Micromonospora yangpuensis sp. nov., isolated from a sponge

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An actinomycete, strain FXJ6.011^T, was isolated from a cup-shaped sponge collected at Dachan reef, Yangpu in the South China Sea. The strain had morphological characteristics of members of the family *Micromonosporaceae*. Phylogenetic analysis of the 16S rRNA gene sequence of strain FXJ6.011^T indicated the highest similarity (98.7%) to *Micromonospora auratinigra* JCM 12357^T, *Micromonospora chaiyaphumensis* JCM 12873^T and *Micromonospora echinofusca* JCM 3327^T. Analysis of the *gyrB* gene sequence also showed that strain FXJ6.011^T should be assigned to the genus *Micromonospora*. It contained DD-diaminopimelic acid as the major cell-wall diamino acid and MK-10(H₂) as predominant menaquinone. The polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidyl mannosides and phosphatidylinositol dimannoside. The major cellular fatty acids were iso-C_{16:0}, C_{17:1ω8c} and C_{16:0}. Physiological and biochemical data and low DNA–DNA relatedness values enabled the strain to be differentiated from members of closely related species. Based on phenotypic and genotypic data, strain FXJ6.011^T represents a novel *Micromonospora* species, for which the name *Micromonospora yangpuensis* sp. nov. is proposed; the type strain is FXJ6.011^T (=CGMCC 4.5736^T=NBRC 107727^T).

Micromonospora, proposed by Ørskov (1923), is the type genus of the family *Micromonosporaceae*. Members of this genus typically exhibit single, non-motile spores on the substrate mycelium and lack aerial mycelium. The spore surface is smooth or ridged. Since Kasai *et al.* (2000) proposed to reclassify the *Micromonospora* species into 14 species based on the *gyrB* gene and DNA–DNA relatedness, a number of novel *Micromonospora* species have been described in the last decade (Hirsch *et al.*, 2004; Thawai *et al.*, 2004, 2005a, b, 2007, 2008; Kroppenstedt *et al.*, 2005; Trujillo *et al.*, 2005, 2006b, 2007; Ara & Kudo, 2007; Jongrungruangchok *et al.*, 2008a, b; Huang *et al.*, 2008; Garcia *et al.*, 2010; Kirby & Meyers, 2010; Songsumanus *et al.*, 2011; Tanasupawat *et al.*, 2010; Wang *et al.*, 2010). At

present, the genus *Micromonospora* contains 44 species and is probably the second richest producer of antibiotics in the order *Actinomycetales* (Bérdy, 2005). During an investigation of unusual actinomycetes in symbiosis with sponges, hundreds of *Micromonospora*-like strains were isolated. The aim of this study was to clarify the taxonomic position of one novel isolate, strain FXJ6.011^T, using a polyphasic approach.

Strain FXJ6.011^T was isolated from a cup-shaped sponge sample collected from Dachan reef at Yangpu in the South China Sea. The sample was homogenized and diluted in series with sterile artificial seawater [artificial sea salt (Instant Ocean), 33.4 g; distilled water, 1 l] to 10^{−3} and then spread onto plates of SMP agar (0.5 g mannitol, 0.1 g peptone, 1000 ml artificial seawater, 15 g agar; pH 7.2) supplemented with nalidixic acid (25 mg l^{−1}), cycloheximide (50 mg l^{−1}) and nystatin (25 mg l^{−1}). After 21 days of aerobic incubation at 28 °C, the isolate was transferred and purified on yeast extract-malt extract agar [International *Streptomyces* Project (ISP) medium 2; Shirling & Gottlieb, 1966].

The morphology of strain FXJ6.011^T was assessed as described by Shirling & Gottlieb (1966) using 21-day-old cultures grown on ISP 2. Spore-surface ornamentation and sporophore morphology were observed by using light microscopy (Zeiss Axioskop) and scanning electron microscopy (FEI Quanta 200). Cultural characteristics were determined using cultures grown at 28 °C for 14 days on

Abbreviations: DPG, diphosphatidylglycerol; ISP, International *Streptomyces* Project; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIMs, phosphatidyl mannosides; PIDM, phosphatidylinositol dimannoside; PL, unidentified phospholipids.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strain FXJ6.011^T and those for the *gyrB* gene sequences of strain FXJ6.011^T, *Micromonospora auratinigra* JCM 12357^T, *Micromonospora chaiyaphumensis* JCM 12873^T, *Micromonospora chokoriensis* JCM 13247^T, *Micromonospora echinofusca* JCM 3327^T, *Micromonospora marina* JCM 12870^T and *Micromonospora tulbaghia* NRRL B-24576^T are GU002071, HM061609, HQ231756, HQ231755, JF272469, HQ231754, JF272468 and JF272467, respectively.

