Actinomadura sediminis sp. nov., a marine actinomycete isolated from mangrove sediment

Jie He,^{1†} Ying Xu,^{1†} Maloy Kumar Sahu,² Xin-Peng Tian,³ Guo-Xing Nie,⁴ Qiong Xie,⁵ Si Zhang,³ Kannan Sivakumar² and Wen-Jun Li^{1,6}

Correspondence

Wen-Jun Li

wjli@ynu.edu.cn

Kannan Sivakumar

oceanactino@gmail.com

¹Key Laboratory of Microbial Diversity in Southwest China, Ministry of Education and Laboratory for Conservation and Utilization of Bio-Resources, Yunnan Institute of Microbiology, Yunnan University, Kunming 650091, PR China

²Centre of Advanced Study in Marine Biology, Faculty of Marine Science, Annamalai University, Parangipettai 608 502, Tamilnadu, India

³CAS Key Laboratory of Marine Bio-Resources Sustainable Utilization, RNAM Center for Marine Microbiology, CAS Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, PR China

⁴College of Life Science, Henan Normal University, Xinxiang 453007, PR China

⁵State Key Laboratory of Space Medicine Fundamentals and Application, China Astronaut Research and Training Center, Beijing 100094, PR China

⁶Key Laboratory of Biogeography and Bioresource in Arid Land, Xingjiang Institute of Ecology and Geography, Chinese Academy Of Sciences, Ürmqi 830011, PR China

In this study, the taxonomic position of an actinobacterium, strain YIM M 10931^T, which was isolated from a mangrove sediment sample collected in Dugong Creek, Little Andaman, India, was determined by a polyphasic approach. This Gram-positive, aerobic strain produced branched substrate mycelium and aerial hyphae, which differentiated into short, hooked or spiral spore chains. The organism contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. The whole cell sugars consisted of mannose, ribose, glucose, galactose and madurose. The cellular fatty acid profile mainly consisted of iso-C_{16:0}, 10-methyl C_{18:0} and C_{16:0}. The quinone system was predominantly composed of MK-9(H₈) (45.5 %) and MK-9(H₆) (39 %). The phospholipids detected were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol mannoside, phosphatidylinositol and two unknown phospholipids. The organism showed a combination of morphological and chemotaxonomic properties typical of members of the genus *Actinomadura*. Moreover, phylogenetic analysis based on a 16S rRNA gene sequence generated from the strain identified its closest relatives as *Actinomadura cremea* DSM 43676^T (98.4 % sequence similarity), *Actinomadura rifamycin* DSM 43936^T (97.4 %) and *Actinomadura apis* IM17-1^T (96.9 %). It was obvious from the resulting phylogenetic trees that strain YIM M 10931^T belongs to a distinct subclade within the evolutionary radiation of the genus *Actinomadura*. DNA–DNA hybridizations of strain YIM M 10931^T with *A. cremea* DSM 43676^T and *A. rifamycin* DSM 43936^T were performed and further confirmed that the isolate represents a separate genomic species. Based on the phenotypic and genotypic characteristics presented, it is proposed that strain YIM M 10931^T represents a novel species within the genus *Actinomadura*, for which the name *Actinomadura sediminis* sp. nov. is proposed; the type strain is YIM M 10931^T (=CCTCC AA 2010009^T=DSM 45500^T).

†These authors contributed equally to this work.

Abbreviations: DAP, diaminopimelic acid; DPG, diphosphatidylglycerol; PI, phosphatidylinositol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM M 10931^T is JF272484.

Two supplementary figures and a supplementary table are available with the online version of this paper.

The mangrove, a typical ecosystem in tropical coastal biomes, is located at the transition area between the land and the sea (Holguin *et al.*, 2001; Kathiresan & Bingham, 2001), which plays a very important role as refuge, feeding, and breeding areas for many organisms and sustains an extensive food web based on detritus. It is distinguished from other ecosystems due to periodic tidal flooding and

variable environmental factors, such as salinity and nutrient availability (Alongi, 1988; Holguin *et al.*, 2006). Mangroves make up approximately 60–75 % of all biotic communities in the world's tropical and subtropical coastlines (Holguin *et al.*, 2001). In these ecosystems, the microbial community diversity has been poorly researched, compared with the well-documented species diversity of larger animals and plants (Xu *et al.*, 2009).

