Actinocatenispora thailandica gen. nov., sp. nov., a new member of the family *Micromonosporaceae*

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Two actinomycete strains, TT2-10^T and TT2-3, which produced long spore chains (more than 10 spores per chain), were isolated from peat swamp forest soil in Pattaloong Province, Thailand. Their taxonomic positions were determined using a polyphasic approach. The chemotaxonomic characteristics of these strains coincided with those of the family *Micromonosporaceae*, i.e. cell-wall chemotype II, muramic acid of the *N*-glycolyl type, whole-cell sugar pattern D and type II phospholipids. Analysis of the 16S rRNA gene sequences also indicated that these strains constitute a distinct lineage within the family *Micromonosporaceae*, sharing 91.3–93.8% sequence similarity with members of this family. On the basis of their phenotypic and genotypic characteristics and their phylogenetic position, these strains represent a novel genus and species, for which the name *Actinocatenispora thailandica* gen. nov., sp. nov. is proposed. The type strain of *Actinocatenispora thailandica* is strain TT2-10^T (=JCM 12343^T =PCU 235^T =DSM 44816^T).

The family *Micromonosporaceae* was first described by Krasil'nikov (1938), and its description has been emended by Koch *et al.* (1996) and Stackebrandt *et al.* (1997), who classified seven genera (*Micromonospora*, *Actinoplanes*, *Dactylosporangium*, *Catellatospora*, *Catenuloplanes*, *Couchioplanes* and *Pilimelia*) within it. Subsequently, the genera *Spirilliplanes*, *Verrucosisporea*, *Virgisporangium*, *Asanoa*, *Longispora* and *Salinispora* have been recognized as novel genera of the family *Micromonosporaceae* on the basis of their chemotaxonomic characteristics and their phylogenetic positions, inferred from 16S rRNA gene sequences and signature nucleotides of this gene (Koch *et al.*, 1996; Stackebrandt *et al.*, 1997). During an investigation of the actinomycete diversity of soils in southern Thailand, we isolated two strains, TT2-10^T and TT2-3, which showed chemotaxonomic characteristics typical of members of the family *Micromonosporaceae* but which represent a novel phylogenetic lineage. In this report, we describe the polyphasic characterization of these strains and propose their classification in a novel genus and species.

Strains TT2-10^T and TT2-3 were isolated from peat swamp forest soil in Pattaloong Province, Thailand. Samples were

taken from the soil surface and were kept at 4 °C. The sampling and isolation methods were as described by Thawai *et al.* (2004), and the pure culture was kept at 4–10 °C on yeast extract-malt extract agar (ISP 2 medium) slants.

Strains TT2-10^T and TT2-3 were grown for 14 days at 30 °C on oatmeal-nitrate agar and observed by using light microscopy and scanning electron microscopy (model JSM-5410 LV; JEOL). Samples for scanning electron microscopy were prepared as described previously (Itoh *et al.*, 1989).

Cultural, physiological and biochemical characteristics were examined by using several standard methods; cultural characteristics were tested using 14 day cultures grown at 30 °C on various agar media. Jacal Colour Card L2200 (Japan Colour Research Institute) was used for determining colour designations and names. The decomposition of various compounds was examined using the basal medium recommended by Gordon *et al.* (1974). Temperature, NaCl and pH tolerances were determined on ISP 2 medium. The utilization of various carbon sources was tested using ISP 9 medium (Shirling & Gottlieb, 1966) supplemented with 1% (final concentration) carbon source and 0.05% Casamino acids. Gelatin liquefaction, peptonization of milk, nitrate reduction, cellulose decomposition and starch hydrolysis were determined through cultivation on various media, as described by Arai (1975) and Williams & Cross (1971). Melanin and hydrogen sulphide production were examined

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TT2-10^T and TT2-3 are AB107233 and AB159211, respectively.

on tyrosine agar and peptone-iron agar slants supplemented with 0.1 % (w/v) yeast extract.

