Leifsonia psychrotolerans sp. nov., a psychrotolerant species of the family Microbacteriaceae from Livingston Island, Antarctica

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A cold-tolerant, yellow-pigmented, Gram-positive, motile, facultatively anaerobic bacterial strain, LI1^T, was isolated from a moss-covered soil from Livingston Island, Antarctica, near the Bulgarian station St. Kliment Ohridski. Comparative 16S rRNA gene sequence-based phylogenetic analysis placed the strain in a clade with the species *Leifsonia kafniensis* KFC-22^T, *Leifsonia pindariensis* PON10^T and *Leifsonia antarctica* SPC-20^T, with which it showed sequence similarities of 99.0, 97.9 and 97.9%, respectively. DNA–DNA hybridization revealed a reassociation value of 2.7% with *L. kafniensis* LMG 24362^T. The DNA G+C content of strain LI1^T was 64.5 mol%. The growth temperature range was –6 to 28 °C, with optimum growth at 16 °C. Growth occurred in 0–5% NaCl and at pH 4.5–9.5, with optimum growth in 1–2% NaCl and at pH 5.5–6.5. The predominant fatty acids were anteiso-C_{15:0}, C_{18:0} and iso-C_{15:0}. The polar lipids were phosphatidylglycerol and diphosphatidylglycerol. Physiological and biochemical tests clearly differentiated strain LI1^T from *L. kafniensis*. Therefore, a novel cold-tolerant species within the genus *Leifsonia* is proposed: *Leifsonia psychrotolerans* sp. nov. (type strain LI1^T=DSM 22824^T=NCCB 100313^T).

The genus *Leifsonia*, originally described by Suzuki *et al.* (1999) and Evtushenko *et al.* (2000), belongs to the family Microbacteriaceae, order Actinomycetales and, at the time of writing, comprised 14 species (<http://www.bacterio.cict.fr/l/leifsonia.html>) isolated from different sources such as roots (Evtushenko *et al.*, 2000; Qiu *et al.*, 2007), plants (Davis *et al.*, 1984), distilled water (Leifson, 1962), soil (Dastager *et al.*, 2008, 2009), various cold habitats in Antarctica (Reddy *et al.*, 2003; Pindi *et al.*, 2009) and close to glaciers (Reddy *et al.*, 2008; Pindi *et al.*, 2009). Very recently, *Leifsonia ginsengi* was reclassified into the novel genus *Herbiconiux* of the family Microbacteriaceae (Behrendt *et al.*, 2011). *L. ginsengi* and *Herbiconiux solani* share chemotaxonomic and phylogenetic features that clearly differentiate them from other members of the genus *Leifsonia*. The genus *Leifsonia* is characterized by

Gram-positive, non-spore-forming, irregular rod- or filament-shaped bacteria that possess MK-10 and/or MK-11 as major menaquinone and 2,4-diaminobutyric acid (DAB), alanine, glycine and glutamic acid as cell-wall amino acids. The DNA G+C contents vary within the genus from 64.0 to 73.4 mol%. At present, five *Leifsonia* species [*Leifsonia antarctica*, *Leifsonia aurea* (recently reclassified as *Rhodoglobus aureus*; An *et al.*, 2010), *Leifsonia kafniensis*, *Leifsonia pindariensis* and *Leifsonia rubra*] are psychrotolerant, i.e. able to grow at 4 °C.

In this study, strain LI1^T, a novel psychrotolerant member of the genus *Leifsonia* isolated from a cold terrestrial environment in the maritime Antarctic, was characterized and proposed as a representative of a novel species, *Leifsonia psychrotolerans* sp. nov.

