Arthrobacter livingstonensis sp. nov. and *Arthrobacter cryotolerans* sp. nov., salt-tolerant and psychrotolerant species from Antarctic soil

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Two novel cold-tolerant, Gram-stain-positive, motile, facultatively anaerobic bacterial strains, LI2^T and LI3^T, were isolated from moss-covered soil from Livingston Island, Antarctica, near the Bulgarian station St Kliment Ohridski. A rod–coccus cycle was observed for both strains. 16S rRNA gene sequence analysis revealed an affiliation to the genus *Arthrobacter*, with the highest similarity to *Arthrobacter stackebrandtii* and *Arthrobacter psychrochitiniphilus* for strain LI2^T (97.8 and 97.7 % similarity to the respective type strains) and to *Arthrobacter kerguelensis* and *Arthrobacter psychrophenolicus* for strain LI3^T (97.4 and 97.3 % similarity to the respective type strains). The growth temperature range was −6 to 28 °C for LI2^T and −6 to 24 °C for LI3^T, with an optimum at 16 °C for both strains. Growth occurred at 0–10 % (w/v) NaCl, with optimum growth at 0–1 % (w/v) for LI2^T and 0.5–3 % (w/v) for LI3^T. The pH range for growth was pH 4–9.5 with an optimum of pH 8 for LI2^T and pH 6.5 for LI3^T. The predominant fatty acids were anteiso-$C_{15:0}$, $C_{18:0}$ and anteiso-$C_{17:0}$ for LI2^T and anteiso-$C_{15:0}$ and $C_{18:0}$ for LI3^T. Physiological and biochemical tests clearly differentiated strain LI2^T from *A. stackebrandtii* and *A. psychrochitiniphilus* and strain LI3^T from *A. kerguelensis* and *A. psychrophenolicus*. Therefore, two novel species within the genus *Arthrobacter* are proposed: *Arthrobacter livingstonensis* sp. nov. (type strain LI2^T =DSM 22825^T =NCCB 100314^T) and *Arthrobacter cryotolerans* sp. nov. (type strain LI3^T =DSM 22826^T =NCCB 100315^T).

Species of the genus *Arthrobacter*, proposed by Conn & Dimmick (1947), have been isolated from very different sources, such as human specimens (Funke *et al.*, 1998; Hou *et al.*, 1998; Wauters *et al.*, 2000; Mages *et al.*, 2008), filtration substrates (Ding *et al.*, 2009), the surfaces of cheese (Irlinger *et al.*, 2005), soil and sediment (Phillips, 1953; Lee *et al.*, 2003; Kageyama *et al.*, 2008) as well as sewage and wastewater reservoir sediment (Kim *et al.*, 2008; Roh *et al.*, 2008). Some isolates are able to degrade complex organic compounds (Kodama *et al.*, 1992; Westerberg *et al.*, 2000; Kotoučková *et al.*, 2004; Kallimanis *et al.*, 2009). Over the last decade, several novel species belonging to the genus *Arthrobacter* have been isolated from cold environments such as an alpine ice cave (Margesin *et al.*, 2004), an alpine soil (Zhang *et al.*, 2010) and various terrestrial and aquatic habitats in the Antarctic (Reddy *et al.*, 2000, 2002; Gupta *et al.*, 2004; Chen *et al.*, 2005; Wang *et al.*, 2009).

In this study, we describe the characterization of two strains from a cold terrestrial environment in the maritime Antarctic and propose to classify them within two novel species of the genus *Arthrobacter*.