Freeze-dried cells used for chemotaxonomic analyses were obtained from cultures grown in yeast extract-malt extract broth (ISP 2 broth) on a rotary shaker at 30 °C. Cell-wall peptidoglycan was prepared and hydrolysed according to the methods of Kawamoto *et al.* (1981), and the amino acid composition was determined with an automatic amino acid analyser. The isomers of diaminopimelic acid in the cell walls were determined by using the method of Stanek & Roberts (1974). The acyl group of the muramic acid in the peptidoglycan was determined by using the method of Uchida & Aida (1984). The reducing sugars from whole-cell hydrolysates were analysed by using the HPLC method of Mikami & Ishida (1983). The phospholipids in the cells were extracted and analysed by using the method of Minnikin *et al.* (1984). Fatty acid methyl ester analysis was performed by means of GLC according to the instructions of the Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Isoprenoid quinones were extracted by using the method of Collins *et al.* (1977) and were analysed by using HPLC [Cosmosil 5C₁₈ 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 using the HPLC method of Tamaoka & Komagata (1984). An equimolar mixture of nucleotides for analysis of the DNA G + C content (Yamasa Shoyu) was digested by bacterial alkaline phosphatase into nucleosides and 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 by Nakajima *et al.* (1999). The 16S rRNA gene sequence was multiply aligned with selected sequences from the GenBank/EMBL/DDBJ databases using CLUSTAL W, version 1.81 (Thompson *et al.*, 1994). Alignments were verified manually and adjusted prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony methods (Kluge & Farris, 1969) in MEGA, version 2.1. Confidence values for the branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. 16S rRNA gene sequence similarities among closely related genera were calculated manually after pairwise alignments had been obtained using CLUSTAL X (Thompson *et al.*, 1997). Gaps and ambiguous nucleotides were eliminated from the calculations.

Strains TT2-10^T and TT2-3 produced well-developed substrate mycelia on ISP 2 agar, oatmeal agar (ISP 3), inorganic

salts-starch agar (ISP 4), peptone-yeast extract-iron agar (ISP 6), tyrosine agar (ISP 7) and glucose-asparagine agar. Aerial mycelia and spores were absent on these media, but white aerial mycelia were formed with cultivation on oatmeal-nitrate agar. Spore chains were borne on the aerial mycelia and each consisted of more than 10 spores (Fig. 1). The spore surface appeared to be smooth. Spores were cylindrical, approximately 0.3–0.4 × 0.5–1.0 µm in size and non-motile. The colonies of strains TT2-10^T and TT2-3 were yellow to vivid yellow–orange in colour; soluble pigments were not produced on most of the media tested, with the exception of a yellow pigment on oatmeal agar. The physiological and biochemical characteristics of the strains are presented in the species description.

Cell-wall hydrolysates of the two strains contained glutamic acid, glycine, alanine and *meso*-diaminopimelic acid, indicating that these strains have wall chemotype II (described by Lechevalier & Lechevalier, 1970); the peptidoglycan was of type A1γ (described by Schleifer & Kandler, 1972). The acyl type of the cell-wall muramic acid was glycolyl. The strains contained glucose, galactose, xylose, arabinose, mannose and ribose as whole-cell sugars, but rhamnose was not detected. This result suggests that the whole-cell sugar profile was pattern D of Lechevalier & Lechevalier (1970). The characteristic phospholipids were diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, phosphatidylethanolamine and unidentified ninhydrin-negative phospholipids, but phosphatidylcholine was not present. This pattern corresponds to phospholipid type II of Lechevalier *et al.* (1977). The cellular fatty acid compositions are shown in Table 1. The major fatty acids of strains TT2-10^T and TT2-3 were iso-C_{16:0}, anteiso-C_{17:0}, iso-C_{15:0} and iso-C_{17:0}. This pattern corresponds to fatty acid type 3b of Kroppenstedt (1985). Mycolic acids were absent. The predominant menaquinones were MK-9(H₄) and MK-9(H₆) and small amounts of MK-9(H₂) and MK-9(H₈) were also present. The DNA G + C contents for strains TT2-10^T and TT2-3 were 72.2 and 72.3 mol%, respectively.

