Actinomadura montaniterrae sp. nov., isolated from mountain soil

Apakorn Songsumanus,¹ Takui Kudo,² Moriya Ohkuma,² Wongsakorn Phongsopitanun¹ and Somboon Tanasupawat¹

¹Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand
²Japan Collection of Microorganisms, RIKEN BioResource Center, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan

The taxonomic position of the mountain soil actinomycete, strain CYP1-1Bᵀ, was clarified by a polyphasic study. The strain produced a single spore, or occasionally a chain of spores, on aerial mycelium. Chemotaxonomic data supported the classification of CYP1-1Bᵀ as representing a member of the genus Actinomadura on the basis of the presence of meso-diaminopimelic acid in the peptidoglycan; galactose, glucose, madurose and ribose as whole cell sugars; MK-9(H₄), MK-9(H₆) and MK-9(H₈) as dominant menaquinones; C₁₆:₀, 10-methylated C₁₈:₀ and C₁₈:₁ω₉c as the major cellular fatty acids; and diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol manosides as the predominant phospholipids. The DNA G+C content was 74.3 mol%. On the basis of the combination of morphological and chemotaxonomic characteristics, CYP1-1Bᵀ was identified as representing a member of the genus Actinomadura. On the basis of the results of 16S rRNA gene analysis, CYP1-1Bᵀ was shown to be closely related to Actinomadura nitritigenes DSM 44137ᵀ (98.9 %). Phenotypic, genotypic and DNA–DNA hybridization data supported the hypothesis that CYP1-1Bᵀ represents a novel species of the genus Actinomadura for which the name Actinomadura montaniterrae sp. nov. is proposed. The type strain is CYP1-1Bᵀ (=JCM 16995ᵀ=KCTC 39784ᵀ=TISTR 2400ᵀ).

The genus Actinomadura was first reported by Lechevalier & Lechevalier (1970) as belonging to the family Thermomonosporaceae, together with other genera such as Thermomonospora, Spirillospora, Actinoallomurus and Actinocorallia. Based on morphological characteristics, members of this genus are aerobic, Gram-positive, non-acid-fast actinomycetes, which form extensive branched and non-fragmenting substrate mycelium. They also display various spore chain morphologies on aerial mycelia. With respect to chemotaxonomic markers, the members of the genus Actinomadura contain meso-diaminopimelic acid as the diagnostic diamino acid and N-acetylated muramic acid in the cell wall. The whole-cell sugars contain galactose, glucose, madurose, mannosé and ribose. The predominant menaquinones are MK-9(H₄), MK-9(H₆) and MK-9(H₈); major phospholipids are diphosphatidylglycerol and phosphatidylinositol (Kroppenstedt et al., 1990; Kroppenstedt & Goodfellow, 2006) and fatty acids mainly contain hexadecanoic (C₁₆:₀), 14-methylpentadecanolic (iso-C₁₆:₀) and 10-methyloctadecanoic acids (10-Me-C₁₈:₀) (Kroppenstedt et al., 1990).

