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Streptomyces humi sp. nov., an actinobacterium isolated from soil of a mangrove forest

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Abstract A novel *Streptomyces* strain, MUSC 119^T, was isolated from a soil collected from a mangrove forest. Cells of MUSC 119^T stained Gram-positive and formed light brownish grey aerial mycelium and grayish yellowish brown substrate mycelium on ISP 2 medium. A polyphasic approach was used to determine the taxonomic status of strain MUSC 119^T, which shows a range of phylogenetic and chemotaxonomic properties consistent with those of the genus *Streptomyces*. The cell wall peptidoglycan consisted of LL-diaminopimelic acid. The predominant menaquinones were identified as MK-9(H_8), MK-9(H_6) and MK-9(H_4). The polar lipid profile consisted of phosphatidylinositol, phosphatidylethanolamine, glycolipids, diphosphatidylglycerol and four phospholipids. The predominant cellular fatty acids were anteiso-$C_{15:0}$, iso-$C_{16:0}$, and anteiso-$C_{17:0}$. The cell wall sugars were glucose, mannose, ribose and rhamnose. The phylogenetic analysis based on 16S rRNA gene sequence similarity showed that strain MUSC119^T to be closely related to *Streptomyces rhizophilus* JR-41^T (99.0 % sequence similarity), *S. panaciradicis* 1MR-8^T (98.9 %), *S. gramineus* JR-43^T (98.8 %) and *S. graminisoli* JR-19^T (98.7 %). These results suggest that MUSC 119^T should be placed within the genus *Streptomyces*. DNA–DNA relatedness values between MUSC 119^T to closely related strains ranged from 14.5 ± 1.3 to 27.5 ± 0.7 %. The G+C content was determined to be 72.6 mol %. The polyphasic study of MUSC 119^T showed that this strain represents a novel species, for which the name *Streptomyces humi* sp. nov. is proposed. The type strain of *S. humi* is MUSC 119^T (=DSM 42174^T = MCCC 1K00505^T).

Keywords *Streptomyces humi* sp. nov · Actinobacteria · Mangrove forest · Malaysia

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Introduction

The mangrove ecosystems have been habitat to diverse flora and fauna of freshwater, marine and terrestrial species (Jennerjahn and Ittekkot 2002). The

information of species diversity of larger animals and plants in mangrove ecosystems are well documented, however little is known about the diversity of microbial communities such as actinobacteria in mangrove environments (Hong et al. 2009; Xu et al. 2009; Lee et al. 2014a; Azman et al. 2015). In recent years, the increased exploitation of mangrove microorganism resources has allowed the discovery of many novel actinobacteria especially of the genus *Streptomyces*, such as the isolation of *Streptomyces avicenniae* (Xiao et al. 2009), *Streptomyces xiamenensis* (Xu et al. 2009), *Streptomyces sanyensis* (Sui et al. 2011), *Streptomyces qinglanensis* (Hu et al. 2012), *Streptomyces pluripotens* (Lee et al. 2014b), *Streptomyces gilvigriseus* (Ser et al. 2015a) and *Streptomyces mangrovisoli* (Ser et al. 2015b).

Waksman and Henrici (1943) proposed the genus *Streptomyces*; members of the genus from various environments have made a significant contribution to mankind due to their ability to produce various natural products of significant importance (Bérdy 2005; Hong et al. 2009). The genus *Streptomyces* is the largest genus in the domain *Bacteria* (Hain et al. 1997), being comprised of 778 species with validly published names (http://www.bacterio.cict.fr/). A study was conducted to investigate the diversity of actinobacteria in mangrove forest. After series of screening, one of these strains was chosen for further study, as it produced significant starch hydrolysis activity. To determine the taxonomic status of strain MUSC 119^T, a polyphasic approach was used to determine the phylogenetic, genomic, chemotaxonomic and phenotypic characteristics of the novel strain. The results indicated that strain MUSC 119^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces humi* sp. nov. is proposed.