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

Fig. 1. Scanning electron micrographs of strain FXJ6.011^T after growth on ISP 2 agar at 28 °C for 14 days. Bars, 2.0 μm.

ISP 2, oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract-iron agar (ISP 6), tyrosine agar (ISP 7), nutrient agar (Waksman, 1967), Bennett's agar (Jones, 1949) and Czapek agar (Waksman, 1967). Strain FXJ6.011^T produced abundant and branched substrate hyphae on ISP 2 medium, but no aerial hyphae. Ovate spores (0.6–0.7 μm in diameter) were singly borne from a short sporophore on substrate hyphae and the spore surface appeared warty (Fig. 1). The strain grew well on ISP 2, Bennett's agar and Czapek agar, and produced brown, soluble pigments on Bennett's agar. Detailed results are shown in Table 1.

Physiological and biochemical characteristics of strain FXJ6.011^T and related strains were determined according to the methods of Shirling & Gottlieb (1966) and Williams *et al.* (1989). The ranges of temperature (4, 10, 15, 20, 25, 30, 37, 40, 45, 50 and 55 °C), pH (4–11, at intervals of 1 pH unit) and NaCl tolerance [0–10 % (w/v), at intervals of 0.5 %] for growth were determined on ISP 2 agar medium. KH₂PO₄/HCl, KH₂PO₄/K₂HPO₄ and K₂HPO₄/NaOH buffer systems were used to maintain the pH values of the medium. Carbon source utilization was tested on basal medium (Shirling & Gottlieb, 1966); each sterile carbon source was added to the medium aseptically to give a concentration of approximately 1 %. Utilization of amino acids as nitrogen sources was tested according to Williams *et al.* (1989). Hydrogen sulfide

production and melanoid pigment production was determined on ISP 6 and ISP 7 media, respectively. Milk peptonization and gelatin liquefaction were tested as described by Arai (1975) and Williams & Cross (1971), respectively. Catalase and oxidase activities were determined according to the method of Trujillo *et al.* (2006a).

For chemotaxonomic analyses, the strain was cultivated in ISP 2 broth for 14 days at 30 °C on rotary shakers to prepare biomass. The isomeric form of diaminopimelic acid and diagnostic sugars in whole-cell hydrolysates were analysed following the procedure of Lechevalier & Lechevalier (1980). Sugars in whole-cell hydrolysates were concentrated, dissolved in 0.2 ml sterile distilled water and subsequently analysed by TLC on microcrystalline cellulose plates (Wang, 1986) using the solvent system n-butyl alcohol/pyridine/methylbenzene/water (10:6:1:6, by vol.). Fatty acids were extracted, methylated and tested using the standard MIDI (Microbial Identification, TSBA6) procedure (Sasser, 1990). Menaquinones were extracted according to the methods of Collins (1985) and Minnikin *et al.* (1984) and were analysed by HPLC with a Cosmosil 5C₁₈ column (4.6 × 250 mm; Thermo), using acetonitrile/2-propanol (3:2, v/v) as the mobile phase. Phospholipids were extracted and identified by two-dimensional TLC (silica gel 60; 10 × 10 cm) as described by Minnikin *et al.* (1984).

Chromosomal DNA for determination of G + C content was extracted using the procedures described by Marmur (1961). The DNA G + C content was determined using the thermal denaturation (*T_m*) method (Mandel & Marmur, 1968) and a Beckman DU-800 spectrophotometer. The modified fluorescent microplate method of Rong & Huang (2010) was used to determine DNA–DNA relatedness values between strain FXJ6.011^T and related strains.