Members of the genus Actinomadura are mostly distributed in terrestrial soils. In addition, many strains are found in other ecosystems such as marine sources (Ara et al., 2008; He et al., 2012) and plant tissues (Qin et al., 2009, 2012). With respect to drug discovery, the members of this genus produce many types of bioactive metabolites, including alkaloid stauroporine analog (Han et al., 2005; Shaaban et al., 2015), diterpene (Takagi et al., 2010), sterol bendigols (Simmons et al., 2011), siderophore madurastatin CI (Mazzei et al., 2012), polypeptide nomimicin (Igarashi et al., 2012), β-carboline (Kornsakulkarn et al., 2013) and 3-oxyanthranilic acid (Intaraudom et al., 2014). During the course of an investigation of rare actinomycetes found in soils in Thailand, an actinomycete strain, CYP1-1Bᵀ, was isolated from mountain soil. This paper reports the taxonomic characterization of this isolate and proposes that it represents a novel species, Actinomadura montaniterrae sp. nov.
CYP1-1B<sup>T</sup> was isolated from mountain soil sample collected from Chaiyaphum Province, Thailand by using starch casein nitrate agar (Tanasupawat et al., 2010), supplemented with nystatin (30 mg l<sup>−1</sup>) and nalidixic acid (20 mg l<sup>−1</sup>) and incubated at 30 °C for 21 days. After that, the single colony was transferred onto yeast extract–malt extract agar (ISP 2) (Shirling & Gottlieb, 1966). Morphological characteristics were observed on ISP2 agar at 30 °C for 30 days using a light microscope and scanning electron microscope (JSM-5410LV, JEOL). The motility of spores was observed after the spore mass was flooded with releasing buffer [0.1 % casamino acids; 0.1 % CaCO<sub>3</sub>; 0.01 % Tween 80 (Matsumoto et al., 2000)] and incubated at 30 °C for 30 min. Cultural characteristics were determined using 14-day cultures grown on several standard agar media at 30 °C as described by Shirling & Gottlieb (1966). The National Bureau of Standards/Inter-Society Color Council (NBS/ISCC) Color System was used for determining the colony color, and carbon utilization of the strain was determined by using carbon utilization medium (ISP 9) supplemented with 1 % sole carbon source (Shirling & Gottlieb, 1966). The tolerance of NaCl and pH and effect of temperature on growth were determined by using growth on ISP 2 medium. Gelatin liquefaction, peptonization of milk, nitrate reduction and starch hydrolysis were determined through cultivation on various media as described by Arai (1975) and Williams & Cross (1971).

For chemotaxonomic analyses, freeze-dried cells were obtained from cultures grown in ISP 2 broth on a rotary shaker (200 rpm) at 30 °C for 7 days. The isomer of diaminopimelic acid in the cell wall was determined by the method of Staneck & Roberts (1974). The whole-cell hydrolysate sugars were analyzed by the cellulose TLC method of Komagata & Suzuki (1987). The N-acetyl type of muramic acids was determined using the method of Uchida & Aida (1984). Polar lipids were extracted and identified by the method of Minnikin et al. (1984). Fatty acid methyl ester analysis was performed by gas–liquid chromatography according to the instructions of the Microbial Identification System (MIDI, version 6.0) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996) with the ACTIN1 MIDI database. Isoprenoid quinones were extracted by the method of Collins et al. (1977) and were analyzed by reversed-phase HPLC.

Genomic DNA was obtained from cells grown in ISP 2 broth according to the method of Tamaoka (1994). The amplification of 16S rRNA gene and nucleotide sequencing of PCR product (Macrogen) was carried out using the methods previously described by Nakajima et al. (1999) and the universal primers as reported by Lane (1991). The BLAST analysis of 16S rRNA gene was determined using the EzTaxon-e database (Kim et al., 2012). The 16S rRNA gene sequence of CYP1-1B<sup>T</sup> was aligned against the 16S rRNA gene sequences of closely related species, obtained from GenBank/EMBL/DDBJ databases, using BioEdit software (Hall, 1999). Phylogenetic trees [neighbor-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981)] were reconstructed using MEGA version 6.0 software (Tamura et al., 2013). Both a neighbor-joining tree and maximum-likelihood tree were reconstructed using the Kimura two-parameter model (Kumura, 1980) with deletion of complete gaps. In addition, nearest-neighbor-interchange (NNI) was used for the heuristic method for the maximum-likelihood tree. Likewise, a maximum-parsimony tree was reconstructed using complete gaps deletion with the subtree pruning–regrafting (SPR) method (number of initial trees=10; MP search level=1; maximum number trees to retain=100). The confidence of branch nodes was evaluated using bootstrap analyses (Felsenstein, 1985) based on 1000 replications. The G+C content of genomic DNA was analyzed using HPLC (Tamaoka & Komagata, 1984). DNA–DNA hybridization was carried out in microdilution well plates, as reported by Ezaki et al. (1989).