The genus *Actinomadura* was established by Lechevalier & Lechevalier (1968) and was placed in the family *Thermomonosporaceae*, which, at the time of writing, contained four other genera, *Actinocorallia*, *Spirillospora*, *Thermomonospora* and *Actinoallomurus* (Zhang *et al.*, 1998; Trujillo & Goodfellow, 2003; Tamura *et al.*, 2009). At present, the genus *Actinomadura* comprises 44 recognized species, including the newly proposed species, *Actinomadura apis* and *Actinomadura rifamycinii* (Promnuan *et al.*, 2011), and *Actinomadura scrupuli* (Lee & Lee, 2010). The emended genus *Actinomadura* represents aerobic, Gram-positive, non-acid-fast, non-motile actinomycetes that generate abundantly branched, non-fragmenting substrate mycelia and aerial hyphae that differentiate into various spore chain morphologies. These spore chains are short to long, straight, hooked or spiral (1–4 turns). Members of the genus *Actinomadura* are characterized by: the presence of meso-diaminopimelic acid (DAP) and madurose in the cell wall; MK-9(H₄), MK-9(H₆) and MK-9(H₈) as the predominant menaquinones; hexadecanoic, 14-methylpentadecanoic and 10-methyloctadecanoic acids as the main fatty acids; and diphosphatidylglycerol (DPG) and phosphatidylinositol (PI) as major phospholipids (Kroppenstedt *et al.*, 1990).

Strain YIM M 10931^T was isolated from a mangrove sediment sample collected from Dugong Creek, Little Andaman, India (10° 48' 43.1" N 92° 34' 59.9" E). Sediment (1 g) was added to 9 ml sterile distilled water and mixed by vortexing. A 10-fold dilution of this soil suspension was prepared in sterilized distilled water and 0.1 ml was spread on Kuster's agar medium (glycerol, 10 g; casein, 0.3 g; KNO₃, 2 g; NaCl, 20 g; K₂HPO₄, 2 g; MgSO₄·7H₂O, 0.05 g; CaCO₃, 0.02 g; FeSO₄·7H₂O, 0.01 g; agar, 15 g; pH 7.0 ± 0.1; distilled water, 1000 ml) and incubated at 28 °C for 21 days. The strain was cultivated and maintained on modified 2216 agar medium containing (l⁻¹): peptone, 1 g; yeast extract, 1 g; FeSO₄·7H₂O, 0.001 g; (NH₄)₂SO₄, 4 g; CaCO₃, 0.1 g; agar, 20 g (pH 7.2). *Actinomadura crenea* DSM 43676^T and *A. rifamycinii* DSM 43936^T were used as reference strains and cultivated under the same conditions.

Strain YIM M 10931^T and other type strains were cultured for 7, 10, 14 and 21 days at 28 °C on modified 2216 agar medium and marine agar medium [NaCl, 19.45 g; MgCl₂·6H₂O, 5.9 g; Na₂SO₄, 3.24 g; CaCl₂, 1.9 g; peptone, 5 g; yeast extract, 1 g; 2 ml solution A containing (l⁻¹ distilled water): ferric citrate, 5 g; KCl, 4 g; SeF, 1.7 g; H₃BO₃, 1.1 g; 1 ml solution B containing (l⁻¹ distilled water): Na₂SiO₃, 4 g; NaF, 1.6 g; NaNO₃, 8 g; Na₂HPO₄, 8 g; and distilled water, 1000 ml] using the coverslip technique of Kawato & Shinobu

(1959). Morphological properties were observed by using a light microscope (BH-2; Olympus) and a scanning electron microscope (Philips XL30; ESEM-TMP).

