Micromonospora pattaloongensis sp. nov., isolated from a Thai mangrove forest

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An actinomycete, designated strain TJ2-2^T, was isolated from soil collected from a mangrove forest in Pattaloong Province, Thailand, and was subjected to morphological and chemotaxonomic analysis and phylogenetic investigation based on 16S rRNA gene sequences. The data from these analyses indicated that the novel strain should be classified as a member of the genus *Micromonospora* and that the closest relative was *Micromonospora olivasterospora* DSM 43868^T (98.7 % gene sequence similarity). The DNA–DNA hybridization data and some physiological and biochemical properties indicated that the novel strain could be readily distinguished from its closest phylogenetic relatives. On the basis of these phenotypic and genotypic data, strain TJ2-2^T represents a novel species of the genus *Micromonospora*, for which the name *Micromonospora pattaloongensis* sp. nov. is proposed. The type strain is TJ2-2^T (=JCM 12833^T=TISTR 1559^T).

The genus *Micromonospora* Ørskov (1923) belongs to the family *Micromonosporaceae* in the order *Actinomycetales* (Stackebrandt *et al.*, 1997). This genus is well established in terms of morphological and chemotaxonomic properties (Lechevalier & Lechevalier, 1970; Lechevalier *et al.*, 1977; Kroppenstedt, 1985) as well as 16S rRNA gene sequence-based phylogeny (Stackebrandt *et al.*, 1997). In 2006, a novel genus in the family *Micromonosporaceae* was proposed, *Polymorphospora*, with the description of *Polymorphospora rubra* (Tamura *et al.*, 2006). This genus is very similar to the genus *Micromonospora* but differs in some morphological and chemotaxonomic characteristics.

As mangrove environments differ greatly from terrestrial habitats, the distribution and biological characteristics of mangrove actinomycetes are expected to be different from those of soil actinomycetes. Studies on the biodiversity of mangrove actinomycetes are important not only in terms of basic research, but also for the biotechnological exploitation of such organisms.

During an investigation of novel actinomycetes from soil collected in a mangrove forest in southern Thailand, we isolated a strain, designated TJ2-2^T, showing morphological and chemotaxonomic characteristics typical of members of the genus *Micromonospora* but which was genotypically and phenotypically distinguishable from all recognized *Micromonospora* species. Here, we describe the polyphasic characterization of strain TJ2-2^T and describe it as a novel species of the genus *Micromonospora*.

Strain TJ2-2^T was isolated from a soil sample collected from a mangrove forest in Pattaloong Province, Thailand. The sample was taken from the soil surface and kept at 4 °C. The sampling and isolation methods were as described by Thawai *et al.* (2004); the purified culture was maintained at 4–10 °C on yeast extract-malt extract (ISP 2) agar slants.

Morphological properties of this strain grown on ISP 2 agar medium were observed by using light microscopy and scanning electron microscopy (JSM-5410 LV; JEOL). The sample used for scanning electron microscopy was prepared as described previously (Itoh *et al.*, 1989).

Phenotypic characteristics were examined by using several standard methods; cultural characteristics were tested using 14 day cultures grown at 30 °C on various agar media. The Jacal Colour Card L2200 (Japan Colour Research Institute) was used for determining colour designations. The decomposition of various compounds was examined using the basal medium recommended by Gordon *et al.* (1974). The temperature, pH and NaCl tolerances were determined on ISP 2 medium. Carbon-source utilization was tested by using

Abbreviation: A_2pm, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TJ2-2^T is AB275607.

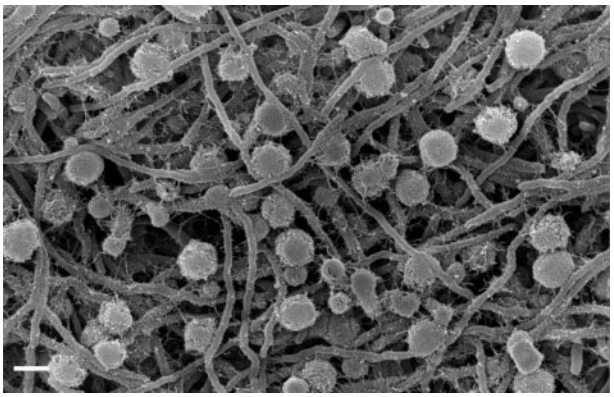

Fig. 1. Scanning electron micrograph of cells of strain TJ2-2^T grown on humic acid-vitamin agar for 4 weeks at 28 °C. Bar, 1 μm.

