Micromonospora chaiyaphumensis sp. nov., isolated from Thai soils

Suchada Jongrungruangchok,¹ Somboon Tanasupawat¹ and Takuji Kudo²

Correspondence

Somboon Tanasupawat

Somboon.T@chula.ac.th

¹Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

²Japan Collection of Microorganisms, RIKEN BioResource Center, Wako, Saitama 351-0198, Japan

Three actinomycete strains, MC5-1^T, MC7-1 and R1-1, were isolated from soil samples collected in Thailand. Their taxonomic positions were determined using a polyphasic approach. The chemotaxonomic characteristics of these strains coincided with those of the genus *Micromonospora*, i.e. meso-diaminopimelic acid and *N*-glycolyl muramic acid were present in the cell-wall peptidoglycan, the whole-cell sugars were of pattern D, the phospholipids were of type II and the cellular fatty acids were of type 3b. Phylogenetic analysis of the 16S rRNA gene sequences revealed a close relationship between strains MC5-1^T, MC7-1 and R1-1 (99.8% sequence similarity) and *Micromonospora auratinigra* JCM 12357^T (99.3%). The three novel strains were clearly distinguishable from *M. auratinigra* JCM 12357^T from the low DNA–DNA relatedness ($\leq 43.4\%$). On the basis of the data presented, strain MC5-1^T represents a novel species of the genus *Micromonospora*, for which the name *Micromonospora chaiyaphumensis* is proposed. The type strain is MC5-1^T (=KCTC 19332^T=JCM 12873^T=PCU 267^T=TISTR 1564^T).

The genus *Micromonospora* was proposed, by Ørskov (1923), for actinomycete strains on the basis of morphological properties, i.e. the absence of true aerial mycelium and the presence of spores borne singly on the substrate mycelium. Kasai *et al.* (2000) suggested that the genus *Micromonospora* should be redefined on the basis of *gyrB* and 16S rRNA gene sequence analyses and DNA–DNA relatedness to include only 14 species. Strains of the genus *Micromonospora* are distributed widely in different environments such as soils, water, sandstone and root nodules (Kawamoto, 1989; Hirsch *et al.*, 2004; Trujillo *et al.*, 2005, 2006; Ara & Kudo, 2007). Recently, the following novel species were assigned to the genus *Micromonospora*: *Micromonospora auratinigra*, *M. eburnea*, *M. endolithica*, *M. mirobrigensis*, *M. siamensis*, *M. citrea*, *M. echinaurantiaca*, *M. echinofusca*, *M. fulviviridis*, *M. inyonensis*, *M. peucetia*, *M. sagamiensis*, *M. viridifaciens*, *M. coriariae*, *M. chokoriensis* and *M. coxensis* (Hirsch *et al.*, 2004; Thawai *et al.*, 2004, 2005a, b; Kroppenstedt *et al.*, 2005; Trujillo *et al.*, 2005, 2006; Ara & Kudo, 2007).

During the course of an investigation into rare actinomycetes from soils in Thailand, we isolated three actinomycetes, designated strains MC5-1^T, MC7-1 and R1-1, which showed the typical morphological characteristics of the genus *Micromonospora*. In this paper, we report the taxonomic characterization and classification of these isolates and propose that they represent a novel species of that genus.

Strains MC5-1^T and MC7-1 were isolated from mountain soil collected in Chaiyaphume Province, Thailand, and strain R1-1 was isolated from soil in Ratchaburi Province, Thailand. Strains were isolated using starch-casein nitrate agar containing (l⁻¹) 10 g starch, 0.3 g sodium caseinate (Difco), 2 g KNO₃ and 15 g agar (pH 7.0–7.4), incubated at 30 °C for 21 days. The isolates were purified and maintained on yeast extract-malt extract agar (ISP 2). They were grown on ISP 2 agar and oatmeal agar (ISP 3) for 2 weeks at 30 °C and were observed by using light microscopy and scanning electron microscopy (model JSM-5410 LV; JEOL). For scanning electron microscopy, samples were prepared as described previously (Itoh *et al.*, 1989).