Strain LI1^T was isolated from a moss-layered soil sample collected in 2005 near the Bulgarian Antarctic station St. Kliment Ohridski (62° 38' 29" S 60° 21' 53" W), located on Livingston Island, South Shetland Islands, Antarctica. For

Abbreviation: DAB, diaminobutyric acid.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of *Leifsonia psychrotolerans* LI1^T is GQ406810.

isolation, 5 g soil was shaken with 10 ml sterile 0.9 % (w/v) NaCl solution for 20 min at 150 r.p.m. Serial dilutions were made with sterile saline solution [0.9 % (w/v) NaCl], plated (0.1 ml) on a modified synthetic BRII agar (Bunt & Rovira, 1955) and incubated at 16 °C for 7–14 days; single colonies were chosen for further purification. The medium used for isolation contained 0.4 % (w/v) $K_2HPO_4 \cdot 3H_2O$, 0.5 % (w/v) $(NH_4)_2HPO_4$, 0.005 % (w/v) $MgSO_4 \cdot 7H_2O$, 0.1 % (w/v) $MgCl_2 \cdot 6H_2O$, 0.001 % (w/v) $FeCl_3 \cdot 6H_2O$, 0.1 % (w/v) $CaCl_2 \cdot 2H_2O$, 0.1 % (w/v) tryptone, 0.1 % (w/v) yeast extract, 0.5 % (w/v) glucose, 0.03 % (w/v) Na_2CO_3 , 25 % (v/v) synthetic stone extract and 1.5 % (w/v) agar in deionized water, pH 8.0. Synthetic stone extract consisted

of 0.0042 % (w/v) NaCl, 0.0013 % (w/v) $AlCl_3 \cdot 6H_2O$, 0.0005 % (w/v) KNO_3 , 0.008 % (w/v) K_2SO_4 , 0.0067 % (w/v) $CaSO_4 \cdot 2H_2O$, 0.0054 % (w/v) $MgSO_4 \cdot 7H_2O$ and 0.1 % (v/v) trace metal mix, pH 7.0, in deionized water. Trace metal mix consisted of 0.286 % (w/v) H_3BO_3 , 0.181 % (w/v) $MnCl_2 \cdot 4H_2O$, 0.022 % (w/v) $ZnSO_4 \cdot 7H_2O$, 0.039 % (w/v) $Na_2MoO_4 \cdot 2H_2O$, 0.008 % (w/v) $CuSO_4 \cdot 5H_2O$ and 0.005 % (w/v) $Co(NO_3)_2 \cdot 6H_2O$ in deionized water. For maintenance and for the determination of morphological, biochemical and physiological characteristics, isolates were grown in half-diluted Luria–Bertani (LB) medium [0.5 % (w/v) tryptone, 0.25 % (w/v) yeast extract, 0.5 % (w/v) NaCl and, if necessary, 1.5 % (w/v) agar, pH 7.2].

Table 1. Differential phenotypic characteristics of strain LI1^T and its closest phylogenetic neighbours

Strains: 1, *L. psychrotolerans* sp. nov. LI1^T; 2, *L. kafniensis* NCCB 100216^T [data from Pindi *et al.* (2009), except sole carbon source utilization data, which are from this study]; 3, *L. pindariensis* LMG 24222^T (Reddy *et al.*, 2008); 4, *L. antarctica* NCCB 100227^T (Pindi *et al.*, 2009); 5, *M. viridarii* DSM 21772^T (Matsumoto *et al.*, 2008); 6, *P. gilvus* DSM 18319^T (Lee *et al.*, 2008). +, Positive; –, negative; +/–, weakly positive; ND, no data.

Characteristic	1	2	3	4	5	6
Colony colour	Translucent yellow	Yellow	Yellow	Pale yellow	Pale yellow	Pale yellow
Motility	+	–	+	–	+	+
Growth temperature (°C):						
Range	–6 to 28	4–30	4–35	4–25	10–38	4–30
Optimum	16	22	25	22	15–30	25
Growth pH:						
Range	4.5–9.5	6.5–7.5	5.0–8.0	6.0–7.5	6.0–11.0	6.1–10.1
Optimum	5.5–6.5	7.5	7.0	7.5	ND	7.1–8.1
NaCl growth range (%, w/v)	0–5	0–5	0–4	0–5	0–3	0–2
Anaerobic growth	+	–	–	–	–	–
Hydrolysis of:						
Gelatin	–	–	–	+	ND	–
Urea	–	–	+	–	ND	–
Starch	+ / –	–	–	–	ND	–
Oxidase	–	–	–	+	ND	–
H ₂ S production	+	–	ND	–	ND	–
Methyl red test	+ / –	–	–	–	ND	ND
Utilization of:						
D-Glucose	+	+	+	+	–	ND
Sucrose	+ / –	+	–	–	–	ND
D-Xylose	+	+	–	–	–	ND
Maltose	+	+	+	–	+	ND
Lactose	+	+	+	–	–	ND
Inulin	+	–	+	–	–	ND
D-Salicin	+	–	+	–	–	ND
Dulcitol	+	–	+	–	–	ND
m-Erythritol	+	–	+	+	–	ND
Sorbitol	+	–	–	ND	–	ND
Trehalose	–	+	–	–	–	ND
L-Rhamnose	+	+	–	ND	–	ND
Succinate	+	–	ND	ND	ND	ND
L-Asparagine	+	+ / –	+	ND	ND	ND
Menaquinones	MK-10, MK-11	MK-11, MK-12	MK-10, MK-11, MK-9	MK-10, MK-11	MK-12, MK-11, MK-13	MK-11, MK-10, MK-12, MK-9
DNA G + C content (mol%)	64.5	ND	ND	ND	70.0	69.8