ISP 9 medium (Shirling & Gottlieb, 1966) supplemented with 1 % (final concentration) carbon source and 0.05 % Casamino acids. Gelatin liquefaction, milk peptonization, nitrate reduction and starch hydrolysis were determined through cultivation on various media as described by Arai (1975) and Williams & Cross (1971). Melanin and hydrogen sulfide production were investigated on slants of tyrosine agar (ISP 7) and peptone iron agar (ISP 6), respectively, supplemented with 0.1 % (w/v) yeast extract.

Freeze-dried cells used for chemotaxonomic analyses were obtained from cultures grown in ISP 2 broth on a rotary shaker at 30 °C for 4 days. Cell-wall peptidoglycan was prepared and hydrolysed by following the methods of Kawamoto *et al.* (1981); the amino acid composition was analysed with an automatic amino acid analyser (L-8500A, Hitachi). The isomers of diaminopimelic acid (A_2pm) present in the cell walls were determined according to the method of Staneck & Roberts (1974). The acyl group of the muramic acid in the peptidoglycan was determined by the method of Uchida & Aida (1984). The reducing sugars from whole-cell hydrolysates were analysed by using HPLC according to Mikami & Ishida (1983). Phospholipids in the cells were extracted and analysed as described by Minnikin *et al.* (1984). Fatty acid methyl ester analysis was performed by using GLC according to the instructions of the Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Isoprenoid quinones were extracted according to Collins *et al.* (1977) and were analysed by using HPLC with a Cosmosil 5C_{18} column (4.6 × 150 mm; Nacalai Tesque). The elution solvent was a mixture of methanol and 2-propanol (2 : 1, v/v).

Chromosomal DNA was isolated from cells grown in ISP 2 broth according to the method of Tamaoka (1994). The G+C content of the DNA was determined by HPLC as described by Tamaoka & Komagata (1984). An equimolar mixture of nucleotides (Yamasa Shoyu) was used as the quantitative standard. DNA–DNA relatedness was measured fluorometrically using the microplate hybridization method devised by Ezaki *et al.* (1989). Hybridization was carried out at 55 °C for 2 h.

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and sequencing of the PCR products were carried out as described previously (Nakajima *et al.*, 1999). The 16S rRNA gene sequence was multiply aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases by using CLUSTAL W, version 1.81 (Thompson *et al.*, 1994). The alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony methods (Kluge & Farris, 1969) in MEGA, version 2.1 (Kumar *et al.*, 2001). The confidence values for the branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The values for sequence

Table 1. Cellular fatty acid contents (%) of strain TJ2-2^T and *M. olivasterospora* JCM 7348^T

Fatty acid	Strain TJ2-2^T	*M. olivasterospora* JCM 7348^T
Saturated fatty acids		
$C_{14:0}$	0.3	0.3
$C_{15:0}$	2.2	0.4
$C_{16:0}$	0.7	1.9
$C_{17:0}$	0.5	0.8
$C_{18:0}$	0.3	1.9
10-Methyl $C_{16:0}$	–	3.0
10-Methyl $C_{17:0}$	2.6	0.9
TBSA 10-methyl $C_{18:0}$	0.1	1.3
Unsaturated fatty acids		
$C_{15:1}\omega6c$	1.0	–
$C_{16:1}$ 2-OH	–	1.0
$C_{17:1}\omega6c$	0.2	–
$C_{17:1}\omega8c$	11.3	1.0
iso-$C_{17:1}\omega9c$	6.5	–
anteiso-$C_{17:1}\omega9c$	3.8	0.6
$C_{18:1}\omega5c$	–	1.3
$C_{18:1}\omega7c$	0.3	–
$C_{18:1}\omega9c$	3.0	0.7
Branched fatty acids		
iso-$C_{13:0}$	0.1	0.1
iso-$C_{14:0}$	6.1	4.0
iso-$C_{15:0}$	24.4	20.2
anteiso-$C_{15:0}$	10.0	6.5
iso-$C_{16:0}$	38.9	36.0
iso-$C_{16:1}$	9.7	1.3
iso-$C_{17:0}$	3.2	6.4
iso-$C_{17:0}$ 3-OH	–	0.4
anteiso-$C_{17:0}$	9.6	7.6
anteiso-$C_{17:1}$	–	0.6
iso-$C_{18:0}$	0.4	1.0
iso-$C_{18:1}$	0.4	–