Cultural characteristics were determined on various agar media after 30 days at 28 °C, according to the methods described by Shirling & Gottlieb (1966). Colours of the aerial and substrate mycelia were determined by using colour chips from the ISCC–NBS colour charts (standard sample no. 2106; Kelly, 1964). Carbon-source utilization tests were performed according to the methods of Shirling & Gottlieb (1966) and Athalye *et al.* (1985) using the basal medium recommended by Pridham & Gottlieb (1948). Growth at various temperatures, pH values and NaCl concentrations was assessed using ISP 2 agar medium at 28 °C for 3 weeks. Degradation of cellulose, gelatin, urea, and Tweens 20, 40 and 80 was determined as described by Athalye *et al.* (1985). Antibiotic susceptibility was determined on ISP 2 agar medium incubated at 28 °C for 7 days by the disc diffusion plate method (Bauer *et al.*, 1966); each antibiotic was tested at 10 µg per disc. Catalase activity was tested using 10 % (v/v) H₂O₂ according the standard method. Other phenotypic characteristics were tested by using standard procedures (Goodfellow, 1971; Athalye *et al.*, 1985).

Biomass for the study of chemotaxonomic characteristics was prepared by cultivation in tryptic soy broth (Difco) in shaken flasks at 170 r.p.m. at 28 °C for 7 days, harvested by centrifugation at 4500 r.p.m. (for 10 min), washed twice with distilled water and then freeze-dried. The DAP isomer was identified by using TLC as described by Stanek & Roberts (1974). The whole cell sugars were separated by HPLC after precolumn derivatization with 1-phenyl-3-methyl-5-pyrazolone (Tang *et al.*, 2009). Phospholipids were determined according to published procedures (Minnikin *et al.*, 1979; Collins & Jones, 1980). Menaquinones were extracted (Collins *et al.*, 1977) and detected by HPLC (Tamaoka *et al.*, 1983). Cellular fatty acids were extracted, methylated and analysed by using the Sherlock Microbial Identification System (MIDI) according to the method of Sasser (1990) and the manufacturer's instructions. Fatty acid methyl esters were then analysed by GC (Agilent Technologies 7890A GC System) by using the Microbial Identification software package (Sherlock Version 6.1; MIDI database TSBA6).

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were done as described by Li *et al.* (2007). Multiple alignments with sequences of the most closely related taxa and calculations of levels of sequence similarity were carried out using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic analyses were performed using three tree-making algorithms, namely, the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A phylogenetic tree and distance matrix were reconstructed using the neighbour-joining method of Saitou & Nei (1987) from *K*_{nuc} values (Kimura, 1980, 1983) using MEGA version 4.0 (Tamura *et al.*, 2007). Topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

Chromosomal DNA for determination of the G + C content was prepared by following the method of Marmur (1961). The G + C content of the genomic DNA was determined by the HPLC method according to Mesbah *et al.* (1989). DNA–DNA hybridization was performed fluorometrically by the methods of Ezaki *et al.* (1989) and He *et al.* (2005) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample and the highest and lowest values in each sample were excluded. The DNA–DNA relatedness values are expressed as the means of the remaining three values.

Strain YIM M 10931^T produced abundantly branched, non-fragmenting substrate mycelium and aerial hyphae, which differentiated into spore chains. Short spore chains were hooked or spiral (1–3 turns) and were composed of 4 to 6 spores, whose surfaces were smooth (Fig. S1, available in IJSEM Online). The aerial mycelium was abundant and white to pale yellow–pink in colour on most media tested. The substrate mycelium was white to grey–brown and produced a deep brown pigment on some media (Table S1, available in IJSEM Online). Strain YIM M 10931^T grew well at pH 7–8 and in up to 7% (w/v) NaCl. The optimum growth temperature was 37 °C. Strain YIM M 10931^T and two reference type strains (*A. cremea* DSM 43676^T and *A. rifamycinii* DSM 43936^T) were positive for utilization of mannitol and negative for utilization of dulcitol, glycerol, lactose, D-mannose, maltose, sodium oxalate, sorbitol, trehalose and xylitol as sole carbon sources. The three strains were positive for utilization of L-alanine, L-arginine, L-glutamic acid, glycine, L-histidine, L-lysine, L-phenylalanine, L-proline, L-serine, L-tyrosine and L-valine as sole nitrogen sources. They were positive for catalase activity and nitrate reduction and negative for degradation of cellulose. However, the novel strain differed from these two reference type strains in some phenotypic characteristics (Table 1).