CYP1-1B<sup>T</sup> formed an extensively branched, non-fragmenting substrate mycelium. The formation of spores was observed on aerial mycelia. The single spores formed along the sporogenous hyphae and its branches. This made the

---

**Fig. 1.** Scanning electron micrographs of spores of CYP1-1B<sup>T</sup> formed along the sporogenous hyphae and its branches (a) and a spore chain of four spores on aerial mycelia (b) grown on ISP2 medium at 30 °C for 30 days. Bars, 1 µm.
A cluster of spores look like a bunch of grapes (Fig. S1, available in the online Supplementary Material). Spores exhibited rough surfaces after cultivation at 30°C for 4 weeks (Fig. 1a). Each spore was oval to ellipsoid in shape (0.9–0.7 µm in size) and not motile. Furthermore, a chain of four spores was observed (Fig. 1b). The colonial appearance and substrate mycelium color were dark grayish yellow on ISP 2 agar plates. The strain grew well on 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) and nutrient agar (Difco). Grayish yellow pigment was observed on ISP2 medium (Table S1). CYP1-1B<sup>T</sup> grew at 0–5.0 % NaCl and pH 4.5–8. Temperature range for growth was 20–45°C.

Starch hydrolysis was positive. Peptonization of milk, gelatin liquefaction and nitrate reduction were negative. The strain could utilize L-arabinose, D-glucose, cellobiose, D-fructose (weakly), lactose, D-mannitol, L-rhamnose, sucrose and D-xylose. Differential characteristics of CYP1-1B<sup>T</sup> and a related species of the genus Actinomadura are shown in Table 1. This strain was screened for antibacterial activities and the crude ethyl acetate extract of the strain cultivated in ISP 2 broth at 30°C for 2 weeks, was found to inhibit Bacillus subtilis ATCC 6633, Pseudomonas aeruginosa ATCC 27853 and Staphylococcus aureus ATCC 25923.

The polar lipids profile comprised diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. The predominant menaquinones were

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CYP1-1B&lt;sup&gt;T&lt;/sup&gt;</th>
<th>Actinomadura nitritigenes JCM 10104&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth on ISP2</td>
<td>Good/Moderate/Very pale green</td>
<td>Good/Abundant/White</td>
</tr>
<tr>
<td>Growth/aerial mass/aerial mass color</td>
<td>Dark grayish yellow</td>
<td>Grayish yellow</td>
</tr>
<tr>
<td>Color of colony</td>
<td>Dark grayish yellow</td>
<td>Grayish greenish yellow</td>
</tr>
<tr>
<td>Color of substrate mycelia</td>
<td>Grayish yellow</td>
<td></td>
</tr>
<tr>
<td>Soluble pigment</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Maximum NaCl tolerance (% w/v)</td>
<td>4.5–8</td>
<td>4–8</td>
</tr>
<tr>
<td>pH ranges</td>
<td>20–45</td>
<td>15–45</td>
</tr>
<tr>
<td>Temperature range (°C)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Peptonization of milk</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Carbon utilization:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Fructose</td>
<td>W</td>
<td>+</td>
</tr>
<tr>
<td>d-Galactose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>d-Melibiose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>L-Raffinose</td>
<td>–</td>
<td>w</td>
</tr>
<tr>
<td>d-Ribose</td>
<td>–</td>
<td>w</td>
</tr>
<tr>
<td>Salicin</td>
<td>–</td>
<td>w</td>
</tr>
</tbody>
</table>

Table 1. Differential characteristics of CYP1-1B<sup>T</sup> and a related species of the genus Actinomadura

+, positive; W, weakly positive; –, negative. All data were determined in this study.

Table 2. Cellular fatty acid profiles of strain CYP1-1B<sup>T</sup> and a related species of the genus Actinomadura

All data were obtained from this study. The fatty acids representing less than 0.5 % in both strains were omitted.
Fig. 2. Neighbor-joining tree based on almost-complete 16S rRNA gene sequences (1488 nt) of CYP1-1B⁺, species of the genus *Actinomadura* and type species of genera of the family *Thermomonosporaceae*. *Streptomyces albifaciens* JCM 4342ᵀ was used as an outgroup. Symbols (*, #) indicate the branches recovered in the maximum-likelihood and maximum-parsimony trees, respectively. The numbers on the branches indicate the percentage bootstrap values of 1000 replicates; only values >50 % are indicated. Bar, 0.01 substitutions per nucleotide position.
MK-9(H₈) (70 %), MK-9(H₇) (18 %) and MK-9(H₆) (12 %). The major cellular fatty acids (>10 %) were C₁₆:0 (31.2 %), 10-methyl C₁₈:0 (19.0 %), C₁₈:1 ω9c (17.1 %) and C₁₆:0 2- OH (10.5 %). This strain showed similar cellular fatty acid profiles to Actinomadura nitritigenes JCM 10104ᵀ. However, they also showed some differences in the amount of minor fatty acids as shown in Table 2. The G+C content of the genomic DNA was 74.3 mol%.