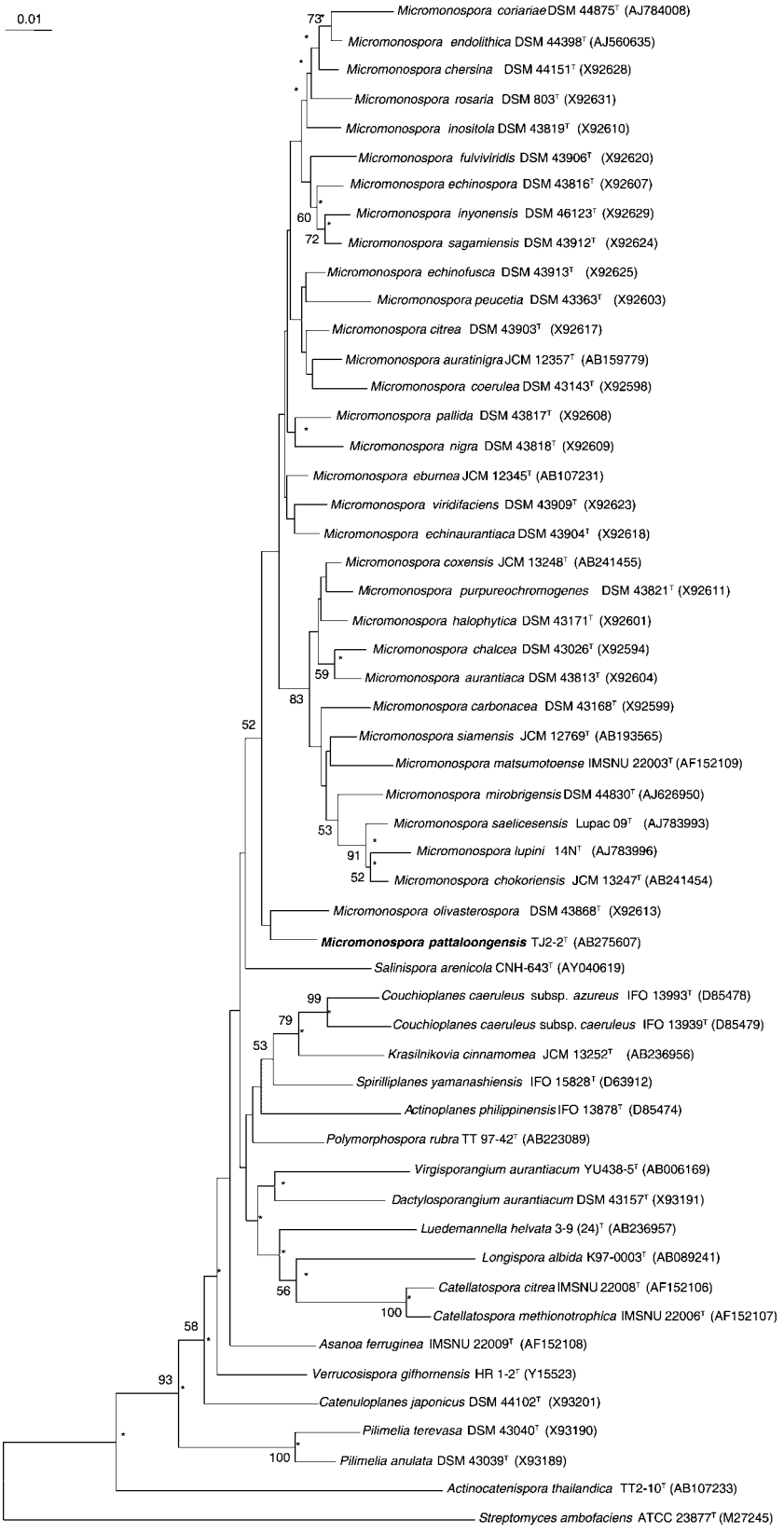

Fig. 2. Neighbour-joining phylogenetic tree (Saitou & Nei, 1987), based on almost-complete 16S rRNA gene sequences, showing the relationships between strain TJ2-2^T, recognized species of the genus *Micromonospora* and members of the family *Micromonosporaceae*. *Streptomyces ambofaciens* ATCC 23877^T was used as an outgroup. Asterisks indicate branches that were also found using the maximum-parsimony (Kluge & Farris, 1969) method. Numbers at branch points indicate bootstrap percentages (based on 1000 replicates); only values >50 % are indicated. Bar, 0.01 substitutions per nucleotide position.

similarity among the closest strains were calculated manually after obtaining pairwise alignments using CLUSTAL_X (Thompson *et al.*, 1997). Gaps and ambiguous nucleotides were eliminated from the calculations.

