Microlunatus endophyticus sp. nov., an endophytic actinobacterium isolated from bark of *Bruguiera sexangula*

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A Gram-stain-positive, aerobic, coccoid, non-motile, non-spore-forming bacterium, designated strain S3Af-1^T, was isolated from surface-sterilized bark of *Bruguiera sexangula* collected from Dongzhaigang National Nature Reserve in Hainan, China, and examined using a polyphasic approach to clarify its taxonomic position. This bacterium did not produce substrate mycelia or aerial hyphae, and no diffusible pigments were observed on the media tested. Strain S3Af-1^T grew optimally without NaCl, at 28–30 °C and at pH 7.0.Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain S3Af-1^T belonged to the genus *Microlunatus* and shared highest similarity with '*Microlunatus terrae*' BS6 (97.43 %) and *Microlunatus soli* CC-12602^T (97.08 %). DNA–DNA hybridization results indicated that the level of relatedness between strain S3Af-1^T and *M. soli* CC-12602^T was less than 70 %. The DNA G+C content of strain S3Af-1^T was 67.1 mol%. The cell-wall peptidoglycan contained LL-2,6-diaminopimelic acid. MK-9(H_6) and MK-9(H_4) were the predominant menaquinones. Phosphatidylglycerol, diphosphatidylglycerol, an unidentified glycolipid, two unidentified phospholipids and other lipids were detected in the polar lipid extracts. The major fatty acids were iso-$C_{16:0}$, anteiso-$C_{17:0}$ and anteiso-$C_{15:0}$.On the basis of phylogenetic analysis, and phenotypic and chemotaxonomic characteristics, strain S3Af-1^T represents a novel species of the genus *Microlunatus*, for which the name *Microlunatus endophyticus* sp. nov. is proposed. The type strain is S3Af-1^T (=DSM 100019^T=CGMCC 4.7306^T).

The genus *Microlunatus*, with *Microlunatus phosphovorus* as the type species, was first proposed by Nakamura *et al.* (1995). At the time of writing, the genus comprised eight species with effectively published names: *M. phosphovorus* (Nakamura *et al.*, 1995), *M. ginsengisoli* (Cui *et al.*, 2007), *M. aurantiacus* (Wang *et al.*, 2008), *M. panaciterrae* (An *et al.*, 2008), *M. soli* (Kämpfer *et al.*, 2010a), *M. parietis* (Kämpfer *et al.*, 2010b), '*M. terrae*' (Lee & Kim, 2012) and '*M. cavernae*' (Cheng *et al.*, 2013); the latter two names have not been validly published. These species were isolated from different habitats, such as activated sludge, ginseng field, growing edible mushrooms, soil, indoor wall material and cave.

During a study on the cultivable actinobacterial diversity of mangrove in Dongzhaigang National Nature Reserve (19° 56′ 59″ N 110° 34′ 32″ E), Hainan, China, strain S3Af-1^T was isolated from surface-sterilized bark of *Bruguiera sexangula*. The plant samples of *Bruguiera sexangula* were washed in running tap water to remove adherent epiphytes and surface-sterilized according to the five-step sterilization procedure (Qin *et al.*, 2008). After drying under a hood, the surface-sterilized bark was ground into powder by using a micromill and distributed on different

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S3Af-1^T is KT749873.

One supplementary table and three supplementary figures are available with the online Supplementary Material.

media plates before being incubated at 28 °C for 4 weeks. Based on phylogenetic analysis, strain S3Af-1^T showed high levels of 16S rRNA gene sequence similarity to members of the genus *Microlunatus*. A polyphasic taxonomic study showed that strain S3Af-1^T differed from previously described species of the genus *Microlunatus* and that it may represent a novel species. The taxonomic position of this strain is reported herein.