Materials and methods

Isolation and maintenance of isolate

A study was conducted to investigate the diversity of actinobacteria in mangrove forest. Strain MUSC 119^T, which produced significant starch hydrolysis activity was chosen for further study. Strains MUSC 119^T was isolated from a soil sample collected at site MUSC-TLS1 (3°48′3.2″N, 103°20′11.0″E) located at the mangrove forest of the Tanjung Lumpur in the State of Pahang, Peninsular Malaysia, in December 2012. Samples of the upper 20 cm topsoil layer (after removing the top 2–3 cm) were collected using an aseptic metal trowel, placed in sterile plastic bags and shored in −20 °C. The selective pretreatment of soil samples was performed using wet heat in sterilized water (15 min at 50 °C) (Takahashi et al. 1996) using a water bath. Five grams of air-dried soil was mixed with 45 ml sterilized water and ground using a mill and then the suspension was spread onto an isolation medium, ISP 2 medium (Shirling and Gottlieb 1966) supplemented with cycloheximide (25 μg ml^{-1}) and nystatin (10 μg ml^{-1}), and incubated at 28 °C for 7–14 days. The pure cultures of isolate were maintained on slants of ISP 2 medium at 4 °C for short term storage and as glycerol suspensions (20 %, v/v) at −20 °C for long term preservation.

Phenotypic characteristics

Cultural characteristics of strain MUSC 119^T were determined following growth on ISP 2, ISP 3, ISP 4, ISP 5, ISP 6 and ISP 7 media (Shirling and Gottlieb 1966), Actinomycetes isolation agar (AIA; Atlas 1993), *Streptomyces* agar (SA; Atlas 1993), starch casein agar (SCA; Küster and Williams 1964), Luria–Bertani agar, tryptic soy agar and nutrient agar (MacFaddin 2000) for 7–14 days at 28 °C. Light microscopy (80i, Nikon) and scanning electron microscopy (JEOL-JSM 6400) were used to observe the morphologies of strains after incubation on ISP 2 medium at 28 °C for 7–14 days. The ISCC-NBS colour charts were used to determine the names and designations of colony colours (Kelly 1964). Growth was tested at 12–48 °C at intervals of 4 °C on ISP 2 medium (solid form). NaCl tolerance was tested using tryptic soy broth (TSB) and salt concentrations of 0-18 % (w/v) at intervals of 2 %. Growth was tested between pH 4.0 and 10.0 at intervals of 2 % using TSB. The pH of TSB was regulated using sodium dihydrogen phosphate and disodium hydrogen phosphate. The responses to temperature, pH and NaCl were observed for 14 days. Gram staining was completed following the standard Gram reaction and was confirmed by using KOH lysis (Cerny 1978). The production of melanoid pigments and catalase activity were determined following protocol as described by Lee et al. (2014c). Haemolytic activity determination was performed on blood agar medium containing 5 %

(w/v) peptone, 3 % (w/v) yeast extract, 5 % (w/v) NaCl and 5 % (v/v) horse blood (Carillo et al. 1996). Plates were examined for haemolysis after incubation at 32 °C for 7–14 days. Lipase, amylolytic, cellulase, chitinase, protease and xylanase activities were determined by growing cells on ISP 2 medium and following protocols as described by Meena et al. (2013). The presence of clear zones around colonies signifies the potential of isolates for surfactant production. Carbon-source utilization and chemical sensitivity assays were determined using Biolog GenIII MicroPlates according to the manufacturer's instructions (Biolog). All of the phenotypic assays mentioned were performed concurrently for strain MUSC 119^T, *Streptomyces rhizophilus* NBRC 108885^T, *Streptomyces gramineus* NBRC 107863^T, *Streptomyces capoamus* NBRC 13411^T and *Streptomyces bungoensis* NBRC 15711^T using the same test media as mentioned above. The physiological characteristics of strain MUSC 119^T and the differences with the closely related type strains are shown in species description and Table 1.

