Description of *Leifsonia kafniensis* sp. nov. and *Leifsonia antarctica* sp. nov.

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Strains KFC-22^T and SPC-20^T are yellow-pigmented, Gram-positive, aerobic, non-motile, rod-shaped bacteria that were isolated from a soil sample near the Kafni glacier in the Himalayan mountain ranges in India, and from a spade core sediment sample from the Antarctic Ocean at Larsemann Hill, respectively. In both cases, the cell-wall peptidoglycan contained 2,4-diaminobutyric acid as the diamino acid, anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} were the predominant fatty acids and MK-11 was the major isoprenoid quinone in the cell membrane. On the basis of the above-mentioned characteristics, both strains can be assigned to the genus *Leifsonia*. The strains share 16S rRNA gene sequence similarity of 97.7 % and DNA relatedness of only 10 %, indicating that they represent different species. A BLAST analysis indicated that *Leifsonia pindariensis* PON10^T was the closest phylogenetic neighbour of strains SPC-20^T and KFC-22^T, showing 16S rRNA gene sequence similarities of 97.3 and 97.7 %, respectively. However, at the whole-genome level, strains KFC-22^T and SPC-20^T shared 42 and 11 % DNA–DNA relatedness, respectively, with *L. pindariensis* PON10^T. In addition, both strains exhibited several phenotypic differences with respect to *L. pindariensis* PON10^T. Thus, on the basis of the differences that the two strains exhibited with respect to *L. pindariensis*, both were identified as representing novel species of the genus *Leifsonia*, for which the names *Leifsonia kafniensis* sp. nov. (type strain KFC-22^T = NCCB 100216^T = LMG 24362^T) and *Leifsonia antarctica* sp. nov. (type strain SPC-20^T = NCCB 100227^T = LMG 24541^T) are proposed.

The genus *Leifsonia* was originally described by Evtushenko *et al.* (2000) and its description was emended by Reddy *et al.* (2008). The genus includes bacteria that are Gram-positive, non-spore-forming, rod-shaped (or filamentous), obligately aerobic and catalase-positive. The cell-wall peptidoglycan is of the B type and contains 2,4-diaminobutyric acid as the diamino acid. MK-11 is the major menaquinone. Phosphatidylglycerol and diphosphatidylglycerol are the principal phospholipids 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%. At the time of writing, 11 species have been described (Evtushenko *et al.*, 2000; Leifson, 1962; Davis *et al.*, 1984; Suzuki *et al.*, 1999; Reddy *et al.*, 2003; Qiu *et al.*, 2007; Reddy *et al.*, 2008; Dastager *et al.*, 2008, 2009). Of the 11

reported species, four (*Leifsonia rubra*, *Leifsonia aurea*, *Leifsonia ginsengi* and *Leifsonia pindariensis*) are psychrotolerant, having a minimum growth temperature of 0–4 °C; the rest are mesophilic. In the present study, two novel psychrotolerant species are described. The strains (designated KFC-22^T and SPC-20^T) representing these two novel species were isolated from two different cold habitats, namely the Kafni glacier of the Himalayas and a sediment from Antarctica, and were characterized using a polyphasic taxonomic approach.

Strain KFC-22^T was isolated from a soil sample collected close to the Kafni glacier, at an altitude of 3500 m in the Himalayan mountain ranges in India. Strain SPC-20^T was isolated from a spade core sediment from the Antarctic Ocean near the Larsemann Hill area (69° 21.950' S 76° 06.296' E). A sample of approximately 200 mg was suspended in 5 ml 0.9 % NaCl, subjected to shaking at 3000 r.p.m. for 1 h at room temperature, allowed to settle and plated (100 µl) either on Luria–Bertani agar (for KFC-22^T) or nutrient agar (for SPC-20^T) (Shivaji *et al.*, 2005). On the basis of colony morphology, two yellow-pigmented strains (KFC-22^T and SPC-20^T) were purified and main-

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KFC-22^T and SPC-20^T are AM889135 and AM931710, respectively.

16S rRNA gene sequence similarities of strains KFC-22^T and SPC-20^T with respect to closely related strains are available as supplementary material with the online version of this paper.

tained on nutrient agar plates (containing 0.3 % beef extract, 0.5 % peptone, 0.8 % sodium chloride 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, biochemical characteristics (Lányi, 1987; Smibert & Krieg, 1994), carbon assimilation and the sensitivity of the cultures to different antibiotics were determined by using previously described methods (Reddy *et al.*, 2004). Biochemical characteristics were determined using the Hi25 *Enterobacteriaceae* identification kit (catalogue no. KB003; Himedia) and parts A, B and C of the HiCarbohydrate kit (catalogue no. KB009; Himedia), according to the manufacturer's protocols.