The morphological and chemical properties of strain TJ2-2^T were consistent with its classification as a member of the genus *Micromonospora* (Kawamoto, 1989). The growth of strain TJ2-2^T was good on ISP 2, oatmeal agar (ISP 3), peptone-yeast extract-iron agar (ISP 6) and nutrient agar (BD, Difco). Colonies on these media were raised and folded; well-developed, branched substrate hyphae were produced but aerial hyphae were not present. Single, non-motile, spherical spores (0.6–0.9 μm) were observed; the spore surface was warty (Fig. 1). The colour of the sporulating colonies was yellowish white to pale orange. A yellow, soluble pigment was produced in ISP 2, ISP 3 and nutrient agar. The cell-wall hydrolysates contained glutamic acid, glycine, alanine and A_2pm; the A_2pm isomer was *meso*, indicating that this strain has wall chemotype II (Lechevalier & Lechevalier, 1970) and peptidoglycan type A1γ (Schleifer & Kandler, 1972). The acyl type of the cell-wall muramic acid was glycolyl. Glucose, xylose, arabinose, galactose, mannose and ribose were found as whole-cell sugars, but rhamnose was not detected (whole-cell sugar pattern D; Lechevalier & Lechevalier, 1970). The characteristic phospholipids were diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and phosphatidylethanolamine, whereas phosphatidylcholine was not present: this pattern corresponds to phospholipid type PII (Lechevalier *et al.*, 1977). The major cellular fatty acids of this strain were iso-$C_{16:0}$, iso-$C_{15:0}$, $C_{17:1}\omega8c$, anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$; 10-methyl $C_{17:0}$ was also present. This pattern corresponds to fatty acid type 3b (Kroppenstedt, 1985) (Table 1). Mycolic acids were absent. The predominant menaquinones were MK-10(H_4) (56.1 %), MK-10(H_6) (22.8 %), MK-10(H_8) (8.6 %); small amounts of MK-9(H_4) (4.7 %), MK-9(H_6) (1.6 %) and MK-9(H_2) (0.4 %) were also present. The G+C content of the DNA was 71.5 mol%.

The 16S RNA gene sequence analysis of strain TJ2-2^T (1511 nucleotides) showed that it exhibited a close relationship with members of the family *Micromonosporaceae*, being located within the clade of the genus *Micromonospora* (Fig. 2). Comparison of the 16S RNA gene sequence of strain TJ2-2^T with corresponding sequences from the type strains of recognized species of the genus *Micromonospora* and from members of the family *Micromonosporaceae* indicated that this strain was closely related to the type strains of *Micromonospora olivasterospora* (Kawamoto *et al.*, 1983) and *Polymorphospora rubra* (Tamura *et al.*, 2006), the highest level of sequence similarity being 98.7 %. The signature nucleotide positions of strain TJ2-2^T were compared with those of its closest relatives (Table 2).

Table 2. Comparison of 16S rRNA gene signature nucleotide positions of strain TJ2-2^T and the most closely related genera

The nucleotide positions of bases or base pairs are given according to *Escherichia coli* numbering (Brosius *et al.*, 1978). The data for the genus *Micromonospora* are taken from Ara & Kudo (2007).

Position	Strain TJ2-2^T	*Micromonospora*	*Polymorphospora*
129	U	C	U
139–224	A–U	A–U	A–U
140–223	G–C	G–U	G–U
144–178	U–G	U–A	U–G
222	C	C	C
232	G	G	G
262	G	G	G
381	G	G	G
415	C	C	C
456	U	U	C
546	G	G	G
594–645	C–G	C–G	C–G
602–636	C–G	C–G	C–G
616–624	G–C	G–C	A–U
615–625	C–G	C–G	C–G
656–750	G–C	G–C	G–C
836–850	G–C	G–C	G–C
859	C	C	C
968	A	A	A
998–1043	G–C	G–C	G–C
1003	A	A	A
1006	U	A	U
1010	G	G	G
1011–1018	C–G	C–G	C–G
1012–1017	A–U	A–U	A–U
1119–1154	U–A	U–A	U–A
1121–1152	G–C	G–C	G–C
1252	U	U	U
1445–1457	C–G	C–G	U–G

The characteristics shown in Table 3 clearly indicate that strain TJ2-2^T possesses some distinct phenotypic and chemotaxonomic profiles that distinguish it from its closest phylogenetic relative, *M. olivasterospora* JCM 7348^T, and members of the genus *Polymorphospora*. In particular, the following features serve to distinguish strain TJ2-2^T from related micro-organisms: the colour of sporulating colonies, the presence/absence of aerial mycelium, the presence/absence of spore chains, the major whole-organism sugars, the fatty acid profile, the type of A_2pm in the cell wall, the liquefaction of gelatin and the utilization profile for melibiose, raffinose, L-rhamnose, D-ribose and salicin. Furthermore, a low level of DNA–DNA relatedness (16.8 %) was observed between strain TJ2-2^T and *M. olivasterospora* JCM 7348^T.