Genomic and phylogenetic characteristics

Genomic DNA extractions for PCR was performed following protocols as described by Hong et al. (2009). PCR amplification of the 16S rRNA gene was done as described by Lee et al. (2014b). The cloning of the 16S rRNA gene was done using the CloneJET PCR cloning kit (Thermo Scientific, USA). The almost complete 16S rRNA gene sequence of strain MUSC 119^T was aligned with sequences of type strains of closely related species of the genus *Streptomyces* that had been retrieved from the GenBank/EMBL/DDBJ databases using CLUSTAL-X software (Thompson et al. 1997). The alignment was manually verified and adjusted prior to the reconstruction of a phylogenetic tree. Phylogenetic trees were constructed with the neighbor-joining (Saitou and Nei 1987) (Fig. 1) and maximum-likelihood (Felsenstein 1981) (Fig. S1) algorithms using MEGA version 6.0 (Tamura et al. 2011). Calculations of levels of sequence similarity were done using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al. 2012). The stability of the resultant tree topologies was evaluated by using the bootstrap resampling method of Felsenstein (1985). Evolutionary distances were computed using Kimura's two-parameter model (Kimura 1980). The genomic DNA for the determination of G+C content was extracted according to Cashion et al. (1977). The G+C content of the DNA was determined by HPLC (Mesbah et al. 1989). The extraction of genomic DNA for DNA–DNA hybridization of strain MUSC 119^T and closely related type strains were carried out by the Identification Service of the DSMZ, Braunschweig, Germany following the protocol of Cashion et al. (1977). DNA–DNA hybridization was carried out as described by De Ley et al. (1970) 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). BOX-PCR fingerprinting was performed using the primer BOX-A1R (5′-CTACGGCAAGGCGACGCT GACG-3′) (Versalovic et al. 1991; Lee et al. 2014d) and PCR condition as described by Lee et al. (2014e). BOX-PCR fingerprint analysis was used to characterize strain MUSC 119^T and its closely related species.

Chemotaxonomic characteristics

Biomass for chemotaxonomic studies was obtained after growing in tryptic soy broth (TSB) at 28 °C for 7–14 days on a rotary shaker. Analysis of peptidoglycan amino-acid composition and sugars was carried out by the Identification Service of the DSMZ using published protocols (Schumann 2011). Analysis of respiratory menaquinones, polar lipids (Kates 1986) and fatty acids (Sasser 1990) were carried out by the Identification Service of the DSMZ. Major diagnostic sugars of strain MUSC 119^T were obtained as described by Whiton et al. (1985) and analyzed by TLC on cellulose plates (Staneck and Roberts 1974).

Results and discussion

The novel isolate formed dark-grayish-yellow pigmented colonies on ISP 2 medium. Good growth was observed on ISP 2 medium, ISP 3 medium, ISP 5 medium, ISP 6 medium, ISP 7 medium, *Streptomyces* agar, starch casein agar, Luria–Bertani agar, tryptic soy agar and nutrient agar after 1–2 weeks at 28 °C, and cells grew moderately on actinomycetes isolation agar, whereas no growth on ISP 4 medium. The morphological observation of a 15-day-old culture grown on ISP 2 medium revealed a straight spore

Table 1 Differentiation characteristics of strain MUSC 119^T and phylogenetically closely related type strains of *Streptomyces* species

Characteristic	1	2	3	4	5	6	7	8
Morphology (on ISP 2):								
Colour of aerial mycelium	Light brownish gray	Grayish yellow	Light yellow	Pale orange yellow	Light greenish yellow	Gray reddish orange	Yellowish white	Brownish gray
Colour of substrate mycelium	Gray yellowish brown	Grayish yellow	Brilliant orange	Deep orange yellow	Grayish yellow	Strong reddish brown	Moderate yellow	Yellowish brown
Soluble pigment	Dark grayish yellow	–	–	Brilliant yellow	–	Pale yellow	Grayish yellow	–
Growth at								
24 °C	(+)	+	(+)	(+)	+	(+)	(+)	(+)
36 °C	+	+	(+)	+$^{\gamma}$	+	+	+	+
pH 4	(+)	−	(+)	(+)$^{\gamma}$	(+)	−	−	(+)
pH 9	(+)	−	(+)	(+)$^{\gamma}$	(+)	(+)	−	(+)
4 % NaCl	+	+	(+)	(+)$^{\gamma}$	(+)	(+)	(+)	+
Catalase	+	+	−	+	−	+	+	+
Haemolytic	−	−	−	−	−	−	−	−
Hydrolysis of								
Starch (amylolytic)	+	+	+	+$^{\gamma}$	+	+	+	+
Carboxymethylcellulose (cellulase)	+	+	+	+	+	+	+	+
Carbon source utilization								
Stachyose	−	+	+	+	+	+	+	+
N-acetyl-β-D-mannosamine	+	−	−	−	−	+	+	+
3-methyl glucose	−	+	−	−	+	+	−	−
D-fucose	−	+	+	−	−	+	+	+
Inosine	−	−	+	+	+	+	+	+
D-sorbitol	−	+	+	−	−	+	+	−
D-fructose-6-PO4	+	+	+	+	+	+	−	+
D-aspartic acid	+	−	−	−	−	+	−	−
Mucic acid	+	−	+	+	−	+	−	−
Chemical sensitivity assays:								
Fusidic acid	−	+	+	+	−	+	−	+
Minocycline	−	+	+	+	+	+	−	+
Lincomycin	−	+	−	+	−	−	−	−
Guanidine HCl	−	+	+	+	+	+	−	−
Niaproof 4	−	+	+	+	−	+	−	−
Vancomycin	−	+	−	+	+	−	−	−
Tetrazolium violet	−	+	+	+	+	+	−	−
Tetrazolium blue	−	+	+	+	+	+	−	−