Strain S3Af-1^T was isolated on chitin agar (containing, per litre distilled water: 2.0 g chitin, 0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgSO_4 . 7H_2O$, 0.01 g $FeSO_4 . 7H_2O$, 0.001 g $ZnSO_4$, 0.001 g $MnCl_2$ and 15.0 g agar, pH 7.2) plates after 4 weeks of incubation at 28 °C. Isolated colonies were transferred and streaked onto ISP 2 agar (Shirling & Gottlieb, 1966) until pure cultures were obtained. The strain was cultivated, maintained on ISP 2 agar slants at 4 °C and stored as aqueous glycerol suspensions (20 %, v/v) at −80 °C.

Cultural, physiological and biochemical characteristics of strain S3Af-1^T were tested by using *M. soli* CC-12602^T (obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ) as a reference strain under the same conditions. Cultural characteristics were determined by observing the growth of the strain at 28 °C for 14 days on ISP 2, ISP 3, ISP 4, ISP 5 and ISP 7 agars (Shirling & Gottlieb, 1966), nutrient agar (Waksman, 1961), R2A agar (Difco), trypticase soy agar (TSA; Bacto) and Luria–Bertani agar. Cell morphology and motility were observed and recorded by transmission electron microscopy (JEM-1400; JEOL) after incubation on R2A agar at 28 °C for 3 days. The Gram-stain test was performed as described by Magee *et al.* (1975). The temperature range for growth was determined by incubation of the strain on R2A agar at 4, 15, 20, 25, 28, 30, 37, 42 and 50 °C for 14 days. The pH range (pH 4.0–13.0, at intervals of 1 pH unit) for growth was measured in R2A broth for 4 weeks using the buffer system described by Xu *et al.* (2005). Salt tolerance was tested on R2A agar supplemented with 0, 1, 2, 3, 4, 5, 6, 7 and 10 % (w/v) NaCl for 14 days. Catalase activity was determined by bubble production in 3 % (v/v) H_2O_2. Oxidase activity was assessed by using 1 % (w/v) tetramethyl-*p*-phenylenediamine (Cappuccino & Sherman, 2002). Hydrolysis of starch, gelatin, casein and Tweens 20, 40 and 80, and production of melanin and H_2S were tested as described by Gonzalez *et al.* (1978). Acid production from carbon sources was examined by using the API 50CH (bioMérieux) system. Oxidation of the carbon sources and sensitivity to antimicrobial compounds were tested by using Biolog GEN III MicroPlates according to the manufacturer's instructions. Other physiological and biochemical characteristics and enzyme activities were tested by using the API 20NE and API ZYM kits (bioMérieux) according to the manufacturer's instructions.

Strain S3Af-1^T was Gram-stain-positive, non-spore-forming and aerobic. Colonies of strain S3Af-1^T grown on ISP 2 agar for 5 days were circular, smooth, entire and yellowish white. Neither substrate nor aerial mycelium was formed, and no diffusible pigments were produced on the media tested. Cells were coccus-shaped (0.5–0.7 μm in diameter) and occurred singly or formed clusters (Fig. S1, available in the online Supplementary Material) after incubation for 3 days at 28 °C on R2A agar. Strain S3Af-1^T grew well on ISP 2 agar, ISP 3 agar, R2A agar and TSA. Poor growth occurred on nutrient agar. No growth occurred on ISP 4, ISP 5, ISP 7 or Luria–Bertani agars. Strain S3Af-1^T grew at 20–42 °C and pH 5.0–9.0 and tolerated 0–2 % (w/v) NaCl. No growth occurred at 15 or 50 °C, at pH 4.0 or 10.0, or in the presence of 3 % (w/v) NaCl. Optimal growth occurred at pH 7.0, at 28–30 °C and without NaCl. The detailed physiological and biochemical characteristics of strain S3Af-1^T are given in Table 1 and the species description.