It is evident from the genotypic and phenotypic data presented above that strain TJ2-2^T is distinguishable from previously described *Micromonospora* species and members of the most closely related genus, *Polymorphospora*. Therefore strain TJ2-2^T represents a novel species of the genus *Micromonospora*, for which the name *Micromonospora pattaloongensis* sp. nov. is proposed.

Description of *Micromonospora pattaloongensis* sp. nov.

Micromonospora pattaloongensis (pat.ta.loong.en′sis. N.L. fem. adj. *pattaloongensis* pertaining to Pattaloong, where the type strain was isolated).

Table 3. Differential characteristics of strain TJ2-2^T and its closest relatives

Strains: 1, TJ2-2^T; 2, *M. olivasterospora* JCM 7348^T; 3, *P. rubra* TT 97-42^T (data from Tamura *et al.*, 2006). +, Positive; −, negative; ND, not determined.

Characteristic	1	2	3
Colony colour on ISP 2 medium	Yellowish white	Greenish black	Red to reddish orange
Aerial mycelium	−	−	+
Spore chains	−	−	+
Cell-wall A_2pm	*meso*-A_2pm	3-OH-*meso*-A_2pm	*meso*-A_2pm
Major whole-organism sugars	Xylose, arabinose	Xylose, arabinose	Xylose
Fatty acid type	3b	3b	2a
Major menaquinones (%)	MK-10(H_4) (56.1), MK-10(H_6) (22.8)	MK-10(H_4) (31.7), MK-10(H_6) (30.8)	MK-10(H_6) (34–35), MK-10(H_4) (20–28)
DNA G+C content (mol%)	71.5	71.9	70.2
Gelatin liquefaction	+	−	ND
Utilization of:			
Melibiose	−	+	+
Raffinose	−	+	+
D-Ribose	+	−	ND
L-Rhamnose	−	+	+
Salicin	+	−	ND

Gram-positive, mesophilic, non-motile actinomycete that forms well-developed and branched substrate hyphae. Colonies are yellowish white to pale orange in ISP 2 medium. Single spores are formed on substrate hyphae. Aerial mycelium is absent. The spore surface appears warty. Spores are non-motile. A yellow soluble pigment is produced in ISP 2, ISP 3 and nutrient agar. Nitrate is reduced to nitrite. Utilizes L-arabinose, D-fructose, D-galactose, D-glucose, lactose, D-ribose, salicin and D-xylose; shows weak utilization of cellobiose, but does not utilize glycerol, D-mannitol, melibiose, raffinose or L-rhamnose. Positive for milk peptonization, starch hydrolysis and gelatin liquefaction, but negative for melanin formation and H_2S production. The optimal temperature for growth is 25–30 °C; no growth occurs above 40 °C. The maximum NaCl concentration for growth is 3 %. The cell wall contains glutamic acid, glycine, alanine and *meso*-A_2pm in the molar ratio 1 : 0.9 : 0.5 : 1.1. The acyl type of the cell-wall muramic acid is glycolyl. The predominant menaquinone is MK-10(H_4). The characteristic whole-cell sugars are xylose and arabinose. The phospholipid profile comprises diphosphatidylglycerol, phosphatidylinositol mannosides, phosphatidylinositol and phosphatidylethanolamine; phosphatidylcholine is not present. The major fatty acids of the type strain are iso-$C_{16:0}$ (38.9 %), iso-$C_{15:0}$ (24.4 %), $C_{17:1}\omega8c$ (11.3 %), anteiso-$C_{15:0}$ (10.0 %), iso-$C_{16:1}$ (9.7 %), anteiso-$C_{17:0}$ (9.6 %) and 10-methyl $C_{17:0}$ (2.6 %). The DNA G+C content is 71.5 mol%.

The type strain, TJ2-2^T (=JCM 12833^T=TISTR 1559^T), was isolated from a soil sample collected from a mangrove forest in Pattaloong Province, Thailand.

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