$^{\gamma}$ Results in accordance with that published for *Streptomyces gramineus* NBRC 107863^T by Lee et al. (2012)

All data were obtained concurrently in this study

All strains were positive for utilization of Dextrin, D-maltose, D-trehalose, D-glucose-6-phosphate, pectin, citric acid, L-malic acid, tween 40, γ-amino-butyric acid, β-hydroxy-D,L-butyric acid, α-keto-butyric acid and acetic acid

Strains 1 Streptomyces humi sp. nov. MUSC 119^T, *2 Streptomyces rhizophilus* NBRC 108885^T, *3 Streptomyces panaciradicis* NBRC 109811^T, *4 Streptomyces gramineus* NBRC 107863^T, *5 Streptomyces graminisoli* NBRC 108883^T, *6 Streptomyces capoamus* NBRC 13411^T, *7 Streptomyces bungoensis* NBRC 15711^T, *8 Streptomyces longwoodensis* JCM 4976^T, + Positive, − negative, *(+)* weak

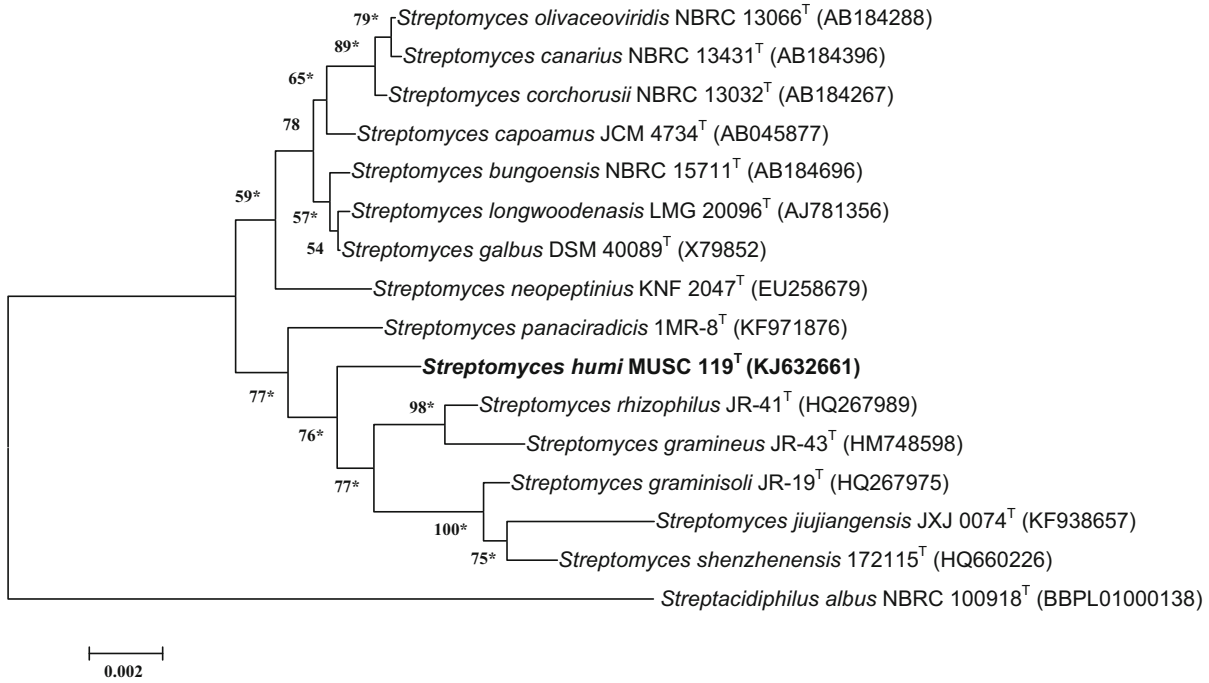

Fig. 1 Neighbour-joining tree (Saitou and Nei 1987) based on almost complete 16S rRNA sequences (1488 nucleotides) showing relationship between strain MUSC119^T and representatives of some other related taxa. Bootstrap values (>50 %) based on 1000 re-sampled datasets are shown at branch nodes. *Bar*, 0.002 substitutions per site. *Asterisks* indicate that the corresponding nodes were also recovered using maximum-likelihood tree-making algorithms

