauer test. Hydrolyses aesculin but not gelatin or starch. Produces acid from maltose and D-mannose but not from D-arabinose, D-fructose, D-galactose, D-glucose, melibiose, salicin, sucrose or D-xylose. Utilizes dulcitol, erythritol,

Table 1. Characteristics that differentiate the genus *Leifsonia* from *Subtercola* and *Agreia*

Additional data were taken from Evtushenko *et al.* (2000) (*Leifsonia*), Männistö *et al.* (2000) (*Subtercola*) and Evtushenko *et al.* (2001) (*Agreia*).

Characteristic	<i>Leifsonia</i>	<i>Subtercola</i>	<i>Agreia</i>
Colony colour*	PY, Y	PY, Y	Y, O, RO, BO
Motility	Variable	—	+
Diamino acid†	DAB	DAB	DAB or Orn
Major menaquinone(s)	MK-11	MK-9, MK-10	MK-10
Polar lipids‡	PG, DPG	PG, DPG, PL, GL	PG, DPG
Cell-wall sugars§	Glc, Gal	Rha, Xyl	Rha, Man, Fuc
DNA G+C content (mol%)	66–73	64–68	67

*BO, Brownish orange; O, orange; PY, pale yellow; RO, red–orange; Y, yellow.

†Orn, Ornithine.

‡DPG, Diphosphatidylglycerol; GL, unidentified glycolipid; PG, phosphatidylglycerol; PL, unknown phospholipid.

§Fuc, Fructose; Gal, galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Xyl, xylose.

Table 2. Characteristics that differentiate strain PON10^T from *L. ginsengi* wged11^T and *L. poae* VKM Ac-1401^T

Strains: 1, strain PON10^T; 2, *L. ginsengi* wged11^T (data from Qiu *et al.*, 2007); 3, *Leifsonia poae* VKM Ac-1401^T (Evtushenko *et al.*, 2000). +, Positive; –, negative; ND, not detected; NR, not reported; S, sensitive; R, resistant.

Characteristic	1	2	3
Motility	+	–	–
Growth temperature range (°C)	4–35	4–37	7–37
Gelatinase	–	–	+
Lipase	–	+	–
Methyl red test	–	–	+
Starch hydrolysis	–	+	–
Production of acid from:			
D-Arabinose	–	+	+
D-Fructose	–	+	+
D-Galactose	–	–	+
Melibiose	–	NR	–
Sucrose	–	+	+
Utilization of carbon compounds			
D-(–)-Arabinose	–	NR	+
Erythritol	+	NR	–
D-Fructose	–	NR	+
D-Galactose	–	–	+
D-Mannitol	–	+	+
Melezitose	–	–	+
Melibiose	–	–	+
Methyl α-D-glucoside	–	–	+
Raffinose pentahydrate	–	–	+
L-(+)-Rhamnose	–	–	+
D-(–)-Ribose	+	+	–
Salicin	+	–	+
Sucrose	–	+	+
D-(+)-Xylose	–	–	+
L-Asparagine	+	–	+
L-Serine	–	–	+
Sensitivity to antibiotics			
Ampicillin	S	R	R
Chloramphenicol	S	S	R
Gentamicin G	S	NR	R
Kanamycin	S	R	NR
Penicillin G	S	R	R
Rifampicin	S	S	R
Tetracycline	S	S	R
Polar lipids*	PG, DPG, (L)	NR	PG, DPG
Menaquinones†	(9), 10, 11	(10), 11, 12 (tr)	10, 11
Major differentiating fatty acids (%)			
iso-C _{15:0}	5.6	NR	1.0
anteiso-C _{15:0}	54.3	19.6	6.3
iso-C _{16:0}	6.8	8.3	15.6
C _{16:0}	1.9	NR	<1.0
iso-C _{17:0}	2.0	NR	<1.0
anteiso-C _{17:0}	19.1	10.5	45.4
Cyclohexylundecanoic acid	ND	52.0	ND

*L, Unknown lipid. Minor components are in parentheses. See Table 1 for other definitions.

†tr, Trace. Minor components are in parentheses.

D-glucose, D-glucuronic acid, glucosamine, glycerol, inulin, lactose, maltose, D-mannose, melibiose, D-ribose, salicin, L-sorbose, L-arginine, L-asparagine, L-cysteine, L-methionine and L-valine, but not N-acetylglucosamine, adonitol, aesculin, D- or L-arabinose, cellobiose, citrate, dextran, D-fructose, galactose, myo-inositol, malonate, maltose, D-mannitol, melezitose, methyl α -D-glucoside, methyl α -D-mannoside, raffinose, rhamnose, sodium gluconate, sorbitol, sucrose, trehalose, xylitol, D- or L-xylose, L-alanine, L-aspartic acid, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine or L-tyrosine. Cells are resistant to (μ g per disc unless indicated) colistin (10), nalidixic acid (30), norfloxacin (10), polymyxin B (50 U) and tobramycin (10) but sensitive to amikacin (30), ampicillin (10), bacitracin (10), carbenicillin (100), cefazolin (30), cefoperazone (75), cefotaxime (30), cefuroxime (30), chloramphenicol (30), ciprofloxacin (5), co-trimoxazole (25), doxycycline (10), erythromycin (15), gentamicin G (30), kanamycin (30), leucomycin (30), lomefloxacin (30), nitrofurantoin (300), novobiocin (30), oleandomycin (15), penicillin G (10), rifampicin (30), roxithromycin (30), spectinomycin (100), streptomycin (10), tetracycline (30) and vancomycin (30). The cell-wall sugars consist of a predominant amount of D-rhamnose and trace amounts of glucose, galactose and mannose. The isoprenoid quinones present are MK-9 (minor), MK-10 and MK-11 (major). Major polar lipids are phosphatidylglycerol and diphosphatidylglycerol; an unknown lipid is present in a minor amount. The fatty acid profile (>0.5 %) includes anteiso-C_{11:0} (0.9 %), anteiso-C_{12:0} (0.6 %), anteiso-C_{13:0} (0.6 %), C_{14:0} (0.7 %), iso-C_{14:0} (0.9 %), iso-C_{15:0} (5.6 %), iso-C_{15:1} G (0.5 %), anteiso-C_{15:0} (54.3 %), iso-C_{16:0} (6.8 %), C_{16:0} (1.9 %), iso-C_{17:0} (2.0 %) and anteiso-C_{17:0} (19.1 %). The methanolic spectrum of the yellow pigment shows absorption peaks at 350, 390, 440, 470 and 500 nm.

The type strain, PON10^T (=LMG 24222^T =MTCC9128^T), was isolated from a soil sample collected at an altitude of approximately 3500 m near the Pindari glacier of the Indian Himalayas.

Acknowledgements

We would like to thank the Department of Biotechnology, Government of India, for financial support to S. S.

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