Fig. 1. Scanning electron micrograph of strain TT2-10^T. Bar, 5 µm.

Table 1. Cellular fatty acid composition of strains TT2-10^T and TT2-3

Values are percentages of total fatty acids. –, Not detected.

Fatty acid	Strain TT2-10 ^T	Strain TT2-3
Saturated fatty acids		
C _{12:0}	0.2	–
C _{13:0}	0.1	0.1
C _{14:0}	0.1	0.1
C _{14:0} 2-OH	1.6	0.6
C _{15:0}	0.3	0.4
C _{16:0}	0.3	0.3
C _{17:0}	0.7	0.5
C _{18:0}	–	0.1
C _{19:0}	0.2	0.2
Unsaturated fatty acids		
C _{16:1} ω11c	0.1	0.2
C _{17:1} ω5c	0.7	0.5
C _{18:1} ω7c	1.8	2.2
C _{18:3} ω6c (6,9,12)	1.2	1.5
Branched fatty acids		
i-C _{13:0}	0.2	0.1
ai-C _{13:0}	0.1	–
i-C _{13:0} 3-OH	0.1	–
i-C _{14:0}	2.6	1.9
i-C _{14:0} 3-OH	3.9	2.2
i-C _{15:0}	20.2	19.2
ai-C _{15:0}	6.0	6.2
i-C _{16:0}	26.2	21.3
i-C _{16:1}	0.9	0.9
i-C _{17:0}	11.3	13.9
ai-C _{17:0}	19.3	25.9
ai-C _{17:1}	0.6	0.6
i-C _{18:0}	0.2	0.2
Summed feature 2b*	0.2	–

*Summed feature 2b contains C_{16:1} and/or C_{14:0} 3-OH.

These chemotaxonomic characteristics, in particular the cell-wall type (type II), the muramic acid acyl type (N-glycolyl), the whole-cell sugar profile (pattern D) and the phospholipid type (type II), suggested that strains TT2-10^T and TT2-3 were members of the family *Micromonosporaceae*. The family *Micromonosporaceae* currently accommodates 13 genera: *Micromonospora* Ørskov 1923, *Actinoplanes* Couch 1950, *Pilimelia* Kane 1966, *Dactylosporangium* Thiemann *et al.* 1967, *Catellatospora* Asano and Kawamoto 1986, *Catenuloplanes* Yokota *et al.* 1993, *Couchioplanes* Tamura *et al.* 1994, *Spirilliplanes* Tamura *et al.* 1997, *Verrucosipora* Rheims *et al.* 1998, *Virgisporangium* Tamura *et al.* 2001, *Asanoa* Lee and Hah 2002, *Longispora* Matsumoto *et al.* 2003 and *Salinispora* Maldonado *et al.* 2005. The morphological characteristics of strains TT2-10^T and TT2-3 were similar to those of members of the genera *Longispora*, *Catellatospora*, *Catenuloplanes*, *Asanoa*, *Couchioplanes* and *Spirilliplanes* in terms of spore-chain

Table 2. Differential characteristics of strains TT2-10^T and TT2-3 (*Actinocatenispora* gen. nov.) and related genera

