Leifsonia soli sp. nov., a yellow-pigmented actinobacterium isolated from teak rhizosphere soil

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Two yellow-pigmented, Gram-stain-positive, aerobic, motile, short rod-shaped bacteria were isolated from natural teak tree rhizosphere soil and their taxonomic positions were determined by using a polyphasic approach. Comparative 16S rRNA gene sequence analysis showed that strains TG-S248^T and TG-S240 formed a distinct phyletic line within the genus *Leifsonia*. 16S rRNA gene sequence analysis of strain TG-S248^T with sequences from *Leifsonia shinshuensis* DB 102^T, *L. poae* VKM Ac-1401^T, *L. naganoensis* DB 103^T, *L. aquatica* DSM 20146^T and *L. xyli* subsp. *cynodontis* JCM 9733^T revealed pairwise similarities ranging from 98.7 to 99.1 %. The major fatty acids were anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The G + C content of the DNA of the type strain was 69.4 mol%. DNA–DNA hybridization experiments revealed low levels of DNA–DNA relatedness (32 % or less) between strain TG-S248^T and its closest relatives. Based on differences in phenotypic and genotypic characteristics, strain TG-S248^T (=LMG 24767^T =JCM 15679^T) is designated the type strain of a novel species of the genus *Leifsonia*, for which the name *Leifsonia soli* sp. nov. is proposed.

The genus *Leifsonia*, which is classified in the class *Actinobacteria* (Evtushenko *et al.*, 2000), currently harbours 12 species and two subspecies, with *Leifsonia aquatica* as the type species (<http://www.bacterio.cict.fr/l/leifsonia.html>). *L. aquatica*, isolated from distilled water, was originally classified as '*Corynebacterium aquaticum*' on the basis of its morphological and physiological characteristics (Leifson, 1962). Subsequently, *Clavibacter xyli* subsp. *cynodontis* (Davis *et al.*, 1984), originally isolated from the xylem of bermudagrass (*Cynodontis dactylon* L. Per.), was reclassified as *Leifsonia xyli* subsp. *cynodontis* (Evtushenko *et al.*, 2000). This bacterium colonized the xylem vessels of maize (*Zea mays* L.) to a high titre when it was artificially inoculated (Lampel *et al.*, 1994). Inoculation with engineered strains of this and other endophytic bacteria into

plants could improve the resistance of plants to disease and insects and also diminish the effect of environmental pollutants (Barac *et al.*, 2004; Fahey *et al.*, 1991). *Leifsonia xyli* subsp. *xyli* is the causative agent of ratoon stunting disease, a major cause of economic loss in sugar cane crops (Sutcliffe & Hutchings, 2007). Members of the genus *Leifsonia* have also been isolated from roots, soil, Himalayan glaciers, cyanobacterial mats and water samples (Evtushenko *et al.*, 2000; Reddy *et al.*, 2003; Dastager *et al.*, 2008, 2009; Hao *et al.*, 2008). This paper presents the formal taxonomic description of a novel species of the genus *Leifsonia* isolated from teak rhizosphere soil, the strains of which produce phytohormones and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which enable them to promote plant growth.

Strains designated TG-S248^T and TG-S240 were isolated from rhizosphere soils of natural teak (*Tectona grandis* Linn.) by the dilution-plating technique on R2A medium (Reasoner & Geldreich, 1985) (Difco) and were maintained as 50 % glycerol stocks at –80 °C. Their carbon source utilization patterns were studied using Biolog GP2 microplates following the manufacturer's instructions and acid production from various sugars was tested by

Abbreviation: ACC, 1-aminocyclopropane-1-carboxylate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TG-S248^T and TG-S240 are EU912483 and EU912481, respectively.

Carbon source utilization patterns and scanning electron micrographs are available as supplementary material with the online version of this paper.

the methods of Smith *et al.* (1952) and Madhaiyan *et al.* (2007b). Colony and cell morphology were determined by using phase-contrast microscopy. Sample preparation and other procedures for scanning electron microscope observations were performed as described by Madhaiyan *et al.* (2007b). The samples were visualized using a Hitachi S-2500C with a Gemini column (Hitachi) equipped with a field emission source. Polar lipids and menaquinones were extracted and analysed according to Minnikin *et al.* (1984) and Kroppenstedt (1982). Preparation of cell walls and determination of peptidoglycan structure were performed as described by Schleifer & Kandler (1972), with the modification that TLC on cellulose sheets (Merck) was used instead of paper chromatography. Cell-wall sugars were identified according to the methods described by Maltsev *et al.* (1992). Cellular fatty acids were extracted from cultures grown in R2A medium at 28 °C for 4–5 days, derivatized to methyl esters and analysed with a gas chromatograph (Hewlett Packard 6890) using the Microbial Identification System software package (Microbial ID), according to standard protocols (Sasser, 1990). The minimal inhibitory concentrations of heavy metals and the antibiotic resistance patterns of the strains were determined as described by Madhaiyan *et al.* (2007a) and Chanprame *et al.* (1996). A quantitative assay for indole-3-acetic acid and plate assays for detecting the presence of siderophore production, phosphate solubilization, ACC deaminase activity and sulfur oxidation were carried out as previously described (Poonguzhali *et al.*, 2006). A gnotobiotic growth pouch assay was performed to measure root elongation induced by ACC deaminase in tomato and canola, which are ethylene-sensitive plants (Glick *et al.*, 1994; Li *et al.*, 2000; Penrose & Glick, 2003; Ghosh *et al.*, 2003; Madhaiyan *et al.*, 2006, 2007b).