Strains LI2^T and LI3^T were 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 in the South Shetland archipelago. The soil was stored at −20 °C for further microbiological investigations. For isolation, 5 g soil was mixed with 10 ml sterile 0.9 % (w/v) NaCl and shaken at 4 °C 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 then chosen for further purification. The medium used for isolation

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LI2^T and LI3^T are GQ406811 and GQ406812.

contained (w/v unless indicated) 0.04 % $K_2HPO_4 . 3H_2O$, 0.05 % $(NH_4)_2HPO_4$, 0.0005 % $MgSO_4 . 7H_2O$, 0.01 % $MgCl_2 . 6H_2O$, 0.0001 % $FeCl_3 . 6H_2O$, 0.01 % $CaCl_2 . 2H_2O$, 0.1 % tryptone, 0.1 % yeast extract, 0.5 % glucose, 0.03 % Na_2CO_3, 25 % (v/v) synthetic stone extract and 1.5 % agar, pH 8.0. The synthetic stone extract consisted of 41.5 mg NaCl, 12.5 mg $AlCl_3 . 6H_2O$, 5.0 mg KNO_3, 80.0 mg K_2SO_4, 67.0 mg $CaSO_4 . 2H_2O$, 54.0 mg $MgSO_4 . 7H_2O$ and 1 ml trace metal mixture in 1000 ml deionized water, pH 7.0. The trace metal mixture contained 2.86 g H_3BO_3, 1.81 g $MnCl_2 . 4H_2O$, 0.22 g $ZnSO_4 . 7H_2O$, 0.39 g $Na_2MoO_4 . 2H_2O$, 0.079 g $CuSO_4 . 5H_2O$ and 0.049 g $Co(NO_3)_2 . 6H_2O$ in 1000 ml deionized water. For maintenance and for determination of morphological, biochemical and physiological characteristics, the isolates were grown at 16 °C on half-strength LB medium (w/v; 0.5 % tryptone, 0.25 % yeast extract, 0.5 % NaCl and, if necessary, 1.5 % agar, pH 7.2). Growth was tested at temperatures from −6 to 28 °C for strain LI3^T and up to 32 °C for strain LI2^T by measuring the OD_{600} over 5–7 days. Salt (NaCl) tolerance was tested from 0 to 10 % (w/v) over 5–7 days. pH tolerance and optimum pH for growth were evaluated from pH 4 to 10 (in increments of 0.5 pH units) over 5–7 days. Anaerobic growth was tested on PYG agar plates (w/v; 0.1 % peptone, 0.1 % yeast extract, 0.2 % glucose, 1.5 % agar, pH 7.2) incubated under a N_2/CO_2 (80 : 20, v/v) atmosphere for 14 days. Colony characteristics were determined visually on agar plates after 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 flagellum and spore detection were carried out by classical procedures described by Süssmuth *et al.* (1999). Susceptibility to antibiotics and lysozyme was examined by a filter disc test (10 μg per disc). Acid production from carbohydrates was tested with peptone water (w/v; 1 % peptone, 0.5 % NaCl) containing solutions of various sugars (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 a 10 % hydrogen peroxide solution. Oxidase activity was analysed with *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) as a redox indicator as described by Kovács (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. To test the utilization of carbon compounds as sole carbon sources, a minimal medium was prepared [w/v unless indicated; 0.1 % NH_4Cl, 0.1 % K_2HPO_4, 0.05 % KH_2PO_4, 0.02 % $MgSO_4 . 7H_2O$, 0.005 % $CaCl_2 . 2H_2O$, 0.2 % (v/v) trace metal mixture, 1.5 % agar] with 0.25 % (w/v) of each carbon compound.