Extraction of genomic DNA for PCR amplification of the 16S rRNA and gyrase B subunit (*gyrB*) genes was performed as described by Kutchma *et al.* (1998). The 16S rRNA gene sequence of the strain was aligned with corresponding sequences of representatives of the genus *Micromonospora* retrieved from GenBank using the program CLUSTAL_X 1.8 (Thompson *et al.*, 1997). MEGA version 4.0 (Tamura *et al.*,

Table 1. Cultural characteristics of strain FXJ6.011^T

Medium	Growth	Colour of colony*	Soluble pigment
Yeast extract-malt extract agar (ISP 2)	Abundant	Apricot orange	None
Oatmeal agar (ISP 3)	Moderate	Amber brown	None
Inorganic salts-starch agar (ISP 4)	Moderate	Brussels brown	None
Glycerol-asparagine agar (ISP 5)	Moderate	Apricot orange	None
Peptone-yeast extract-iron agar (ISP 6)	Poor	Mars yellow	None
Tyrosine agar (ISP 7)	Poor	Argus brown	None
Bennett's agar	Abundant	Apricot orange	Brown
Czapek agar	Abundant	Brussels brown	None
Nutrient agar	Poor	Amber brown	None

*Color Standards and Color Nomenclature (Ridgway, 1912) was used to determine colony colours.

2007) was used for both multiple alignment and for phylogenetic tree construction using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) tree-making algorithms. Evolutionary distance matrices were calculated according to the algorithm of the neighbour-joining method. The robustness of the tree

topology was evaluated by bootstrap analysis (Felsenstein, 1993) based on 1000 resamplings. The EzTaxon server was used to calculate 16S rRNA gene sequence similarities (<http://www.eztaxon.org/>; Chun *et al.*, 2007). The *gyrB* gene sequence was amplified to obtain two overlapping fragments (a final composite sequence of about 1120 bp) using primers

Fig. 2. Neighbour-joining tree based on almost complete 16S rRNA gene sequences, showing the relationship between strain FXJ6.011^T and members of all species of the genus *Micromonospora*. Numbers at branch nodes are bootstrap values (%) from 1000 resamplings; only values over 40 % are given. Filled circles indicate branches that were also recovered by the maximum-parsimony method. Bar, 0.005 substitutions per nucleotide position.

GYF1/GYR1B and GYF3/GYR3B based on the method of Garcia *et al.* (2010). Each PCR mixture (50 µl final volume) contained 5 µl 10 × PCR buffer (with Mg²⁺), 1 µl dNTP (10 mM), 1 µl each primer (GYF1/GYR1B, GYF3/GYR3B, 20 µM), 3 µl DMSO, 2.5 U *Taq* DNA polymerase and 100 ng template DNA. Phylogenetic analysis of *gyrB* gene sequences was performed as described above.

An almost complete 16S rRNA gene sequence (1477 bp) for strain FXJ6.011^T was obtained. A preliminary comparison of the sequence with those in GenBank indicated that strain FXJ6.011^T belonged to the genus *Micromonospora*. It was evident from the phylogenetic analysis that the strain formed a distinct phyletic line loosely related to recognized *Micromonospora* species (Fig. 2). The closest relatives of strain FXJ6.011^T were *Micromonospora auratinigra* JCM 12357^T, *Micromonospora chaiyaphumensis* JCM 12873^T and

Micromonospora echinofusca JCM 3327^T, which all shared 98.7 % gene sequence similarity with the isolate.

To further clarify the affiliation of strain FXJ6.011^T to its closest relatives, the *gyrB* gene sequences for strain FXJ6.011^T (1118 bp), *M. auratinigra* JCM 12357^T (1121 bp), *M. chaiyaphumensis* JCM 12873^T (1123 bp) and *M. echinofusca* JCM 3327^T (1123 bp) were obtained and compared. The sequence distances between strain FXJ6.011^T and the above three type strains were 0.064, 0.060 and 0.070, respectively (Fig. 3). Levels of *gyrB* gene sequence distance between strain FXJ6.011^T and other *Micromonospora* species ranged from 0.046 to 0.124. Kasai *et al.* (2000) studied *gyrB* gene sequences and DNA–DNA reassociation of type strains of the genus *Micromonospora*, and determined that, within this genus, a *gyrB* genetic distance of around 0.014 corresponds to 70 % DNA–DNA reassociation value and that *gyrB* gene sequence