The cells of strains TG-S248^T and TG-S240 were aerobic, motile, non-endospore forming, Gram-stain-positive, short rods (0.4–0.5 × 0.6–0.9 µm), occurring singly or in pairs, in yellow, circular and opaque colonies with entire margins on R2A agar. Scanning electron micrographs are shown in Supplementary Fig. S1 (available in IJSEM Online). The major fatty acids of strain TG-S248^T were anteiso-C_{15:0} (40.1 %), iso-C_{16:0} (14.3 %), anteiso-C_{17:0} (34.7 %), iso-C_{15:0} (7.9 %) and iso-C_{17:0} (2.6 %). The nutritional and physiological characteristics of strains TG-S248^T and TG-S240 are listed in the species description and the characteristics that differentiate them from their closest relatives are presented in Table 1. Strains TG-S248^T and TG-S240 differed from their closest relatives in utilization of several carbon sources (Supplementary Table S1). Both strains exhibited ACC deaminase activity, phosphate solubilization and sulfur oxidation when examined by plate assays, but were negative for siderophore production. Strains TG-S248^T and TG-S240 produced, respectively, 2.1 and 3.2 µg indole-3-acetic acid ml⁻¹ and 16.2 and 21.3 nmol α-ketobutyrate min⁻¹ (mg protein)⁻¹ (as an indication of ACC deaminase activity). In the gnotobiotic

growth pouch assay, the root lengths of the *Leifsonia*-treated tomato and canola seeds were comparatively greater when compared with the uninoculated control (data not shown).

Chromosomal DNA was extracted according to the kit instructions (QIAamp DNA mini kit; Qiagen). The 16S rRNA gene was amplified using the universal primers 27F and 1492R as described by Madhaiyan *et al.* (2009). The partial gene sequence was determined by the fluorescent dye terminator method using an ABI Prism BigDye Terminator cycle sequencing ready reaction version 3.1 kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. The resultant sequences were compared with sequences from *Leifsonia* and related genera in GenBank and aligned by using CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic relationships were determined by the neighbour-joining method (Saitou & Nei, 1987) with the program MEGA version 3.1 (Kumar *et al.*, 2004). Bootstrap confidence values were obtained using 1000 resamplings. DNA–DNA hybridizations were carried out on nitrocellulose membranes according to the method of Seldin & Dubnau (1985). The DIG-High Prime system and DIG luminescent detection kit (Roche Diagnostics) were used for labelling the probe DNA and visualization. Hybridization was carried out at 60 and 65 °C, and DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad Laboratories). The G+C content of the genomic DNA was determined by HPLC as described by Mesbah *et al.* (1989) using a Supelcosil LC-18 S reversed-phase column (Supelco).

The 16S rRNA gene sequence phylogenetic tree placed strains TG-S248^T and TG-S240 in the genus *Leifsonia*, grouping with *L. shinshuensis* DB 102^T, *L. poae* VKM Ac-1401^T, *L. naganoensis* DB 103^T, *L. aquatica* DSM 20146^T and *L. xyli* subsp. *cynodontis* JCM 9733^T (Fig. 1). Pairwise sequence similarities ranged from 98.7–99.1 %. Strains TG-S248^T and TG-S240 showed a high level of DNA–DNA relatedness (83 ± 7.9 %) with each other and low levels of relatedness with *L. shinshuensis* JCM 10591^T (21 ± 1.7 %), *L. poae* DSM 15202^T (26 ± 2.2 %), *L. naganoensis* JCM 10592^T (31 ± 2.9 %), *L. aquatica* DSM 20146^T (29 ± 2.6 %) and *L. xyli* subsp. *cynodontis* JCM 9733^T (32 ± 2.7 %). Based on the DNA–DNA hybridization data and the recommendation of a threshold value of 70 % DNA–DNA relatedness for species definition (Wayne *et al.*, 1987), it is clear that strains TG-S248^T and TG-S240 do not belong to any of the previously described species of *Leifsonia*. The DNA G+C content of strain TG-S248^T was 69.4 mol%, which falls within the range described for the genus *Leifsonia* (Evtushenko *et al.*, 2000).