For quantitative analysis of cellular fatty acid compositions, 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 was conducted according to Zink & Mangelsdorf (2004). Isoprenoid quinones were extracted using the small-scale integrated procedure of Minnikin *et al.* (1984). Menaquinones were analysed with 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 the mobile phase at 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). Identified fatty acids were anteiso-$C_{15:0}$ (36.4 %), $C_{18:0}$ (23.0 %), anteiso-$C_{17:0}$ (22.1 %), $C_{16:0}$ (10.5 %), iso-$C_{17:0}$ (2.3 %), iso-$C_{15:0}$ (2.0 %), iso-$C_{16:0}$ (1.5 %), $C_{18:1}\omega 9c$ (1.5 %) and $C_{14:0}$ (0.7 %) for LI2^T and anteiso-$C_{15:0}$ (40.1 %), $C_{18:0}$ (23.8 %), $C_{16:0}$ (10.8 %), $C_{18:2}$ (7.9 %), $C_{18:1}\omega 9c$ (5.8 %), iso-$C_{15:0}$ (4.1 %), anteiso-$C_{17:1}\omega 8$ (2.9 %), anteiso-$C_{17:0}$ (1.8 %), $C_{20:0}$ (1.1 %), $C_{18:1}\omega 7c$ (0.6 %), $C_{14:0}$ (0.5 %), iso-$C_{16:0}$ (0.4 %) and $C_{17:0}$ (0.3 %) for LI3^T. The polar lipids were characterized by the presence of phosphatidylglycerol only. Identified menaquinones were MK-9(H_2) (69 %), MK-7(H_2) (22 %) and MK-8(H_2) (9 %) for strain LI2^T and MK-9 (47 %), MK-10 (20 %), MK-8 (17 %), MK-7 (12 %) and MK-6 (4 %) for strain LI3^T. For strain LI2^T, peptidoglycan structure analyses revealed type A3α with a Lys–Thr–Ala interpeptide bridge and a substitution of the α-carboxyl group of D-glutamic acid by alanine amide, type A11.26 (DSMZ, 2001). Amino acid analyses of cell walls showed the presence of alanine, threonine and glutamic acid, with lysine as the diagnostic diamino acid. The molar ratio of Ala/Glu/Thr/Lys was 3.9 : 1.0 : 0.6 : 0.6. For strain LI3^T, the cell-wall peptidoglycan was characterized by the presence of glutamic acid, alanine and lysine as the diagnostic diamino acid, at a molar ratio of 1.7 : 1.2 : 1.0. The peptidoglycan type was A4α with a Lys–Glu interpeptide bridge, with glutamic acid at the N terminus (after Schleifer, 1985), type A11.54 (DSMZ, 2001).

Isolation of DNA from strains LI2^T and LI3^T was done using a Microbial DNA isolation kit (MoBio Laboratories) according to the manufacturer's protocol. For 16S rRNA gene amplification, general bacterial primers 8F (Ravenschlag *et al.*, 1999) and 1492R (Dojka *et al.*, 1998) were used. Sequencing (by GATC Biotech, Konstanz, Germany) resulted in a 1379 bp gene product for LI2^T and a 1364 bp gene product for LI3^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 and to construct a phylogenetic tree by the neighbour-joining method (Saitou & Nei, 1987; Fig. 1) using the correction of Jukes & Cantor (1969) and a termini filter that is implemented in the ARB program. To evaluate the tree

topologies, a bootstrap analysis with 1000 replications was performed. For strain LI2^T, highest 16S rRNA gene sequence similarity was found to the type strains of *Arthrobacter stackebrandtii* (97.8 %) and *Arthrobacter psychrochitiniphilus* (97.7 %), whereas strain LI3^T showed the highest sequence similarity to the type strains of *Arthrobacter kerguelensis* (97.4 %) and *Arthrobacter psychrophenolicus* (97.3 %). 16S rRNA gene sequence similarity between LI2^T and LI3^T was only 95.5 %. As the 16S rRNA gene sequence similarity between the two novel strains was well below the value of 98.5 % defined by Stackebrandt & Ebers (2006) as the threshold for requiring DNA–DNA hybridization experiments, we did not carry out this analysis. Determination of G+C content of DNA was done by HPLC according to the method of Mesbah *et al.* (1989).