For chemotaxonomic studies of polar lipids and fatty acids, strain S3Af-1^T was analysed together with the reference strain. The isomers of diaminopimelic acid in whole-cell hydrolysates of strain S3Af-1^T were identified by TLC as described by Schleifer & Kandler (1972). Menaquinone analysis of strain S3Af-1^T was carried out by the Identification Service of the DSMZ. The polar lipids were extracted and analysed by two-dimensional TLC on a silica gel 60 F_{254} plate (Merck) as described by Minnikin *et al.* (1984). The solvent systems of the first and the second dimension were chloroform/methanol/water (64 : 27 : 5, by vol.) and chloroform/methanol/acetic acid/water (80 : 18 : 12 : 5, by vol.), respectively. For the analysis of whole-cell fatty acids, cell mass of strain S3Af-1^T and the reference strain were harvested from R2A agar at 28 °C, when the bacterial communities had reached the late-exponential stage of growth. The whole-cell fatty acids were saponified, methylated and extracted according to the standard protocol described by Sasser (1990), and analysed by using an Agilent 7890A gas chromatograph coupled with an Agilent 5975C single quadrupole mass spectrometer equipped with Nist08 Library software database (Tuo *et al.*, 2015).

For the determination of DNA–DNA hybridization and G+C content, genomic DNA of strain S3Af-1^T was prepared according to the methods described by Marmur (1961). DNA–DNA hybridization was determined following the thermal renaturation method (De Ley *et al.*, 1970). The G+C content was determined by reversed-phase HPLC as described by Mesbah *et al.* (1989).

The whole-cell hydrolysate of strain S3Af-1^T contained LL-2,6-diaminopimelic acid, and the menaquinones comprised MK-9(H_6) (78 %), MK-9(H_4) (10 %), MK-9(H_8) (4 %) and some minor components. The polar lipids comprised phosphatidylglycerol, diphosphatidylglycerol, an unidentified glycolipid, two unidentified phospholipids and other lipids (Fig. S2). The whole-cell fatty acid profile contained large amounts of iso-$C_{16:0}$ (44.27 %), anteiso-$C_{17:0}$ (37.17 %) and anteiso-$C_{15:0}$ (12.68 %) with minor amounts of $C_{16:0}$ (5.88 %) and iso-$C_{15:0}$

Table 1. Differential characteristics between strain S3Af-1^T and its closest phylogenetic neighbour, *M. soli* CC-12602^T

All data were obtained from this study unless indicated otherwise. Both strains were Gram-stain-positive, positive for catalase, and hydrolysis of Tweens 20 and 40, weakly positive for casein, but negative for nitrate reduction, hydrolysis of urease and production of H_2S. In API 20NE kits, both strains were negative for assimilation of potassium gluconate, caprate, malic acid, trisodium citrate, phenylacetate, D-glucose, D-mannose, D-mannitol and adipate. In API ZYM kits, both strains were positive for *N*-acetyl-β-glucosaminidase, acid phosphatase, esterase lipase, esterase, α-fucosidase, naphthol-AS-BI-phosphohydrolase, leucine arylamidase, valine arylamidase, β-galactosidase, α-chymotrypsin, α-mannosidase, α-glucosidase and β-glucosidase, but negative for lipase, β-glucuronidase and trypsin. +, Positive; −, negative; W, weakly positive; ND, not determined.

Characteristic	S3Af-1^T	*M. soli* CC-12602^T
Cell size (μm)	0.5–0.7	1.0–1.5*
Motility	−	ND*
Growth pH range (optimum)	5.0–9.0 (7.0)	5–10 (7.0)
Temperature range for growth (optimum) (°C)	20–42 (28–30)	15–37 (28–30)
NaCl tolerance range (optimum) (%, w/v)	0–2 (0)	0–7 (0)
Oxidase	+	−
Hydrolysis of:		
Tween 80	−	+
Gelatin	−	+
Starch	+	W
Carbon source utilization:		
L-Arabinose	−	+
Maltose	−	+
N-Acetyl-D-glucosamine	−	+
API ZYM results:		
Alkaline phosphatase	−	+
Cystine arylamidase	+	−
α-Galactosidase	+	−
Major menaquinone(s)	MK-9(H_6), MK-9(H_4)	MK-9(H_4)*
Major fatty acids (>10 %)	iso-$C_{16:0}$ (44.27 %), anteiso-$C_{17:0}$ (37.17 %), anteiso-$C_{15:0}$ (12.68 %)	anteiso-$C_{15:0}$ (33.11 %), iso-$C_{16:0}$ (33.04 %), iso-$C_{15:0}$ (19.37 %)
DNA G+C content (mol%)	67.1	66.4

*Data from Kämpfer *et al.* (2010a).