chain with smooth surface, an abundant well developed aerial and vegetative hyphae (Fig. S2a, S2b and S3c). These morphological characteristics are consistent with assignment of the strain to the genus *Streptomyces* (Williams et al. 1989). Growth was found to occur at pH 5.0-8.0 (optimum pH 6.0-7.0), with 0–4 % NaCl tolerance (optimum 0–4 %) and at 24–40 °C (optimum 28–36 °C). Cells were found to be catalase positive but negative for haemolytic activity and melanoid pigment production. Strain MUSC 119^T was positive for hydrolysis of soluble starch and carboxymethylcellulose; but negative for hydrolysis of tributyrin (lipase), casein, chitin and xylan. Strain MUSC 119^T could be differentiated from closely related members of the genus *Streptomyces* member based on a range of phenotypic properties (Table 1).

The almost-complete 16S rRNA gene sequence was determined for strain MUSC 119^T (1488 bp). Phylogenetic trees were reconstructed based on 16S rRNA gene sequences to determine the phylogenetic position of this strain (Fig. 1; Fig S1). Phylogenetic analysis revealed strain MUSC 119^T to be closely related to the type strains *S. rhizophilus* JR-41^T, *S. gramineus* JR-43^T, *S. graminisoli* JR-19^T, *S. shenzhenensis* 172115^T and *Streptomyces panaciradicis* 1MR-8^T (Fig. 1). This association was also supported in the phylogenetic tree reconstructed using the maximum-likelihood algorithm (Fig. S1). Strain MUSC 119^T shows high 16S rRNA gene sequence similarity to *S. rhizophilus* JR-41^T (99.0 %), which corresponds to 15 nt differences at 1457 locations with gaps, followed by *S. panaciradicis* 1MR-8^T (98.9 %), *S. gramineus* JR-43^T (98.8 %), *Streptomyces graminisoli* JR-19^T (98.7 %), *S. capoamus* JCM 4734^T (98.6 %), *S. bungoensis* NBRC 15711^T (98.6 %) and *Streptomyces longwoodensis* LMG 20096^T (98.6 %).

The DNA–DNA relatedness values between strain MUSC 119^T and *S. rhizophilus* NBRC 108885^T (27.5 ± 0.7 %), *S. panaciradicis* NBRC 109811^T (9.8 ± 0.2 %), *S. gramineus* NBRC 107863^T (20.3 ± 0.7 %), *S. graminisoli* NBRC 108883^T (14.5 ± 1.3 %), *S. capoamus* NBRC 13411^T (19.0 ± 0.5 %), *S. bungoensis* NBRC 15711^T (15.6 ± 3.2 %) and *S. longwoodensis* JCM 4976^T (15.3 ± 3.9 %) were significantly below 70 %, the threshold value for the delineation of genomic

species (Wayne et al. 1987). These results support the conclusion that strain MUSC 119^T represents a novel *Streptomyces* species. Also BOX-PCR fingerprinting results (Fig. S3) of strain MUSC119^T exhibited a unique DNA profile compare to related type strains.

Chemotaxonomic analyses showed that the cell wall of strain MUSC 119^T is of cell wall type I (Lechevalier and Lechevalier 1970) as it contains LL-di-diaminopimelic. The presence of LL-di-di-aminopimelic has been observed in all described species of the genus *Streptomyces* (Xu et al. 2009; Hu et al. 2012; Lee et al. 2014b; Zhang et al. 2014). The cell wall sugars detected were glucose, mannose, ribose and rhamnose. Glucose and ribose were detected in other members of the genus *Streptomyces* such as *S. rhizophilus* JR-41^T and *S. graminisoli* JR-19^T (Lee and Whang 2014), *S. gramineus* JR-43^T (Lee et al. 2012), *Streptomyces shenzhenensis* 172115^T (Hu et al. 2011) and *S. pluripotens* (Lee et al. 2014b).