For quantitative analysis of whole-cell fatty acids, cells of KFC-22^T and SPC-20^T were grown on tryptic soy agar medium (Reddy *et al.*, 2008) at 25 °C for 2 days and analysed using the Sherlock Microbial Identification System (MIDI), according to the protocols described by Agilent Technologies. Lipids and pigments were extracted and analysed as described previously (Reddy *et al.*, 2007). 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 were separated using HPLC with an isocratic solvent system consisting of methanol/isopropyl ether (3:1) (Tamaoka *et al.*, 1983; Tamaoka, 1986).

DNA from strains KFC-22^T and SPC-20^T was isolated according to the method of Marmur (1961) and the 16S rRNA gene was PCR-amplified and sequenced as described previously (Reddy *et al.*, 2000). The almost-complete sequences (1518 and 1516 nt for KFC-22^T and SPC-20^T, respectively) of the 16S rRNA genes were aligned with closely related sequences belonging to 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, namely neighbour joining, minimum evolution, the unweighted pair group method with arithmetic averages and maximum parsimony, by using the MEGA3 software package (Kumar *et al.*, 2004). Bootstrap analysis, based on 1000 replicate datasets, was performed to assess stability among the clades recovered in the phylogenetic tree.

Strains KFC-22^T and SPC-20^T were assigned to the genus *Leifsonia* on the basis of characteristics described for the genus *Leifsonia* (Evtushenko *et al.*, 2000; Reddy *et al.*, 2008). Both isolates consisted of Gram-positive, catalase-positive, curved rods that contained 2,4-diaminobutyric acid in the peptidoglycan, had MK-11 as the major menaquinone, contained phosphatidylglycerol and diphosphatidylglycerol as the polar lipids and had high levels of fatty acids anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. In addition, the BLAST sequence similarity results, based on

16S rRNA gene sequences, also supported the affiliation of KFC-22^T and SPC-20^T to the genus *Leifsonia* (Evtushenko *et al.*, 2000; Suzuki *et al.*, 1999; Reddy *et al.*, 2008). The two strains shared a base-to-base 16S rRNA gene sequence similarity of 97.7 % (Supplementary Table S1, available in IJSEM Online) and a DNA–DNA relatedness of approximately 10 %. They also exhibited many phenotypic differences (Table 1), supporting their assignment to separate novel species. Strains KFC-22^T and SPC-20^T exhibited 16S rRNA gene sequence similarity of 95.0–98.0 % with respect to representatives of recognized species of the genus *Leifsonia* (Supplementary Table S1). The other phylogenetically related genera are *Subtercola* (96.0 % sequence similarity) (Männistö *et al.*, 2000), *Okibacterium* (96.0–97.0 %) (Evtushenko *et al.*, 2002), *Salinibacterium* (96.0 %) (Han *et al.*, 2003), *Rhodoglobus* (95.0–96.0 %) (Sheridan *et al.*, 2003), *Plantibacter* (96.0–97.0 %) (Behrendt *et al.*, 2002), *Agrococcus* (96.0–97.0 %) (Groth *et al.*, 1996) and *Agreia* (96.0–97.0 %) (Evtushenko *et al.*, 2001). Although the 16S rRNA gene sequence similarities were high with respect to some members of these genera, phylogenetic analyses (Fig. 1) showed that the two strains should be assigned to the genus *Leifsonia*. Interestingly, phylogenetic analysis based on neighbour joining, minimum evolution and maximum parsimony (DNAPARS) algorithms indicated that the genus *Leifsonia* is polyphyletic, with the species clustering into three clades (Fig. 1). Despite the polyphyletic nature of the genus, all of the species share common generic characteristics, thus overcoming the ambiguity caused by the phylogenetic analyses. Of all the species of the genus *Leifsonia*, it was the type strain of the recently described species *L. pindariensis* (Reddy *et al.*, 2008) with which KFC-22^T and SPC-20^T exhibited the highest 16S rRNA gene sequence similarity (97.7 and 97.3 %, respectively). This was further confirmed by phylogenetic analyses using minimum evolution and the unweighted pair group method with arithmetic averages, in which strains KFC-22^T and SPC-20^T clustered with *L. pindariensis* PON10^T at a bootstrap value greater than 50 %; with neighbour joining, the bootstrap value was 46 %. Phylogenetic trees constructed using *Arthrobacter globiformis* DSM 20124^T (GenBank accession no. X80736), *Corynebacterium diphtheriae* NCTC 11397^T (X84248), *Bacillus subtilis* DSM 10^T (AJ276351) and *Agrococcus jenensis* DSM 9580^T (X92492) as outgroups showed no change in the topology of the trees (not shown). This suggests that the two isolates could be strains of previously described species. However, strains KFC-22^T and SPC-20^T showed DNA–DNA relatedness of 42 and 11 %, respectively, with *L. pindariensis* PON10^T, clearly showing that both strains represent novel species. In addition, KFC-22^T and SPC-20^T exhibited phenotypic differences with respect to *L. pindariensis* PON10^T (Table 1). On the basis of the above characteristics, strains KFC-22^T and SPC-20^T represent novel species, for which the names *Leifsonia kafniensis* sp. nov. and *Leifsonia antarctica* sp. nov., respectively, are proposed.