Growth was tested at temperatures from -6 to 32 °C by measuring the OD₆₀₀ over 5–7 days. Tolerances to salt (NaCl) and pH were tested in media containing 0–7 % (w/v) NaCl and at pH 4.0–10.0 (in 0.5 pH unit increments), respectively, over 5–7 days. Anaerobic growth was tested on PYG agar plates [0.1 % (w/v) peptone, 0.1 % (w/v) yeast extract, 0.2 % (w/v) glucose, 1.5 % (w/v) agar, pH 7.2] incubated under an N₂/CO₂ (80:20, v/v) atmosphere for 14 days. Colony characteristics were determined visually on agar plates between 7 and 14 days of bacterial growth. Cell morphology was examined by light microscopy of cells grown for 2 and 9 days. Gram staining, and flagella and spore detection were carried out by classical procedures as described by Süssmuth *et al.* (1999). Susceptibility to antibiotics was examined by a filter disc test (10 µg per disc). Acid production from carbohydrates was tested with peptone water [1 % (w/v) peptone, 0.5 % (w/v) NaCl] containing different sugar solutions (1 %, w/v) and bromothymol blue as an indicator according to Hugh & Leifson (1953). The methyl red test was performed according to Schröder (1991). Catalase activity was determined by bubble production in 10 % hydrogen peroxide solution. Oxidase activity was analysed with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine as a redox indicator as described by Kovacs (1956). Hydrolysis of starch and casein and the production of urease, hydrogen sulfide and indole from tryptophan were determined as described by Schröder (1991). Hydrolysis of gelatin was tested by flooding gelatin agar plates with saturated ammonium sulfate solution after incubation. Utilization of various compounds as sole carbon sources was tested using a minimal medium [0.1 % (w/v) NH₄Cl, 0.1 % (w/v) K₂HPO₄, 0.05 % (w/v) KH₂PO₄, 0.02 % (w/v) MgSO₄·7H₂O, 0.001 % (w/v) CaCl₂·2H₂O, 0.2 % (v/v) trace metal mix, 1.5 % (w/v) agar] prepared with 0.25 % (w/v) of each carbon compound. Phenotypic characteristics of strain LI1^T are presented in Table 1.

For quantitative analysis of cellular fatty acid composition, cells were grown in half-strength LB medium (pH 7.2) at 16 °C for 3 days. Extraction and analysis of polar lipids and fatty acid methyl esters were conducted according to the protocol of Zink & Mangelsdorf (2004). Isoprenoid quinones were extracted using the small-scale integrated procedure of Minnikin *et al.* (1984). Menaquinones were analysed by a Hewlett Packard series 1050 HPLC equipped with an ODS Hypersil column and a diode-array detector. Methanol/isopropyl ether (9:2, v/v) was used as mobile phase with a flow rate of 1.0 ml min⁻¹ and a column temperature of 30 °C (Hu *et al.*, 1999). To determine the peptidoglycan structure, cell-wall extracts were prepared according to the method of Schleifer & Kandler (1972). After derivatization (MacKenzie, 1987), the molar ratio of the amino acids was determined by GC (Groth *et al.*, 1996). Fatty acids of strain LI1^T were identified as follows: anteiso-C_{15:0} (48.6 %), C_{18:0} (17.2 %), iso-C_{15:0} (10.9 %), C_{16:0} (8.8 %), anteiso-C_{17:0} (6.7 %), C_{18:2} (1.8 %), C_{18:1ω9c} (1.7 %), iso-C_{16:0} (1.3 %), C_{14:0} (0.8 %), iso-C_{17:0} (0.7 %), C_{18:1ω7c} (0.6 %), C_{20:0} (0.5 %) and C_{15:0}