Strain YIM M 10931^T exhibited chemical markers that are typical of members of the genus *Actinomadura*, i.e. it contained: *meso*-DAP as the cell wall diamino acid; galactose, glucose, madurose, mannose and ribose as whole cell sugars; DPG and PI as major polar lipids (Fig. S2, available in IJSEM Online); MK-9(H₈) and MK-9(H₆) as the predominant menaquinones; and iso-C_{16:0}, 10-methyl C_{18:0} and C_{16:0} as the major fatty acids.

An almost complete 16S rRNA gene sequence (1537 nt) was generated for isolate YIM M 10931^T. Comparative 16S rRNA gene sequence analysis showed that strain YIM M 10931^T was phylogenetically related to members of the genus *Actinomadura* (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strain YIM M 10931^T clustered with the type strains of *A. cremea*, *A. rifamycinii* and *A. apis*. This relationship was supported by all tree-making methods used in this study (data not shown). The similarity values of strain YIM M 10931^T with *A. cremea* DSM 43676^T, *A. rifamycinii* DSM 43936^T and *A. apis* IM17-1^T were 98.4, 97.4 and 96.9%, respectively. The results of the

16S rRNA gene sequence comparisons further demonstrated that strain YIM M 10931^T is a member of the genus *Actinomadura*.

The genomic DNA G + C content of strain YIM M 10931^T was 69.7 mol%. The levels of DNA–DNA relatedness of strain YIM M 10931^T with *A. cremea* DSM 43676^T and *A. rifamycinii* DSM 43936^T were 49.1% and 50.8% (standard deviations were 2.9% and 3.3%, respectively). These values are well below the 70% cut-off point recommended for the delineation of genomic species (Wayne *et al.*, 1987).

Strain YIM M 10931^T should be placed in the genus *Actinomadura* based on its morphological characteristics, chemotaxonomic data and relatively high 16S rRNA gene sequence similarities with other members of the genus. However, the isolate can be distinguished from its nearest phylogenetic neighbours, *A. cremea* DSM 43676^T and *A. rifamycinii* DSM 43936^T, on the basis of cultural characteristics (data not shown), physiological properties (Table 1) and low DNA–DNA hybridization values. Therefore, the results of the polyphasic taxonomic study presented here allow us to assign the isolate to a novel species, for which we propose the name *Actinomadura sediminis* sp. nov.

Description of *Actinomadura sediminis* sp. nov.

Actinomadura sediminis (se.di'mi.nis. L. gen. n. *sediminis* of sediment).

Aerobic, Gram-positive. Forms abundantly branched substrate mycelium; aerial hyphae differentiate into hooked or spiral spore chains. Spore surface is smooth. Aerial mycelia are white to pale yellow–pink on most media, but grey–brown on potato–glucose agar; substrate mycelia are white to grey–brown and produce a deep brown diffusible pigment on ISP 2 and nutrient agar. Growth occurs at 28–45 °C and pH 7.0–8.0. Tolerates up to 7% (w/v) NaCl. Urea is hydrolysed, but cellulose, gelatin, starch, and Tweens 20, 40 and 80 are not. Negative for milk coagulation and peptonization. Positive for catalase, oxidase, H₂S production and nitrate reduction. Utilizes L-arabinose, D-fructose, D-galactose, mannitol, L-rhamnose, ribose and D-xylose as sole carbon sources. Dulcitol, cellobiose, glucose, inositol, lactose, mannose, maltose, raffinose, sodium citrate, sodium oxalate, sorbitol, sucrose, trehalose and xylitol are not utilized. L-Alanine, L-arginine, L-asparagine, L-glutamic acid, glycine, L-histidine, L-hydroxyproline, hypoxanthine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-valine and xanthine are used as sole nitrogen sources. The cell wall contains *meso*-DAP and the whole cell sugars are mannose, ribose, glucose, galactose and madurose. The major phospholipids are DPG, phosphatidylglycerol, PI, phosphatidylinositol mannosides and unknown phospholipids. MK-9(H₈) and MK-9(H₆) are the major components of the quinone system, with minor amounts of MK-9(H₄) and MK-9(H₁₀). The major fatty acids (>5%) are C_{16:0}, 10-methyl C_{18:0}, iso-C_{16:0}, C_{18:1}ω9c and C_{18:0}.