Based on differences in their morphological, physiological and biochemical characteristics, strains LI2^T and LI3^T can

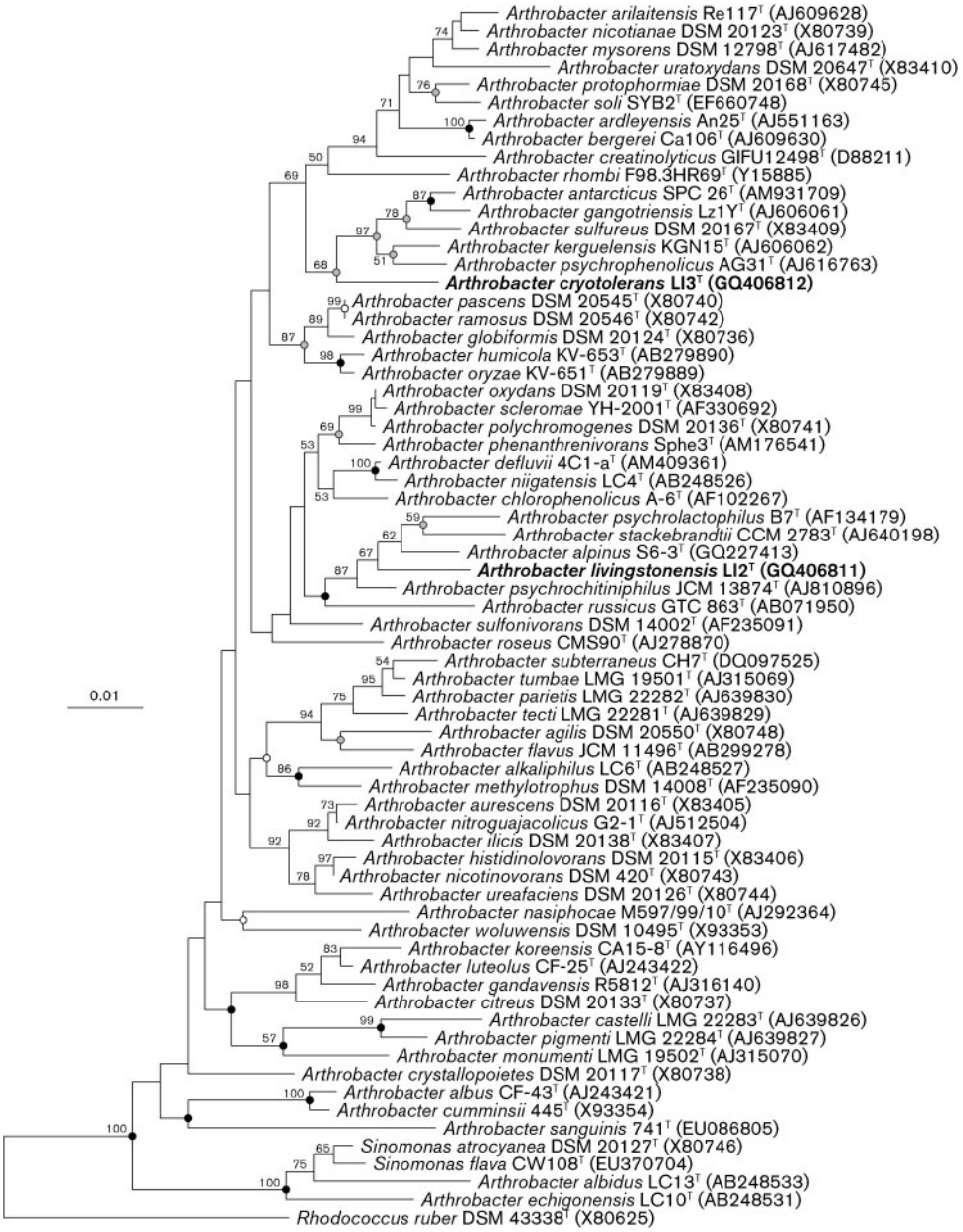

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strains LI2^T and LI3^T within the genus *Arthrobacter*. Open circles indicate branches that were also found in maximum-parsimony trees (Fitch, 1971) and shaded circles indicate branches that were also found in maximum-likelihood trees (Felsenstein, 1981); filled circles indicate branches found in both. Numbers at nodes indicate bootstrap percentages (Felsenstein, 1985) based on a neighbour-joining analysis of 1000 replications; only values $\geqslant$50 % are shown. Bar, 0.01 substitutions per nucleotide position.

be differentiated from the most closely related neighbours within the genus *Arthrobacter* (Table 1). We therefore propose the novel species *Arthrobacter livingstonensis* sp. nov. and *Arthrobacter cryotolerans* sp. nov., respectively, to accommodate the two strains.