(0.4 %). This differed from the fatty acid composition of the closest relative, *Leifsonia kafniensis*, when grown under identical growth conditions (Table 2). The polar lipids of strain LI1^T were phosphatidylglycerol and diphosphatidylglycerol. Menaquinones were MK-10 (56 %) and MK-11 (44 %). For strain LI1^T, peptidoglycan structure analysis revealed type B2γ with a D-Glu–D-DAB interpeptide bridge, type B7 according to the DSMZ (2001). Amino acid analyses of cell walls showed the presence of DAB, glycine, alanine and glutamic acid with a DAB:Gly:Ala:Glu ratio of 1.5:0.9:0.4:1.0.

DNA–DNA hybridization was performed by isolating DNA from strain LI1^T and *L. kafniensis* LMG 24362^T using a French pressure cell (Thermo Spectronic) for the disruption of cells. DNA was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) using a model Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). Reassociation experiments were performed in 2× SSC plus 10 % formamide at 69 °C. Determination of DNA G+C content was done by HPLC according to the method of Mesbah *et al.* (1989). DNA isolation from strain LI1^T was conducted using the Microbial DNA isolation kit (MoBio Laboratories). For 16S rRNA gene amplification, general bacterial primers 8F (Ravenschlag *et al.*, 1999) and 1492R (Dojka *et al.*, 1998) were used. Sequencing (GATC Biotech, Konstanz, Germany) resulted in a 1357 bp gene

Table 2. Fatty acid compositions (%) of strain LI1^T and *L. kafniensis* NCCB 100216^T under identical growth conditions.

–, Not detected.

Fatty acids	LI1 ^T	<i>L. kafniensis</i>
C _{14:0}	–	0.1
iso-C _{14:0}	–	0.1
C _{15:0}	0.4	0.1
iso-C _{15:0}	10.9	0.5
anteiso-C _{15:0}	48.6	65.1
C _{16:0}	8.8	1.7
iso-C _{16:0}	1.3	4.1
C _{16:1ω7c}	–	0.1
C _{16:1ω9c}	–	0.1
C _{17:0}	–	0.1
iso-C _{17:0}	0.7	0.2
anteiso-C _{17:0}	6.7	27.1
C _{18:0}	17.2	0.4
C _{18:1ω7c}	0.6	–
C _{18:1ω9c}	1.7	0.2
C _{18:2}	1.8	–
anteiso-C _{19:0}	–	0.1
C _{20:0}	0.5	–

Fig. 1. Neighbour-joining tree of *Microbacteriaceae* species showing the phylogenetic position of the novel isolate LI1^T within this genus. *, Branches that were also found in maximum-parsimony trees (Fitch, 1971); +, branches that were also found in maximum-likelihood trees (Felsenstein, 1981). Numbers at nodes indicate bootstrap-support values (Felsenstein, 1985) based on a neighbour-joining analysis of 1000 replications; only values $\geq 50\%$ are shown. Bar, 0.01 substitutions per nucleotide position.

product for LI1^T. Alignments were done with closely related sequences obtained from GenBank using the integrated SINA alignment tool from the ARB-SILVA website (Pruesse *et al.*, 2007) and were checked manually. The ARB program (Ludwig *et al.*, 2004) was used for calculation of evolutionary distances. 16S rRNA gene sequence analysis placed the isolate in a clade with *L. kafniensis* KFC-22^T, *L. pindariensis* PON10^T and *L. antarctica* SPC-20^T, with sequence similarities of 99.0, 97.9 and 97.9 %, respectively. Strain LI1^T shared the highest 16S rRNA gene sequence similarity with the type strain of *L. kafniensis* (99.0 %).