On the basis of the results presented, strains TG-S248^T and TG-S240 are members of a novel species of *Leifsonia*, for which we propose the name *Leifsonia soli* sp. nov.

Table 1. Physiological and biochemical properties of strains TG-S248^T and TG-S240 that differentiate them from their closest phylogenetic neighbours in the genus *Leifsonia*

Strains: 1, *Leifsonia soli* sp. nov. TG-S248^T; 2, *Leifsonia soli* sp. nov. TG-S240; 3, *L. shinshuensis* JCM 10591^T; 4, *L. poae* DSM 15202^T; 5, *L. aquatica* DSM 20146^T; 6, *L. xyli* subsp. *cynodontis* JCM 9733^T; 7, *L. naganoensis* JCM 10592^T; 8, *L. bigeumensis* DSM 19322^T; 9, *L. kribbensis* DSM 19272^T. Data in columns 7–9 were obtained from Suzuki *et al.* (1999), Reddy *et al.* (2003) and Dastager *et al.* (2008, 2009). All other data were determined in this study. +, Positive; w, weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9
Colony colour*	Y	Y	WH	Y	Y	Y	WH	PY	Y
Motility	+	+	–	+	+	–	–	+	+
Oxidase	w	–	–	–	+	–	ND	–	–
Catalase	+	+	+	+	+	+	+	–	–
Growth temperature (°C)	10–35	10–35	7–42	4–37	7–37	7–35	7–37	20–37	20–37
Growth with 5% NaCl	–	w	–	–	+	–	–	–	–
Methyl red test	w	+	+	+	–	–	ND	ND	ND
Voges–Proskauer test	–	–	–	–	–	+	ND	ND	ND
H ₂ S production	–	–	–	–	+	–	ND	–	–
Hydrolysis of:									
Starch	+	+	+	–	+	+	+	+	w
Aesculin	+	+	w	+	–	–	ND	–	–
Gelatin	–	–	–	+	–	–	ND	–	–
Acid production from:									
L-Arabinose	+	+	–	+	–	–	ND	–	–
D-Fructose	+	+	+	+	+	+	+	–	–
D-Galactose	–	–	–	+	+	–	+	–	–
D-Glucose	+	+	–	–	+	+	+	–	–
Maltose	+	+	–	–	–	–	+	–	–
D-Mannose	+	+	–	+	–	+	+	–	–
Utilization as sole carbon source of:									
L-Arabinose	w	w	–	+	–	–	ND	–	–
Cellobiose	+	+	–	+	+	–	+	+	+
D-Fructose	w	w	+	+	+	+	+	–	–
D-Galactose	w	w	w	+	+	–	+	–	–
D-Glucose	+	+	–	w	+	+	+	–	–
myo-Inositol	–	–	+	–	+	–	+	+	+
D-Mannitol	w	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	w	+	+	–	–
Melibiose	–	–	+	+	+	–	w	+	+
Raffinose	–	–	–	+	–	–	ND	+	+
D-Rhamnose	–	–	w	–	+	–	–	+	+
D-Ribose	–	w	w	–	–	–	w	–	–
Trehalose	+	+	+	–	+	–	w	+	+
Fatty acids (% of total content)									
C _{16:0}	–	3.0	0.8	2.8	0.6	1.8	3.4	1.5	–
iso-C _{14:0}	0.4	0.3	0.4	0.3	0.1	0.6	0.6	1.9	7.8
iso-C _{15:0}	7.9	5.6	1.5	1.2	1.1	0.8	1.4	2.4	5.3
anteiso-C _{15:0}	40.1	35.7	45.2	35.2	28.3	33.5	41.5	36.0	19.7
iso-C _{16:0}	14.3	15.5	17.2	14.9	15.2	11.5	17.5	38.9	43.4
iso-C _{17:0}	2.6	2.5	2.4	2.1	1.5	0.9	1.8	1.1	2.1
anteiso-C _{17:0}	34.7	37.4	32.4	43.4	53.1	50.6	33.6	18.2	6.8
anteiso-C _{17:1ω9c}	–	–	–	–	–	–	–	–	8.7
DNA G + C content (mol%)	69.4	69.5	70.7	69.5	70.3	69.4	70.5	69	73.4
Major menaquinone(s)	11	11	11, 12	11	11, 10	11, 12	11, 10	11	11, 12

*PY, Pale yellow; WH, white; Y, yellow.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence comparisons showing the positions of strains TG-S248^T and TG-S240 in the genus *Leifsonia*. Percentages at branch nodes indicate levels of bootstrap support based on 1000 resampled datasets. Bar, 0.005 substitutions per nucleotide position.