Table 1. Differential characteristics of strain YIM M 10931^T, *A. cremea* DSM 43676^T and *A. rifamycin* DSM 43936^T

Strains: 1, *A. sediminis* sp. nov. YIM M 10931^T; 2, *A. cremea* DSM 43676^T; 3, *A. rifamycin* DSM 43936^T. Data are from this study except where marked.

Characteristic	1	2	3
pH range	7–8	6–8	5–7
Temperature range (°C)	28–45	28–37	28–45
Milk coagulation	–	+	–
Milk peptonization	–	+	–
H ₂ S production	+	–	–
Degradation of:			
Gelatin	–	+	–
Starch	–	+	–
Urea	+	+	–
Tween 20	–	+	+
Tween 40	–	+	+
Tween 80	–	+	+
Sensitivity to antibiotics:			
Erythromycin	–	+	–
Norfloxacin	–	–	+
Gentamicin	–	–	+
Ampicillin	+	–	+
Vancomycin	–	–	+
Chloramphenicol	–	+	–
Novobiocin	–	–	+
Rifampicin	–	+	–
Utilization as sole carbon source:			
L-Arabinose	+	+	–
Cellobiose	–	+	–
D-Fructose	+	+	–
D-Galactose	+	–	–
Glucose	–	+	–
Inositol	–	+	–
Raffinose	–	+	–
L-Rhamnose	+	+	–
D-Ribose	+	+	–
Sucrose	–	+	–
Sodium citrate	–	–	+
D-Xylose	+	+	–
Utilization as sole nitrogen source:			
L-Hydroxyproline	+	–	+
Xanthine	+	–	+
L-Threonine	+	–	+
Major (>5%) fatty acids (%)	C _{16:0} (28.05), 10-methyl C _{18:0} (20.75), iso-C _{16:0} (16.18), C _{18:1ω9c} (9.03), C _{18:0} (8.20)	C _{16:0} (41.2), C _{18:1ω9c} (14.6), 10-methyl C _{18:0} (13.4)	C _{16:0} (40.9), C _{18:1ω9c} (25.6), 10-methyl C _{18:0} (9.4)
Menaquinones (%)	MK-9(H ₈) (45.5), MK-9(H ₆) (39.0), MK-9(H ₁₀) (5.6)	MK-9(H ₈) (6), MK-9(H ₆) (83), MK-9(H ₄) (11)*	MK-9(H ₈) (48.1), MK-9(H ₆) (44.4)
Whole cell sugars†	Man, Rib, Glc, Gal, Mad	Man, Rib, Glc, Mad*	Man, Rib, Glc, Gal, Mad, Ara*
DNA G + C content (mol%)	69.7	71.1*	73.4*

*Data from Kroppenstedt *et al.* (1990).

†Man, Mannose; Rib, ribose; Glc, glucose; Gal, galactose; Mad, madurose; Ara, arabinose.

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on nearly complete 16S rRNA gene sequences (1500 bp length) showing relationships between strain YIM M 10931^T and other members of the genus *Actinomadura*. Asterisks indicate branches of the tree that were also found using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) tree-making algorithms. Numbers on branch nodes are bootstrap values (1000 resamplings; only values over 50% are given). The sequence of *Streptosporangium album* DSM 43023^T was used as the outgroup. Bar, 0.005 substitutions per nucleotide position.

The type strain, YIM M 10931^T (=CCTCC AA 2010009^T =DSM 45500^T), was isolated from a mangrove sediment collected from Dugong Creek, Little Andaman, India. The genomic DNA G+C content of the type strain is 69.7 mol%.

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