Cultural characteristics were determined using 14 day-cultures grown at 30 °C on ISP media, as described by Shirling & Gottlieb (1966). The *Color Harmony Manual* (Jacobson *et al.*, 1958) was used for determining the colours of colonies. The decomposition of various

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains MC5-1^T, MC7-1, and R1-1 are AB196710, AB196709 and AB196711, respectively.

A maximum-parsimony phylogenetic tree and cellular fatty acid compositions, DNA G+C contents and DNA–DNA relatedness values for strains R1-1, MC5-1^T and MC7-1 and related strains are presented as supplementary material available with the online version of this paper.

compounds was examined by using the basal media recommended by Gordon *et al.* (1974). The utilization of carbohydrates as sole carbon sources was tested using neutralized yeast nitrogen base without amino acids (Difco) as a basal medium, supplemented with 1 % (w/v) each carbon source and 0.05 % (w/v) each amino acid according to the method of Stevenson (1967). Tolerance of NaCl and the effect of temperature and pH on growth were determined on Sucrose Bennett's Agar (Arai, 1975). Gelatin liquefaction, milk peptonization, nitrate reduction and starch hydrolysis were determined as described by Arai (1975) and Williams & Cross (1971). In addition, tyrosine agar (ISP 7) and peptone-iron agar (ISP 6) supplemented with 0.1 % (w/v) yeast extract were used to determine melanoid pigment production.

Freeze-dried cells for chemotaxonomic analyses were obtained from cultures grown in ISP 2 broth incubated on a rotary shaker at 30 °C for 14 days. Cell-wall peptidoglycan was prepared and hydrolysed using the method described by Kawamoto *et al.* (1981). The isomers of diaminopimelic acid in the cell walls were determined as described by Stanek & Roberts (1974). Reducing sugars from whole-cell hydrolysates were analysed by using the HPLC method of Mikami & Ishida (1983). The acyl group of the muramic acid in the peptidoglycan was determined using the method of Uchida & Aida (1984). Phospholipids were extracted and identified as described by Minnikin *et al.* (1984). Methyl esters of the cellular fatty acids were prepared by using the direct transmethylation method with methanolic hydrochloride (Suzuki & Komagata, 1983) and were analysed with GLC according to the instructions of the Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996) as described previously (Kudo *et al.*, 1993). The identification of each peak was carried out by comparison with commercially available standards and by using GC-MS. Isoprenoid quinones were extracted using the method of Collins *et al.* (1977) and were analysed by HPLC with a Cosmosil 5C₁₈ column (4.6 × 150 mm; Nacalai Tesque). Methanol/2-propanol (2:1, v/v) was used as an eluant.

DNA extraction from cells grown in ISP 2 broth was performed according to the method of Tamaoka (1994). The G+C content of the DNA was determined using HPLC as described by Tamaoka & Komagata (1984). DNA–DNA relatedness was measured fluorometrically using the microplate hybridization method devised by Ezaki *et al.* (1989). 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 sequences of the three novel strains were multiply aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases by using the CLUSTAL W program package (Thompson *et al.*, 1994). A phylogenetic tree was constructed using the Kimura two-parameter model in the neighbour-joining method (Saitou & Nei, 1987) and the maximum-parsimony method with MEGA, version 3.1 (Kumar *et al.*, 2004). The

confidence values of the branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Gaps and ambiguous nucleotides were eliminated from the calculations.