Taxa: 1, strains TT2-10^T and TT2-3; 2, *Longispora*; 3, *Virgisporangium*; 4, *Asanoa*; 5, *Actinoplanes*; 6, *Spirilliplanes*; 7, *Micromonospora*; 8, *Dactylosporangium*; 9, *Catellatospora*; 10, *Catenuloplanes*; 11, *Couchioplanes*; 12, *Pilimelia*; 13, *Verrucosipora*; 14, *Salinispora*. Data for reference taxa were taken from Matsumoto *et al.* (2003), Tamura *et al.* (1994, 1997, 2001), Stackebrandt & Kroppenstedt (1987), Vobis (1989), Rheims *et al.* (1998), Lee *et al.* (2000), Lee & Hah (2002), Kudo *et al.* (1999), Goodfellow *et al.* (1990) and Maldonado *et al.* (2005). Cell wall, phospholipid and fatty acid types are given according to the respective classification schemes of Lechevalier & Lechevalier (1970), Lechevalier *et al.* (1977) and Kroppenstedt (1985). ND, No data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Spore chains	+	+	–	+	–	+	–	–	+	+	+	–	–	–
Spore motility	–	–	+	–	+	+	–	+	–	+	+	+	–	–
Cell-wall type	II	II	II	II	II	II	II	II	II	VI	VI	II	II	II
Major menaquinones	MK-9(H _{4,6})	MK-10(H _{4,6})	MK-10(H _{4,6})	MK-10(H _{4,6})	MK-9(H ₄)	MK-10(H _{4,6})	MK-10(H _{4,6})	MK-9(H _{4,6})	MK-10(H _{8,6})	MK-9(H ₈), MK-10(H ₈)	MK-9(H ₄)	MK-9(H _{2,4})	MK-9(H ₄)	MK-9(H ₄)
Phospholipid type	PII	PII	PII	PII	PII	PII	PII	PII	PII	PIII	PII	PII	PII	PII
Fatty acid type	3b	2d	2d	2d	2d	2d	3b	3b	3b	2c	2c	2d	2b	3a
Characteristic whole-cell sugars*	Ara, Gal, Xyl	Ara, Gal, Xyl	Ara, Gal, Xyl	Ara, Gal, Xyl	Ara, Xyl	Gal, Xyl	Ara, Xyl	Ara, Xyl	Ara, Gal, Xyl/Xyl only	Xyl	Ara, Xyl, Gal	Ara, Xyl	Man, Xyl	Ara, Gal, Xyl
DNA G + C content (mol%)	72	70	71	71–72	72–73	69	71–73	72–73	71–72	71–73	70–72	ND	70	70–73

*Ara, Arabinose; Gal, galactose; Man, mannose; Xyl, xylose.

production, but these genera (except *Longispora*) could be distinguished on the basis of spore motility and true aerial mycelium production. The genus *Longispora* seems to be the genus most closely related to strains TT2-10^T and TT2-3, but the major menaquinones and fatty acid composition of its members distinguished them from strains TT2-10^T and TT2-3. These results indicated that strains TT2-10^T and TT2-3 do not belong to any known genera in the family *Micromonosporaceae*. Differential characteristics for the isolates and members of related genera in the family *Micromonosporaceae* are shown in Table 2.

Almost complete 16S rRNA gene sequences (1444 and 1434 nt for strains TT2-10^T and TT2-3, respectively) were used for phylogenetic analysis with those of members of the order *Actinomycetales*. Phylogenetic analysis of this large dataset revealed that strains TT2-10^T and TT2-3 were positioned within the cluster of the family *Micromonosporaceae* (data not shown). When the sequences of strains TT2-10^T and TT2-3 were compared with corresponding sequences from all of the type species of recognized members of the family *Micromonosporaceae* and selected sequences from members of related families, they were found to form a distinct phylogenetic lineage within the family *Micromonosporaceae* and were also found to be separate from closely related families in the neighbour-joining analysis (with high levels of bootstrap support) (Fig. 2). 16S rRNA gene sequence similarities between strain TT2-10^T and members of the family *Micromonosporaceae* ranged from 91.3% (with *Longispora albida*) to 93.8% (with *Salinispora arenicola*), and strain TT2-10^T showed 99.7% sequence similarity to strain TT2-3; these similarities represent 127, 96 and 4 nucleotide differences, respectively. Additionally, strains TT2-10^T and TT2-3 had no more than 93% sequence similarity with members of any of the other families with which they were compared. Furthermore, the 16S rRNA gene sequences of strains TT2-10^T and TT2-3 contained the

majority of the signature nucleotides of members of the family *Micromonosporaceae* (Stackebrandt *et al.*, 1997), except for variations at some positions (position 502: 543, U→G; position 747, G; and position 811, C; *Escherichia coli* numbering). On the basis of phenotypic and genotypic data, strains TT2-10^T and TT2-3 are readily distinguishable from the members of all established genera in the family *Micromonosporaceae*. Therefore, we propose that strains TT2-10^T and TT2-3 should be classified in a novel genus and species, for which the name *Actinocatenispora thailandica* gen. nov., sp. nov. is proposed.