Fig. 3. Neighbour-joining tree based on *gyrB* gene sequences of strain FXJ6.011^T, *M. echinofusca* JCM 3327^T, *M. chaiyaphumensis* JCM 12873^T, *M. auratinigra* JCM 12357^T and other members of the family *Micromonosporaceae*. Bootstrap values (%) are based on 1000 resampled datasets; only values greater than 40 % are given. Filled diamonds represent branches that were also recovered by the maximum-parsimony method. Bar, 0.02 substitutions per nucleotide position.

analysis is superior for inferring intragenetic relationships. Moreover, the DNA–DNA relatedness values between strain FXJ6.011^T and the related strains *M. auratinigra* JCM 12357^T, *M. chaiyaphumensis* JCM 12873^T and *M. echinofusca* JCM 3327^T were 15.3, 10.7 and 28.9 %, respectively. These results demonstrate that strain FXJ6.011^T represents a separate genomic species of the genus *Micromonospora*.

Chemotaxonomic analyses were carried out to confirm the above results. The cell-wall diamino acid of strain FXJ6.011^T contained DD-diaminopimelic acid and a small amount of meso-diaminopimelic acid, whereas the related type strain *M. auratinigra* JCM 12357^T contained only meso-diaminopimelic acid (Supplementary Fig. S1, in IJSEM Online). The whole-cell sugars of strain FXJ6.011^T were galactose, xylose, glucose, mannose, arabinose, ribose and rhamnose. The major polar

lipids were diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylmannosides (PIMs) and phosphatidylinositol dimannoside (PIDM). Furthermore, two minor unidentified phospholipids (PL) were detected (Supplementary Fig. S2, in IJSEM Online). The menaquinones of strain FXJ6.011^T contained MK-10(H₂) (64.6 %), MK-10(H₄) (16.3 %), MK-10(H₆) (8.8 %), MK-9(H₄) (7.1 %) and a small amount of MK-10 (3 %). The major cellular fatty acids were iso-C_{16:0} (40.0 %), C_{17:1}ω8c (14.5 %), C_{16:0} (10.2 %), C_{18:1}ω9c (9.4 %), iso-C_{15:0} (4.7 %), C_{18:0} (3.9 %) and 10-methyl-C_{17:0} (3.1 %). The genomic DNA G + C content was 71.3 mol%.

Strain FXJ6.011^T also differed from related strains in some physiological and biochemical characteristics. In particular, the growth temperature range and utilization of cellobiose,

Table 2. Differential characteristics of the novel strain FXJ6.011^T and related members of the genus *Micromonospora*

Strains: 1, *Micromonospora yangpuensis* sp. nov. FXJ6.011^T; 2, *M. auratinigra* JCM 12357^T; 3, *M. chaiyaphumensis* JCM 12873^T; 4, *M. citrea* JCM 3256^T; 5, *M. chokoriensis* JCM 13247^T; 6, *M. eburnea* JCM 12345^T; 7, *M. echinofusca* JCM 3327^T; 8, *M. marina* JCM 12870^T; 9, *M. nigra* JCM 8973^T; 10, *M. tulbaghia* NRRL B-24576^T. All data are from this study. +, Positive; w, weak; –, negative.