Strains MC5-1^T, MC7-1 and R1-1 had morphological, cultural and chemotaxonomic properties consistent with their classification in the genus *Micromonospora* (Ørskov, 1923). They formed well-developed and branched substrate hyphae. Aerial mycelium was absent. At maturity, spores were spherical to oval, appeared to be rough and were non-motile (Fig. 1). Their phenotypic characteristics are given in the species description, in Tables 1 and 2 and in Supplementary Table S1 (available with the online version of this paper). The strains contained *meso*-diaminopimelic acid in the cell wall. The acyl type of the cell-wall peptidoglycan was determined to be of the glycolyl type. The whole-cell sugars detected were glucose, xylose, mannose, ribose, galactose and arabinose (pattern D of Lechevalier & Lechevalier, 1970). The polar lipid profile comprised diphosphatidylglycerol, phosphatidylinositol, phosphatidylmannosides and phosphatidylethanolamine (the PII pattern of Lechevalier *et al.*, 1977). The significant fatty acids of strains MC5-1^T and MC7-1 were iso-C_{15:0} (29.3–31.4 %), iso-C_{16:0} (14.6–31.3 %), iso-C_{17:0} (8.1–11.3 %), iso-C_{17:1ω9c} (5.6 %), anteiso-C_{17:0} (5.5–8.3 %) and 10-methylated C_{17:0} (5.8–6.1 %) (Supplementary Table S1). This pattern corresponds to fatty acid type 3b of Kroppenstedt (1985). Mycolic acids were absent. The predominant menaquinones were MK-10(H₈) (20.0–51.1 %), MK-10(H₆) (15.5–30.6 %), MK-9(H₈) (9.2–19.0 %), and MK-9(H₆) (9.2–17.7 %). Small amounts (<3.1 %) of MK-9(H₂), MK-9(H₄) and MK-10(H₄) were also detected. The DNA G+C contents were 72.7–72.9 mol% (see Supplementary Table S2 in IJSEM Online). Our isolates contained roughly the same fatty acids, menaquinones and DNA G+C contents as the closest relative, *M. auratinigra* JCM 12357^T, as reported by Thawai *et al.* (2004).

The analysis of the almost-complete 16S rRNA gene sequences (1436 nt) of strains MC5-1^T, MC7-1 and R1-1 indicated that they were 99.8 % related to each other and

Fig. 1. Scanning electron micrograph of strain MC5-1^T grown on ISP 2 agar medium incubated at 30 °C for 14 days. Bar, 1 µm.

Table 1. Cultural characteristics of strains MC5-1^T, MC7-1 and R1-1

Media	MC5-1 ^T		MC7-1		R1-1	
	Growth	Colony colour	Growth	Colony colour	Growth	Colony colour
Yeast extract-malt extract agar (ISP 2)	Abundant	Orange to brownish black	Abundant	Orange to brownish black	Abundant	Orange to brownish black
Oatmeal agar (ISP 3)	Abundant	Greyish black	Abundant	Greyish black	Abundant	Greyish black
Inorganic salts-starch agar (ISP 4)	Abundant	Beige brown	Abundant	Melon yellow	Abundant	Dull gold
Glycerol-asparagine agar (ISP 5)	Poor	Light grey	Poor	Light grey	Poor	Light grey
Peptone-yeast extract iron agar (ISP 6)	Moderate	Honey gold	Moderate	Honey gold	Moderate	Greyish black
Tyrosine agar (ISP 7)	Moderate	Greyish black	Moderate	Greyish black	Moderate	Greyish black
Nutrient agar	Poor	Light wheat	Poor	Light wheat	Poor	Light wheat
Glucose-asparagine agar (ISP 5)	Poor	White	Poor	White	Moderate	White

that they formed a monophyletic clade with the closest relatives, *M. auratinigra* JCM 12357^T (99.3 %) and *Micromonospora coerulea* JCM 3175^T (98.6 %), as shown in Fig. 2 and Supplementary Fig. S1 (tree constructed with the maximum-parsimony method). Comparison with the descriptions of previously characterized species of *Micromonospora* showed that the novel isolates were distinguishable from the related species *M. auratinigra* JCM 12357^T (=DSM 44815^T) and *M. coerulea* JCM 3175^T (=DSM 43143^T) on the basis of a combination of biochemical and physiological properties, in particular, tyrosine decomposition, weak utilization of glycerol and raffinose, growth at pH 4 and a maximum NaCl tolerance of 3.0–3.5 % (Table 2). In addition, the amounts of cellular fatty acids served to differentiate the isolates from the phylogenetically closest species, *M. auratinigra* JCM 12357^T (Supplementary Table S1).