The fatty acids profiles of strain MUSC 119^T and the closely related type strains are given in Table S1. The major cellular fatty acids of strain MUSC 119^T were anteiso-$C_{15:0}$ (25.1 %), iso-$C_{16:0}$ (24.5 %) and anteiso-$C_{17:0}$ (12.8 %), $C_{16:0}$ (8.1 %), iso-$C_{15:0}$ (7.2 %) and trace amounts of $C_{15:0}$, iso-$C_{16:1}$ H, anteiso-$C_{17:1}$w9c and iso-$C_{17:0}$ (Table S1). The fatty acids profile of MUSC 119^T was consistent with those of closely related type strains, which also contained the fatty acids anteiso-$C_{15:0}$ (21.4–28.0 %), iso-$C_{16:0}$ (15.4–24.5 %) and anteiso-$C_{17:0}$ (9.6–16.7 %) (Table S1). However, the fatty acids profile of strain MUSC 119^T was quantitatively different from those of these type strains; for example, though fatty acid iso-$C_{16:0}$ (24.5 %) was found to be predominant in strain MUSC 119^T, the amount of iso-$C_{16:0}$ was notably lower (15.4 %) in *S. rhizophilus* NBRC 108885^T (Table 1 and S1).

Strains MUSC 119^T contained MK-9(H_8) (51 %) and MK-9(H_6) (37 %) as predominant menaquinones. Minor amounts of MK-9(H_4) (6 %), MK-10(H_2) (2 %) and MK-10 (1 %) were also detected. This menaquinone composition is in agreement with other reports that the predominant menaquinones of members of the genus *Streptomyces* are MK-9(H_6) and MK-9(H_8) (Lee et al. 2014b; Ser et al. 2015a, b). The polar lipids consisted of phosphatidylinositol, phosphatidylethanolamine, glycolipid, diphosphatidylglycerol and four unidentified phospholipids. Differences in polar lipids indicated that MUSC 119^T is distinct from related type strains; for example, strain MUSC 119^T (Fig. S4a) was found to contain glycolipid that was not detected in *S. rhizophilus* NBRC 108885^T (Fig. S4b) and *S. gramineus* NBRC 107863^T (Fig. S4c). Also *S. rhizophilus* NBRC 108885^T contains phosphoglycolipid and phosphatidylglycerol that was not detected in strain MUSC 119^T.

The DNA G+C content of strain MUSC 119^T was found to be 72.6 mol %. Based on its distinct phylogenetic position, together with genomic, chemotaxonomic and phenotypic characteristics, strain MUSC 119^T should be classified as representing a novel species of the genus *Streptomyces*, for which the name *Streptomyces humi* sp. nov. is proposed.

Description of *Streptomyces humi* sp. nov.

Streptomyces humi (hu'mi. L. gen. n. humi of soil, ground)

Cells form grayish yellow soluble pigment, light brownish gray aerial and grayish yellowish brown substrate mycelium on ISP 2 medium. Cells are Gram-positive. Good growth is observed on ISP 2, ISP 3, ISP 5, ISP 6, ISP 7 media (agar form), *Streptomyces*, starch casein, Luria–Bertani, tryptic soy and nutrient agars after 1–2 weeks at 28 °C, while cells grow moderately on actinomycetes isolation agar, whereas cells do not grow on ISP 4 medium. The colours of the aerial and substrate mycelium are media-dependent (Table S2). Cells grow at pH 5.0–8.0 (optimum pH 6.0–7.0), with 0–4 % NaCl tolerance (optimum 0–4 %) and at 24–40 °C (optimum 28–36 °C). Positive for catalase but negative for haemolytic activity and melanoid pigment production. Positive for hydrolysis of soluble starch and carboxymethylcellulose, but negative for hydrolysis of tributyrin (lipase), casein, chitin and xylan. The peptidoglycan contains LL-di-di-aminopimelic acid as the diagnostic diamino acid. The predominant menaquinones are MK-9(H_8), MK-9(H_6) and MK-9(H_4). The polar lipids are four unidentified phospholipids, phosphatidylinositol, phosphatidylethanolamine, glycolipid and diphosphatidylglycerol. The major cellular fatty acids are anteiso-$C_{15:0}$, iso-$C_{16:0}$, and anteiso-$C_{17:0}$. The cell wall sugars are glucose, mannose, ribose and rhamnose. The G+C content of the genomic DNA of the type strain is 72.6 mol %.

The type strain is MUSC 119^T (=DSM 42174^T = MCCC 1K00505^T), which was isolated from a mangrove soil collected from the Tanjung Lumpur Mangrove forest located in state of Pahang, Peninsular of Malaysia. The 16S rRNA gene sequence of strain MUSC 119^T has been deposited in GenBank/EMBL/DDBJ under the accession number KJ632661.

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