Nonetheless, DNA–DNA hybridization between the two strains showed a relatedness of only 2.7 % (repeat value 2.6 %), indicating that strain LI1^T represents a novel species. For phylogenetic tree construction, the neighbour-joining method (Saitou & Nei, 1987; Fig. 1), with the correction of Jukes & Cantor (1969) and a termini filter that is implemented in the ARB program, was used. To evaluate tree topologies, bootstrap analysis with 1000 replications was performed (Felsenstein, 1985). Additionally, maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) trees were calculated to support the

branching of the neighbour-joining algorithm. The compared trees showed some differences in the position of single species, but the overall topology was similar among the different trees (data not shown). Phylogenetic analyses revealed the polyphyletic structure of the genus *Leifsonia*, where the species cluster in three clades (in accordance with Pindi *et al.*, 2009). Although strain LI1^T, *L. antarctica*, *L. kafniensis* and *L. pindariensis* form a phylogenetic cluster with two other genera, i.e. *Microterricola* (Matsumoto *et al.*, 2008) and *Phycicola* (Lee *et al.*, 2008), they share specific biochemical properties that are typical for the genus *Leifsonia*, such as MK-10 and/or MK-11 as major menaquinones and 2,4-DAB, alanine, glycine and glutamic acid as cell-wall amino acids. On the other hand, *Phycicola gilvus* is characterized by the formation of budding-like structures, the presence of phosphatidylcholine and phosphatidylinositol in the polar lipid fraction and a rod-coccus cycle. *Microterricola viridarii* exhibits very similar biochemical features to those of the genus *Leifsonia*, but the major menaquinone is MK-12.

Therefore, based on morphological, physiological and biochemical characteristics, strain LI1^T can be clearly differentiated from its most closely related neighbours (see Table 1) and is proposed as a representative of a novel species of the genus *Leifsonia*.

Description of *Leifsonia psychrotolerans* sp. nov.

Leifsonia psychrotolerans (psy.chro.to'le.rans. Gr. adj. *psychros* cold; L. part. adj. *tolerans* tolerating; N.L. part. adj. *psychrotolerans* cold-tolerating).

Cells are Gram-positive, motile, non-spore-forming short rods (0.3 × 0.9 µm). Colonies are circular, convex, translucent and yellow in colour. Grows at −6 to 28 °C (optimum 16 °C) and at pH 4.5–9.5 (optimum pH 5.5–6.5). Tolerates up to 5 % NaCl (optimum 1–2 % NaCl). Positive for catalase, H₂S production and methyl red test (weak), but negative for oxidase, urease and indole production. Hydrolyses starch (weak), but not casein, Tween 20 or gelatin. Grows on nutrient, LB and tryptic soy agar media, but not on mineral medium. Unlike other members of the genus *Leifsonia*, can grow anaerobically with glucose as carbon source. Produces acid from D-galactose, D-glucose, L-rhamnose, salicin (weak) and sucrose (weak), but not from lactose, maltose, D-mannitol, adonitol, L-arabinose, dulcitol, *myo*-inositol, D-sorbitol, trehalose or D-xylose. Can utilize L-arabitol (weak), cellobiose, dulcitol (weak), *m*-erythritol, D-fructose (weak), L-fucose, D-glucose, *myo*-inositol, inulin, lactose, maltose, D-mannose, melezitose, L-rhamnose, D-ribose, sucrose (weak), D-salicin, sorbitol, D-xylose, acetate, L-asparagine, glycerol, glycogen (weak), pyruvate and succinate, but not adonitol, formate, glycine, lactate, melibiose, raffinose or trehalose as sole carbon source. Sensitive to penicillin, neomycin, streptomycin, oxytetracycline, novobiocin, rifampicin and kanamycin. Resistant to ampicillin and erythromycin. Major menaquinone is

MK-10. Major fatty acid (>20 % of total composition) is anteiso-C_{15:0}.

The type strain is LI1^T (=DSM 22824^T=NCCB 100313^T), isolated from moss-covered soil from Livingston Island, South Shetland Islands, Antarctica. The genomic DNA G + C content of LI1^T is 64.5 mol%.

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