(<0.5 %). The cellular fatty acid content of strain S3Af-1^T and the reference strain are given in Table S1. The DNA G+C content of strain S3Af-1^T was 67.1 mol%. The major fatty acids and polar lipids of the reference strain were similar to those previously reported (Kämpfer *et al.*, 2010a). Differences in the proportion of the fatty acids and slight differences in the types of polar lipids may be due to the different experimental conditions used.

The extraction of genomic DNA from strain S3Af-1^T and PCR amplification of the 16S rRNA gene were performed as described by Li *et al.* (2007). The purified PCR products were cloned by using the pEASY-T1 Cloning kit (TransGen Biotech) and sequenced at Sangon Biotech. The 16S rRNA gene sequence similarity values between strain S3Af-1^T and related species were determined by using the EzTaxon server (http://eztaxon-e.ezbiocloud.net/; Chun *et al.*, 2007). Multiple alignments were made using CLUSTAL X (Thompson *et al.*, 1997). Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods with MEGA version 5.0 software (Tamura *et al.*, 2011). The topologies of the phylogenetic trees were evaluated by using the bootstrap method with 1000 replications (Felsenstein, 1985).

An almost-complete sequence of the 16S rRNA gene of strain S3Af-1^T (1478 bp) was obtained. A BLAST search showed that strain S3Af-1^T exhibited the highest levels of 16S rRNA gene sequence similarity with '*M. terrae*' BS6 (97.43 %) and *M. soli* CC-12602^T (97.08 %). Levels of similarity with other species of the genus *Microlunatus* were all less than 97.0 %. The phylogenetic trees based on 16S rRNA gene sequences generated by using all three tree-making methods showed that strain S3Af-1^T formed a distinct cluster with *M. soli* CC-12602^T (Figs. 1 and S3) within the genus *Microlunatus*, which indicated that strain S3Af-1^T was phylogenetically affiliated to the genus *Microlunatus*. Meanwhile, the mean DNA–DNA hybridization value between strain S3Af-1^T and the phylogenetically related strain *M. soli* CC-12602^T was 45.6±12.7 %, which was below the 70 % cut-off value generally recommended for the delineation of genomic species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Thus, strain S3Af-1^T

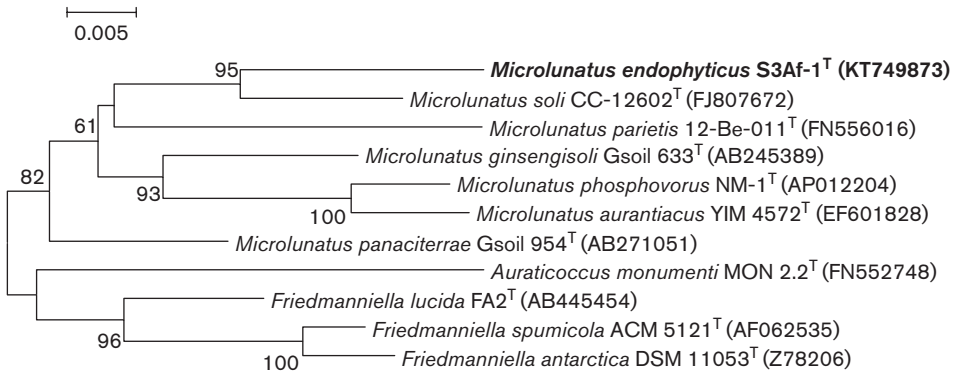

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strain S3Af-1^T and related species of the genus *Microlunatus*. Numbers at nodes indicate the level of bootstrap support (>50 %) based on 1000 replications. Bar, 5 nt substitutions per 1000 nt.

is considered to represent a novel genomic species within the genus *Microlunatus*.