Description of *Leifsonia soli* sp. nov.

Leifsonia soli (so'li. L. gen. n. *soli* of soil, referring to the isolation of the first strains from teak tree rhizosphere soil).

Colonies are circular, convex, smooth, yellow and 1.0–2.8 mm in diameter on R2A medium after 96 h at 28 °C. Cells are aerobic, Gram-stain-positive, motile, short rods, 0.4–0.5 µm wide by 0.6–0.9 µm long, occurring singly or in pairs. Tolerates up to 2.0% NaCl. Grows at 10–35 °C, with optimum growth at 30 °C, and at pH 5.0–9.0, with optimum growth at pH 6.8. Negative for degradation of cellulose, gelatin and pectin. Positive for decomposition of casein, tributyrin, starch and aesculin. Urease, indole production, H₂S production and reduction of nitrate are negative. Positive for the methyl red test and negative for the Voges–Proskauer test. Produces acid from L-arabinose, D-fructose, D-glucose, maltose and D-mannose but not from D-galactose. The yellow pigment is water-insoluble and has absorption maxima at 210, 253, 450 and 480 nm in methanol. Grows on R2A agar, TSA and NA, but not on MacConkey agar. Tolerates up to 0.5 mM NiCl₂ and <0.5 mM CdCl₂ in plate assays: no growth observed at higher concentrations. With the Biolog GP2 MicroPlate system, utilizes dextrin, Tweens 40 and 80, L-arabinose, cellobiose, D-fructose, D-galactose, D-gluconic acid, α-D-glucose, maltose, maltotriose, D-mannitol, D-mannose, melezitose, palatinose, salicin, D-sorbitol, sucrose, trehalose, turanose, D-xylose, pyruvic acid methyl ester, succinic acid monomethyl ester, glycerol and adenosine, but not α- or β-cyclodextrin, inulin, mannan, N-acetyl-D-galactosamine, N-acetyl-β-D-mannosamine, D-arabitol, D-galacturonic acid, myo-inositol, α-lactose, lactulose, melibiose, methyl α-D-galactoside, methyl β-D-galactoside, 3-methyl D-glucose, methyl β-D-glucoside, methyl α-D-mannoside, raffinose, L-rhamnose, sedoheptulosan, stachyose, D-tagatose, xylitol, α-, β- or γ-hydroxybutyric acid, p-hydroxyphenylacetic acid, α-ketobutyric acid, α-ketovaleric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D- or L-malic acid, propionic acid, succinamic acid, succinic acid, N-acetyl-L-glutamic acid, D- or L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-pyrogutamic acid, L-serine, putrescine, 2,3-butanediol, 2'-deoxyadenosine, inosine, uridine, adenosine 5'-monophosphate, thymidine 5'-monophosphate, uridine 5'-

monophosphate, D-fructose 6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate or DL-α-glycerol phosphate. The type strain is highly resistant (up to 500 µg ml⁻¹) to ampicillin, carbenicillin, nalidixic acid and bacitracin but sensitive to (lowest concentration; µg ml⁻¹) gentamicin (25), kanamycin (300), cefotaxime (50), chloramphenicol (300), spectinomycin (500), doxycycline (100), erythromycin (25), penicillin G (100), polymyxin B (500), rifampicin (25), novobiocin (100), tetracycline (500), cephalosporin (25), vancomycin (25) and trimethoprim (25). The cell-wall peptidoglycan contains alanine, 2,4-diaminobutyric acid, glutamic acid and glycine (molar ratio 1 : 2 : 1 : 1). The cell-wall sugars are galactose, glucose, mannose and rhamnose. Phospholipids detected are diphosphatidylglycerol, phosphatidylglycerol and several unidentified lipids. Major fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The major menaquinone is MK-11. The G+C content of the DNA of the type strain is 69.4 mol%.

The type strain, TG-S248^T (=LMG 24767^T =JCM 15679^T), was isolated from rhizosphere soil of natural teak (*Tectona grandis* Linn.), the sample being collected from Field No. 37, Tamilnadu Agricultural University (TNAU), Coimbatore, Tamilnadu, India.

Acknowledgements

We wish to thank Yoshimasa Kosako, Japan Collection of Microorganisms, RIKEN, Wako, Japan, and ir. Claudine Vereecke, BCCM/LMG Bacteria Collection, Belgium, for their valuable advice. We also thank Dr Jean P. Euzéby for his valuable advice on nomenclature. M. M. was supported through funds provided by the Indian Council of Agricultural Research (ICAR), New Delhi, India.

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