Table 2. Differential characteristics of the novel strains and related species of the genus *Micromonospora*

Strains: 1, MC5-1^T; 2, MC7-1; 3, R1-1; 4, *M. auratinigra* JCM 12357^T; 5, *M. coerulea* JCM 3175^T. +, Positive; –, negative; w, weakly positive. All strains were positive for growth at pH 8 and 15 °C and for the utilization of L-arabinose, D-fructose, D-galactose, D-glucose, lactose, melibiose, salicin and D-xylose. All were negative for nitrate reduction and for the utilization of D-mannitol and rhamnose.

Characteristic	1	2	3	4	5
Decomposition of tyrosine	+	+	w	–	–
Growth at:					
pH 4	+	+	+	–	–
45 °C	+	+	+	+	–
Maximum NaCl tolerance (%)	3	3.5	3	2.5	1.5
Utilization of:					
Glycerol	w	w	w	–	–
Raffinose	w	w	w	+	+
D-Ribose	–	–	–	+	w

The levels of DNA–DNA relatedness among strains MC5-1^T, MC7-1 and R1-1 ranged from 72.5 to 88.5 % while the levels of DNA–DNA relatedness between our strains and closely related species *M. auratinigra* JCM 12357^T and *M. coerulea* JCM 3175^T were 32.7–43.4 % (Supplementary Table S2), i.e. below the threshold value (70 %) suggested for species definition (Wayne *et al.*, 1987). It is evident from the genotypic and phenotypic data that strains MC5-1^T, MC7-1 and R1-1 represent a novel species within the genus *Micromonospora*, for which the name *Micromonospora chaiyaphumensis* sp. nov. is proposed.

Description of *Micromonospora chaiyaphumensis* sp. nov.

Micromonospora chaiyaphumensis (chai.ya.phum.en'sis. N.L. fem. adj. *chaiyaphumensis* pertaining to Chaiyaphume Province, the source of the soil from which the type strain was isolated).

Aerobic, Gram-positive, mesophilic, non-motile actinomycete that forms a well-developed and extensively branched substrate mycelium. No aerial mycelium is produced. The colour of the vegetative mycelium on ISP 2 medium is orange, turning to greyish black or black upon sporulation. Brown, diffusible pigment is produced in ISP 2 and ISP 7 media. Spores are oval and have a rough surface. Utilizes L-arabinose, D-fructose, D-galactose, D-glucose, lactose, melibiose, raffinose, salicin and D-xylose, but not D-mannitol, L-rhamnose or D-ribose, as sole carbon sources for energy. Positive for starch hydrolysis, gelatin liquefaction and milk peptonization. Decomposes L-tyrosine, but not adenine, hypoxanthine or xanthine. The optimal temperature for growth is 25–30 °C. No growth occurs above 45 °C. Grows at pH 4–8 (weakly at pH 9). Strain MC5-1^T grows in the presence of 3 % NaCl but not 3.5 % NaCl. Cell-wall peptidoglycan contains glutamic acid, alanine and *meso*-diaminopimelic acid. The acyl type of the cell wall is glycolyl. The predominant menaquinones are MK-10(H₈), MK-10(H₆) and MK-9(H₈). Major cellular fatty acids are iso-C_{15:0}, iso-C_{16:0}, iso-C_{17:0}, anteiso-C_{17:0}, and iso-C_{17:1ω9c}. The DNA G+C content of the type strain is 72.8 mol%.

Fig. 2. Neighbour-joining phylogenetic tree, based on almost-complete 16S rRNA gene sequences, showing relationships among strains R1-1, MC5-1^T and MC7-1, species of the genus *Micromonospora* and related *Actinoplanes* species. Bootstrap percentages (based on 1000 replications) >50 % are shown. Bar, 0.005 substitutions per nucleotide position.

The type strain, MC5-1^T (=KCTC 19332^T=JCM 12873^T=PCU 267^T=TISTR 1564^T), was isolated from soil collected in Thailand.

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