Additionally, chemotaxonomic characteristics of strain S3Af-1^T support the above conclusion. Strain S3Af-1^T shared the common chemotaxonomic characteristics of recognized members of the genus *Microlunatus*, in containing LL-2,6-diaminopimelic acid as the diagnostic diamino acid of the cell-wall peptidoglycan, and iso-$C_{16:0}$ and anteiso-$C_{15:0}$ as the major cellular fatty acids (Hanada & Nakamura, 2012). Strain S3Af-1^T differed from its phylogenetically related species in some chemotaxonomic characteristics. Regarding the polar lipid profiles, strain S3Af-1^T shared the same polar lipids of phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid with the reference strain. However, there were significant differences in the unidentified phospholipids between S3Af-1^T and the reference strain. Strain *M. soli* CC-12602^T contained unidentified phospholipids PL3 and PL5, which were absent from strain S3Af-1^T (Fig. S2). The major menaquinones of strain S3Af-1^T were MK-9(H_6) and MK-9(H_4), whereas the major menaquinone of strain *M. soli* CC-12602^T was MK-9(H_4). The dominant fatty acids (>10 %) of strain S3Af-1^T were iso-$C_{16:0}$ (44.27 %), anteiso-$C_{17:0}$ (37.17 %) and anteiso-$C_{15:0}$ (12.68 %), which differentiated it from *M. soli* CC-12602^T [anteiso-$C_{15:0}$ (33.11 %), iso-$C_{16:0}$ (33.04 %), iso-$C_{15:0}$ (19.37 %)] (Table S1). Other morphological and biochemical properties that differentiated strain S3Af-1^T from *M. soli* CC-12602^T are shown in Table 1.

In summary, based on phylogenetic analysis, and phenotypic and chemotaxonomic characteristics, strain S3Af-1^T represents a novel species of the genus *Microlunatus*, for which the name *Microlunatus endophyticus* sp. nov. is proposed.

Description of *Microlunatus endophyticus* sp. nov.

Microlunatus endophyticus (en.do.phy′ti.cus. Gr. pref. *endo* within; Gr. neutr. *phyton* plant; L. masc. suff. *-icus* adjectival suffix used with the sense of belonging to; N.L. masc. adj. *endophyticus* within plant, endophytic, pertaining to the original isolation from plant tissues).

Cells are aerobic, Gram-stain-positive, non-spore-forming, non-motile, coccus-shaped and 0.5–0.7 μm in diameter after incubation for 3 days at 28 °C on R2A agar. Neither substrate nor primary mycelium is formed, and no diffusible pigments are produced on any of the test media. Positive for oxidase and catalase. Colonies grown on ISP 2 agar for 5 days are smooth, circular, entire and yellowish white. Grows well on ISP 2 agar, ISP 3 agar, TSA and R2A agar, poor growth occurs on nutrient agar, and no growth occurs on ISP 4, ISP 5, ISP 7 or Luria–Bertani agars. Growth occurs at 20–42 °C (optimum, 28–30 °C), at pH 5.0–9.0 (optimum, pH 7.0) and with 0–2 % (w/v) NaCl (optimum, 0 %). No growth occurs at 15 or 50 °C, at pH 4.0 or 10.0, or in the presence of 3 % (w/v) NaCl. Cells are positive for hydrolysis of starch, Tween 20 and Tween 40. Weakly positive for hydrolysis of casein. Hydrolysis of Tween 80 and gelatin, nitrate reduction, and urease and H_2S production are negative. According to the API ZYM test, positive for *N*-acetyl-β-glucosaminidase, acid phosphatase, α-chymotryspin, cystine arylamidase, esterase (C4), esterase lipase (C8), α-fucosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, leucine arylamidase, α-mannosidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase. Negative for alkaline phosphatase, β-glucuronidase, lipase (C14) and trypsin. In API 50CH strips, acid is produced from *N*-acetylglucosamine, aesculin, amygdalin, D-arabinose, L-arabinose, arbutin, cellobiose, D-galactose, gentiobiose, D-glucose (weakly), lactose (weakly), maltose, D-mannose (weakly), melezitose, melibiose, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, raffinose, D-ribose, sucrose, salicin, starch (weakly), trehalose, turanose and D-xylose, but not from D-adonitol, D-arabitol, L-arabitol, dulcitol, erythritol, D-fructose, L-fucose, D-fucose, gluconate, glycogen, glycol, inositol, inulin, 2-ketogluconate, 5-ketogluconate, D-lyxose, mannitol, L-rhamnose, sorbitol, L-sorbose,