Description of *Arthrobacter livingstonensis* sp. nov.

Arthrobacter livingstonensis (li.ving.sto.nen′sis. N.L. masc. adj. *livingstonensis* pertaining to Livingston Island, Antarctica, the sampling location of the soil from which the type strain was isolated).

Colonies are off-white, opaque, round, slightly convex and glossy with entire margins. Cells are facultatively anaerobic, psychrotolerant, Gram-stain-positive, motile, non-spore-forming and exhibit a rod–coccus cycle. Growth occurs from −6 to 28 °C, at pH 4.0–9.5 and in the presence of 0–10 % (w/v) NaCl, with optimum growth at 16 °C, pH 8.0 and 0–1 % (w/v) NaCl. Positive for catalase, H_2S production and urease and negative for oxidase, indole production and the methyl red test. Does not hydrolyse starch. Casein hydrolysis is weak. Acid is produced from D-glucose and D-mannitol and is produced weakly from D-galactose and sucrose. No acid is produced from adonitol, L-arabinose, L-arabitol, cellobiose, dulcitol, *meso*-erythritol, D-fructose, L-fucose, inulin, lactose, maltose, D-mannose, melibiose, melezitose, raffinose, L-rhamnose, D-ribose, salicin, D-sorbitol, trehalose or D-xylose. Can utilize

Table 1. Phenotypic characteristics that differentiate isolates LI2^T and LI3^T from the type strains of related *Arthrobacter* species

Strains: 1, *Arthrobacter livingstonensis* sp. nov. LI2^T; 2, *Arthrobacter cryotolerans* sp. nov. LI3^T; 3, *A. psychrochitiniphilus* JCM 13874^T (data from Wang *et al.*, 2009); 4, *A. stackebrandtii* DSM 16005^T (Tvrzová *et al.*, 2005); 5, *A. psychrophenolicus* DSM 15454^T (Margesin *et al.*, 2004); 6, *A. alpinus* S6-3^T (Zhang *et al.*, 2010); 7, *A. kerguelensis* DSM 15797^T (Gupta *et al.*, 2004). +, Positive; −, negative; W, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6	7
Colony colour	Off-white	Yellow	Yellow	ND	Yellow	Yellow	Yellow
Motility	+	+	+	−	−	−	−
Growth temperature (°C)							
Range	−6 to 28	−6 to 24	0–25	4–30	1–25	1–25	4–30
Optimum	16	16	20	ND	25	20–25	22
pH for growth							
Range	4–9.5	4–9.5	ND	5.7–9.1	6–10	6–9	ND
Optimum	8	6.5	6–8	ND	ND	ND	7
NaCl growth range (%, w/v)	0–10	0–10	0–3	⩽5	1–5	0–5	⩽6
Obligately aerobic	−	−	+	+	+	+	ND
Hydrolysis of:							
Gelatin	−	−	−	+	−	ND	+
Casein	W	−	ND	ND	ND	−	+
Urea	+	−	−	+	+	+	+
Starch	−	−	+	+	ND	+	−
H_2S production	+	+	−	ND	−	−	ND
Utilization of:							
D-Glucose	+	+	+	+	−	+	+
Glycerol	+	−	+	+	+	ND	−
L-Fucose	+	−	−	+	ND	ND	ND
Sucrose	+	−	+	+	ND	ND	+
Inulin	+	−	−	−	ND	ND	+
Glycogen	+	+	−	−	ND	ND	ND
Lactose	+	−	+	+	ND	+	+
D-Mannose	+	W	+	+	−	+	+
Melezitose	+	W	+	−	ND	ND	ND
D-Xylose	+	−	+	+	ND	ND	+
Peptidoglycan type	A3α Lys–Thr–Ala	A4α Lys–Glu	A3α	A3α Thr–Ala_3	A4α Lys–Glu	A3α Lys–Thr–Ala_3	A4α Lys–Glu
Menaquinone(s)	9(H_2), 7(H_2), 8(H_2)	9, 10, 8, 7, 6	9(H_2)	9(H_2), 10(H_2), 11(H_2)	10, 9, 11	9(H_2), 8(H_2), 10(H_2)	9, 8, 10
DNA G+C content (mol%)	64.7	64.5	58.5	ND	ND	61.9	58