Description of *Actinocatenispora* gen. nov.

Actinocatenispora [Ac.ti.no.ca.te.ni.spo'ra. Gr. n. *actinos* ray; L. n. *catena* chain; Gr. n. *spora* seed; N.L. fem. n. *Actinocatenispora* spore chain-producing ray (fungus)].

Gram-positive, non-acid-fast, non-motile and aerobic organisms with branching substrate hyphae. Aerial hyphae are formed and bear spore chains each consisting of more than 10 spores. Spores are cylindrical (0.3–0.4 × 0.5–1.0 µm) and the spore surface is smooth. In general, substrate mycelia are yellow to vivid orange in colour. Cell wall contains glutamic acid, glycine, alanine and *meso*-diaminopimelic acid. The *N*-acyl group of the cell-wall muramic acid is glycolyl. Galactose, xylose, arabinose, glucose, mannose and ribose are detected as whole-cell sugars. Contains phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, phosphatidylglycerol and unidentified ninhydrin-negative phospholipids as cellular phospholipids (phospholipid pattern type II). Predominant menaquinones are MK-9(H₄) and MK-9(H₆); MK-9(H₂) and MK-9(H₈) are minor components. Mycolic acids are not detected. The DNA G + C content is 72 mol%. Habitat is soil. The type species is *Actinocatenispora thailandica*.

Fig. 2. Neighbour-joining tree (Saitou & Nei, 1987), based on almost complete 16S rRNA gene sequences, showing relationships among strains TT2-10^T and TT2-3, all of the type species of recognized representative members of the family *Micromonosporaceae* and selected sequences of members of closely related families. The 16S rRNA gene sequence of *Bacillus subtilis* subsp. *subtilis* NCDO 1769^T was used as an outgroup. Asterisks indicate branches of the tree that were also found using the maximum-parsimony method (Kluge & Farris, 1969). Numbers on branches indicate percentage bootstrap values from 1000 replicates; only values greater than 50% are indicated. Bar, 0.02 substitutions per nucleotide position.

Description of *Actinocatenispora thailandica* sp. nov.

Actinocatenispora thailandica (thai.lan'di.ca. N.L. fem. adj. *thailandica* of Thailand, where the type strain was isolated).

The description of the morphological, chemotaxonomic and general characteristics are as described for the genus. White aerial mycelia are formed on oatmeal-nitrate agar. Soluble yellow pigment present on oatmeal agar. The type strain utilizes D-glucose, D-mannitol, D-melibiose, D-raffinose, glycerol, *myo*-inositol, salicin and cellobiose but not D-ribose, L-rhamnose, lactose, D-galactose, L-arabinose or D-fructose. Positive for reduction of nitrate, weakly positive for peptonization of milk and gelatin liquefaction and negative for hydrolysis of starch, formation of melanin and H₂S production. The optimal temperature for growth is 25–30 °C. No growth occurs above 40 °C. Minimum pH tolerated is 4.5. Maximum NaCl concentration for growth is 7 %. The major cellular fatty acid components are iso-C_{16:0}, anteiso-C_{17:0}, iso-C_{15:0} and iso-C_{17:0}. The DNA G + C content of the type strain is 72 mol%.

The type strain, TT2-10^T (= JCM 12343^T = PCU 235^T = DSM 44816^T), was isolated from peat swamp forest soil in Pattaloong Province, Thailand.

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