D-tagatose, L-xylose or xylitol. In the Biolog system, positive for oxidation of acetic acid, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, *N*-acetylneuraminic acid, D-alanine, γ-aminobutyric acid, D-arabitol, L-arginine, D-aspartic acid, L-aspartic acid, bromosuccinic acid, cellobiose, citric acid, dextrin, formic acid, D-fructose, D-fructose 6-phosphate, D-fucose, L-fucose, D-galactose, galacturonic acid, D-galactonic acid lactone, gelatin, gentiobiose, D-gluconic acid, α-D-glucose, D-glucose 6-phosphate, glucuronamide, D-glucuronic acid, L-glutamic acid, glycerol, glycyl L-proline, L-histidine, α-hydroxybutyric acid, β-hydroxy-DL-butyric acid, *p*-hydroxyphenylacetic acid, inosine, α-ketobutyric acid, α-ketoglutaric acid, L-lactic acid, D-lactic acid methyl ester, α-lactose, D-malic acid, L-malic acid, maltose, D-mannitol, D-mannose, melibiose, 3-methyl D-glucose, methyl β-D-glucoside, methyl pyruvate, mucic acid, *myo*-inositol, pectin, L-pyroglutamic acid, quinic acid, raffinose, L-rhamnose, D-saccharic acid, salicin, L-serine, D-sorbitol, stachyose, sucrose, trehalose, turanose and Tween 40, but negative for acetoacetic acid, propionic acid and D-serine. Cells are sensitive to aztreonam, fusidic acid, guanidine HCl, lincomycin, lithium chloride, minocycline, niaproof 4, sodium bromate, sodium butyrate, tetrazolium blue, tetrazolium violet, troleandomycin, vancomycin and 1 % sodium lactate but resistant to nalidixic acid, potassium tellurite and rifamycin SV. The cell-wall peptidoglycan contains LL-2,6-diaminopimelic acid as the diagnostic diamino acid. The predominant menaquinones are MK-9(H_6) and MK-9(H_4). The polar lipids comprise phosphatidylglycerol, diphosphatidylglycerol, an unidentified glycolipid, two unidentified phospholipids and other lipids. The major fatty acids are iso-$C_{16:0}$, anteiso-$C_{17:0}$ and anteiso-$C_{15:0}$.

The type strain, S3Af-1^T (=DSM 100019^T=CGMCC 4.7306^T), was isolated from surface-sterilized bark of *Bruguiera sexangula* collected from Dongzhaigang National Nature Reserve in Hainan, China. The G+C content of the genomic DNA of the type strain is 67.1 mol%.

Acknowledgements

We are grateful to Cai-Rong Zhong from Hai Nan Dong Zhai Gang National Nature Reserve Authority for his assistance with collecting samples. This research was partly supported by the National Natural Sciences Foundation of China (NSFC, Grant nos. 81172963, 31260004 and 81321004) and the National Science and Technology Major Project (Grant no. 2012ZX09301-002-001-018) from the Ministry of Science and Technology of China.

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