L-arabitol, cellobiose, dulcitol, D-fructose, L-fucose, D-glucose, inulin, lactose, maltose, D-mannose, melibiose, melezitose, raffinose, D-ribose, D-salicin, sorbitol, sucrose, trehalose, D-xylose, glycerol, glycogen, L-asparagine, glycine, acetate, pyruvate and succinate as sole carbon sources, but not adonitol, *meso*-erythritol, formate, lactic acid or L-rhamnose. Sensitive to (10 μg per disc) penicillin, ampicillin, kanamycin, neomycin, streptomycin, erythromycin, oxytetracycline, novobiocin and rifampicin. Major fatty acids (>20 % of total fatty acids) are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and $C_{18:0}$. The major menaquinone is MK-9(H_2). The G+C content of the genomic DNA of the type strain is 64.7 mol%.

The type strain is LI2^T (=DSM 22825^T =NCCB 100314^T), isolated from a moss-covered soil from Livingston Island, South Shetland Islands, Antarctica.

Description of *Arthrobacter cryotolerans* sp. nov.

Arthrobacter cryotolerans (cry.o.to′ler.ans. N.L. *cryo* from Gr. adj. *krýos* cold; L. pres. part. *tolerans* tolerating, enduring; N.L. part. adj. *cryotolerans* cold-tolerating).

Colonies are yellow, opaque, round, convex and glossy with a slimy consistency and entire margins. Cells are facultatively anaerobic, psychrotolerant, Gram-stain-positive, motile, non-spore-forming and exhibit a rod–coccus cycle. Growth occurs from −6 to 24 °C, at pH 4.0–9.5 and in the presence of 0–10 % (w/v) NaCl, with optimum growth at 16 °C, pH 6.5 and 0.5–3.0 % (w/v) NaCl. Positive for catalase and H_2S production, and negative for oxidase, urease, indole production and the methyl red test. Does not hydrolyse starch or casein. Acid is produced weakly from D-fructose and L-rhamnose. No acid is produced from adonitol, L-arabinose, L-arabitol, cellobiose, dulcitol, *meso*-erythritol, L-fucose, D-glucose, D-galactose, inulin, lactose, maltose, D-mannose, D-mannitol, melibiose, melezitose, raffinose, D-ribose, salicin, D-sorbitol, sucrose, trehalose or D-xylose. Can utilize cellobiose (weakly), dulcitol (weakly), D-fructose (weakly), D-glucose, maltose (weakly), D-mannose (weakly), melezitose (weakly), raffinose, D-salicin (weakly), trehalose, glycine and glycogen as sole carbon sources, but not adonitol, L-arabitol, *meso*-erythritol, L-fucose, inulin, lactose, melibiose, L-rhamnose, D-ribose, sorbitol, sucrose, D-xylose, glycerol, L-asparagine, lactic acid, acetate, formate, pyruvate or succinate. Sensitive to (10 μg per disc) penicillin, ampicillin, kanamycin, neomycin, streptomycin, erythromycin, oxytetracycline, novobiocin and rifampicin. Shows weak sensitivity to lysozyme (10 μg per disc). Major fatty acids (>20 % of total fatty acids) are anteiso-$C_{15:0}$ and $C_{18:0}$. The major menaquinone is MK-9. The G+C content of the genomic DNA of the type strain is 64.5 mol%.

The type strain is LI3^T (=DSM 22826^T =NCCB 100315^T), isolated from a moss-covered soil from Livingston Island, South Shetland Islands, Antarctica.

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