Characteristic	1	2	3	4	5	6	7	8	9	10
Utilization of:										
L-Arabinose	–	–	+	+	+	–	w	–	w	w
Cellobiose	–	+	+	+	+	–	+	–	+	–
D-Fructose	–	+	+	–	+	–	+	–	w	–
Glycerol	+	–	–	w	–	–	–	–	–	–
α-Lactose	–	+	–	w	+	–	+	w	+	–
Maltose	–	w	+	+	+	–	+	–	+	w
Mannitol	+	+	–	w	–	–	w	–	–	w
L-Rhamnose	–	–	w	w	w	–	+	–	–	+
Raffinose	+	–	+	–	–	–	–	+	+	–
Ribose	–	+	+	w	–	–	+	w	–	+
Salicin	–	+	+	+	–	w	+	+	+	–
Sucrose	+	+	–	+	+	–	+	–	+	–
D-Xylose	+	–	–	+	+	–	+	–	+	–
Alanine	+	+	+	+	+	+	+	–	–	+
Cysteine	–	–	–	+	+	–	–	–	+	–
Glycine	–	–	+	+	+	w	–	–	w	–
Proline	w	+	+	+	+	–	w	w	–	–
Serine	+	+	+	+	+	–	+	w	w	+
Degradation of:										
Casein	+	w	+	+	+	+	+	+	+	w
Tween 20	+	+	–	+	+	+	+	+	–	–
Tween 60	+	+	–	+	+	+	+	+	+	–
Tween 80	+	–	–	–	–	+	+	+	–	+
Hydrolysis of:										
Starch	+	–	+	–	–	–	+	–	+	+
Aesculin	+	–	–	–	+	–	+	–	–	–
Milk peptonization	+	–	–	–	+	–	–	+	+	–
Gelatin liquefaction	–	–	+	+	–	–	–	+	–	+
Nitrite from nitrate	+	–	–	+	+	–	+	–	+	–
Maximum NaCl tolerance (%)	3.0	2.5	3.0	4.5	3.0	4.0	3.5	6.5	4.0	5.0
Growth temperature (°C)	10–45	15–40	15–40	10–40	20–37	20–45	15–40	15–45	15–40	4–37
Growth pH range	6–11	5–8	4–9	5–10	5–10	5–9	5–11	5–9	6–10	6–9

D-fructose, glycerol and salicin effectively discriminated strain FXJ6.011^T from the phylogenetically closely related type strains. Detailed physiological and biochemical properties of strain FXJ6.011^T are given in Table 2 and the species description.

In summary, phenotypic and genotypic characteristics showed that strain FXJ6.011^T was a member of the genus *Micromonospora* but that it can be differentiated from other species by its genetic, physiological and chemotaxonomic characteristics. Based on all these results, it is proposed that strain FXJ6.011^T represents a novel species of the genus *Micromonospora* with the name *Micromonospora yangpuensis* sp. nov.

Description of *Micromonospora yangpuensis* sp. nov.

Micromonospora yangpuensis (yang.pu.en'sis. N.L. fem. adj. *yangpuensis* pertaining to Yangpu district, in Hainan Province, China, the source of the sample from which the type strain was isolated).

Aerobic, Gram-positive. Aerial mycelium is not formed. Well-developed and branched substrate mycelium bears single spores with warty surfaces. Growth is good on yeast extract-malt extract agar, Bennett's agar and Czapek agar, moderate on oatmeal agar, inorganic salts-starch agar and glycerol-asparagine agar, and poor on peptone-yeast extract-iron agar, tyrosine agar and nutrient agar; colony colours are indicated in Table 1. Brown, diffusible pigment is produced on Bennett's agar. The growth temperature range is 10–45 °C. Grows at pH 6–11 and in the presence of 3 % NaCl. Utilizes fucose, D-galactose, glucose, glycerol, inositol, mannitol, D-mannose, raffinose, sucrose, trehalose and D-xylose, but not L-arabinose, cellobiose, D-fructose, α -lactose, maltose, L-rhamnose, ribose and salicin as sole carbon sources. Utilizes alanine, glutamic acid, proline and serine, but not cysteine or glycine as sole nitrogen sources. Positive for catalase, nitrite from nitrate, milk peptonization, and hydrolysis of casein, aesculin, starch, and Tweens 20, 60 and 80. Negative for oxidase, gelation liquefaction, and degradation of adenine, allantoin, arbutin, chitosan, guanine, hypoxanthine and xanthine. Whole-cell hydrolysates contain DD-diaminopimelic acid, meso-diaminopimelic acid, galactose, xylose, glucose, mannose, arabinose, ribose and rhamnose. The predominant menaquinones are MK-10(H₂), MK-10(H₄) and MK-10(H₆). The major cellular fatty acids are iso-C_{16:0}, C_{17:1} ω 8 ϵ , C_{16:0}, C_{18:1} ω 9 ϵ , iso-C_{15:0}, C_{18:0} and 10-methyl-C_{17:0}. The polar lipids are DPG, PE, PI, PIMs and PIDM.

The type strain is FXJ6.011^T (=CGMCC 4.5736^T=NBRC 107727^T), isolated from a cup-shaped sponge collected at Dachan reef in the South China Sea. The genomic DNA G + C content is 71.3 mol%.

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