Table 1. Characteristics that serve to distinguish strains KFC-22^T and SPC-20^T from *L. pindariensis* PON10^T

Data for *L. pindariensis* PON10^T are from Reddy *et al.* (2008). –, Absent; +, present; ND, not done; S, sensitive; R, resistant.

Characteristic	Strain SPC-20 ^T	Strain KFC-22 ^T	<i>L. pindariensis</i> PON10 ^T
Temperature for growth (°C)			
Range	4–25	4–30	4–35
Optimum	22	22	25
pH for growth			
Range	6.0–7.5	6.5–7.5	5.0–8.0
Optimum	7.5	7.5	7.0
Maximum NaCl concentration for growth (%)	5	5	4
Oxidase	+	–	–
Urease	–	–	+
Gelatinase	+	–	–
β-Galactosidase	+	–	+
Citrate utilization	+	+	–
Phosphatase	+	–	–
Nitrate reduction	+	–	–
Malonate utilization	–	+	ND
Utilization of amino acids			
L-Arginine	–	+	+
L-Asparagine	–	+	+
L-Aspartic acid	–	+	–
L-Cysteine	+	–	+
L-Glutamic acid	–	+	ND
L-Isoleucine	+	+	–
L-Lysine	+	+	–
L-Serine	–	+	–
L-Threonine	+	–	–
L-Tyrosine	+	–	–
L-Valine	–	+	+
Production of acid from:			
D-Arabinose	–	+	–
L-Arabinose	+	–	ND
Dulcitol	+	–	ND
Erythritol	+	–	ND
D-Fructose	+	+	–
Lactose	+	–	ND
Maltose	–	–	+
D-Mannose	+	–	+
D-Ribose	+	–	ND
Trehalose	+	–	ND
Carbon-source utilization			
N-Acetyl-D-glucosamine	+	–	–
Glucose	+	–	–
Dulcitol	–	+	+
Erythritol	+	–	+
D-Fructose	+	+	–
D-Galactose	+	+	–
Inulin	–	–	+
D-Lactose	–	–	+
Maltose	–	–	+
Mannitol	+	–	–
Melibiose	–	–	+

Table 1. cont.

Characteristic	Strain SPC-20 ^T	Strain KFC-22 ^T	<i>L. pindariensis</i> PON10 ^T
Raffinose	–	+	–
D-Rhamnose	+	+	–
Salicin	–	+	+
L-Sorbose	–	–	+
Xylitol	–	+	–
Sensitivity to antibiotics (per disc)			
Amikacin (30 µg)	R	S	S
Ampicillin (10 µg)	S	R	S
Cefoperazone (75 µg)	R	S	S
Lincomycin (2 µg)	S	R	ND
Nalidixic acid (30 µg)	S	R	R
Nitrofurantoin (300 µg)	R	S	S
Novobiocin (30 µg)	S	R	S
Norfloxacin (10 µg)	S	R	R
Polymyxin B (50 U)	R	S	R
Tobramycin (10 µg)	R	S	R
Menaquinones*	MK-10, MK-11	MK-11, MK-12	(MK-9), MK-10, MK-11
Polar lipids†	PE, PG, DPG, 3ULs	PE, PG, DPG, PA	PG, DPG, (UL)
Cell-wall sugars‡	Gal, (Man, Rha, US)	Rha, Man, Glc	Rha, (Glc, Gal, Man)
Fatty acids (%)			
iso-C _{15:0}	6.62	3.15	5.6
anteiso-C _{15:0}	30.26	58.21	54.3
anteiso-C _{15:1}	9.37	2.20	–
C _{16:0}	1.86	1.55	1.9
iso-C _{16:0}	11.38	8.99	6.8
anteiso-C _{16:0}	0.92	–	–
iso-C _{16:1}	0.50	–	–
C _{17:0}	2.75	–	–
C _{17:0} cyclo	1.72	–	–
iso-C _{17:0}	2.51	1.25	–
anteiso-C _{17:0}	10.88	22.36	19.1
anteiso-C _{17:1}	1.57	–	–
C _{17:0} 2-OH	1.33	–	–
C _{18:0}	1.46	–	–
iso-C _{18:1} H	2.76	–	–

*Minor components given in parentheses.

†DPG, Diphosphatidylglycerol; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; UL, unknown lipid. Minor components given in parentheses.

‡Gal, Galactose; Glc, glucose; Man, mannose; Rha, rhamnose; US, unknown sugar. Minor or trace components are given in parentheses.

Description of *Leifsonia kafniensis* sp. nov.

Leifsonia kafniensis (kaf.ni.en'sis. N.L. fem. adj. *kafniensis* pertaining to the Kafni glacier in the Himalayas of India, where the type strain was isolated).

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strains KFC-22^T and SPC-20^T and related taxa. Bootstrap percentages (based on 1000 replications) greater than 40% are given at nodes. Asterisks indicate branches that were conserved in the trees constructed using neighbour joining, minimum evolution and maximum parsimony (DNAPARS). The three clades containing *Leifsonia* strains are indicated. Bar, 5 substitutions per 1000 nucleotide positions.

Cells are Gram-positive, non-motile, aerobic, rod-shaped (0.4 × 1.5 µm) and yellow-pigmented. Positive for catalase and aesculin hydrolysis, but negative for lipase, hydrolysis of casein and starch, H₂S production, lysine, arginine and ornithine decarboxylases, indole production, the methyl red test, the Voges–Proskauer test and for phenylalanine deamination. Does not produce acid from *myo*-inositol, D-rhamnose or D-xylose. Utilizes glycerol, but not creatinine, L-histidine, hydroxyproline, L-leucine, L-methionine, sucrose, trehalose, L-tryptophan or D-xylose. The type strain is 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) and tetracycline (30); resistant to colistin (10 µg per disc). Absorption maxima of the yellow pigment in methanol at 360, 370, 440, 450, 470 and 480 nm. The other characteristics of the species are listed in Table 1.

The type strain, KFC-22^T (=NCCB 100216^T =LMG 24362^T), was isolated from a soil sample collected close to the Kafni glacier in the Himalayan mountain ranges of India.

Description of *Leifsonia antarctica* sp. nov.

Leifsonia antarctica (an.tarc'ti.ca. L. fem. adj. *antarctica* southern, by extension pertaining to the Antarctic, where the type strain was isolated).

Cells are Gram-positive, non-motile, aerobic, rod-shaped (0.5 × 0.7 µm) and pale-yellow-pigmented. Positive for catalase and aesculin hydrolysis, but negative for lipase, hydrolysis of casein and starch, H₂S production, lysine, arginine and ornithine decarboxylase, indole production, in the methyl red test, in the Voges–Proskauer test and for phenylalanine deamination. Does not produce acid from *myo*-inositol, D-rhamnose or D-xylose. Utilizes glycerol, but not creatinine, L-histidine, hydroxyproline, L-leucine, L-methionine, sucrose, trehalose, L-tryptophan or D-xylose.

The type strain is 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) and tetracycline (30); resistant to colistin (10 µg per disc). Contains three unknown lipids, with *R_f* values of 0.63, 0.66 and 0.73, in addition to the lipids listed in Table 1. The other characteristics of the species are listed in Table 1. Absorption maxima of the yellow pigment in methanol at 350, 360, 370, 430, 440, 450, 470 and 475 nm.

The type strain, SPC-20^T (=NCCB 100227^T =LMG 24541^T), was isolated from a sediment from the Antarctic Ocean, near the Larsemann Hill area of Antarctica.

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