An almost-complete 16S rRNA gene sequence (1488 nt) of CYP1-1Bᵀ was determined and compared with the corresponding sequences of the members of the genus Actinomadura. This strain exhibited the highest 16S rRNA gene sequence similarity with *A. nitritigenes* DSM 44137ᵀ (98.9 %). In addition, neighbor-joining tree analysis of CYP1-1Bᵀ and other members of the genus *Actinomadura* as well as type species of genera of the family Thermomonosporaceae revealed that CYP1-1Bᵀ formed a cluster with *Actinomadura rudentiformis* HMC1ᵀ, *Actinomadura fibrosa* IMSNU 22177ᵀ, *A. nitritigenes* DSM 44137ᵀ, *Actinomadura fulvescens* DSM 43923ᵀ and *Spirillospora albida* IFO 12248ᵀ (Figs 2, S2 and S3). CYP1-1Bᵀ formed an abundance of monomeric spores that could be used to distinguish it from closely related species of the genus *Actinomadura*. *A. fibrosa* IMSNU 22177ᵀ (Mertz & Yao, 1990) and *A. rudentiformis* HMC1ᵀ (Rose & Meyers, 2007) did not produce spores or sporangia on agar media. In addition, *A. nitritigenes* DSM 44137ᵀ (Lipski & Altendorf, 1995) and *A. fulvescens* DSM 43923ᵀ (Terekhova et al., 1982) produced spore chains; however, their spores formed a smooth surface. Interestingly, although CYP1-1Bᵀ formed a sub-cluster with *S. albida* IFO 12248ᵀ (Figs 2, S2 and S3), its spores showed no motility and sporangia were absent.

Stackebrandt & Ebers (2006) recommended that the 16S rRNA gene sequence similarity range above 97.7–99 % should be mandatory for testing the genomic uniqueness of a novel isolate. On the basis of 16S rRNA gene analysis, only three species of the genus *Actinomadura*, including *A. nitritigenes* DSM 44137ᵀ, *Actinomadura madurae* DSM 43067ᵀ and *Actinomadura darangshiensis* DLS-70ᵀ, showed 16S rRNA gene sequence similarity higher than 98.7 %. Nevertheless, *A. madurae* DSM 43067ᵀ and *A. darangshiensis* DLS-70ᵀ formed a clearly separate cluster from strain CYP1-1Bᵀ (Figs 2, S2 and S3). Furthermore, unlike strain CYP1-1Bᵀ, *A. madurae* DSM 43067ᵀ (Trujillo & Goodfellow, 2012) and *A. darangshiensis* DLS-70ᵀ (Lee & Kim, 2015) produced a hooked or curled spore chain with warty spores. On the basis of these findings, only *A. nitritigenes* DSM 44137ᵀ (=JCM 10104ᵀ) was selected for DNA–DNA hybridization study.

The levels of DNA–DNA relatedness values between CYP1-1Bᵀ and *A. nitritigenes* JCM 10104ᵀ were 30.3 %. These values were below the threshold value of 70 % for distinguishing genomic species (Wayne et al., 1987). Moreover, CYP1-1ᵀ could be distinguished from *A. nitritigenes* JCM 10104ᵀ using phenotypic characteristics, in particular NaCl tolerance, pH and temperature range for growth, milk peptonization, liquefaction of gelatin, utilization of D-fructose, D-galactose, glycerol, D-melibiose, l-raffinose, D-ribose and salicin. It was evident from the genotypic and phenotypic data that CYP1-1Bᵀ should represent a novel species of the genus *Actinomadura*.

On the basis of morphological and cultural characteristics, chemotaxonomic data and 16S rRNA gene sequence of strain CYP1-1Bᵀ, it is suggested that this strain represents a member of the genus *Actinomadura*. Therefore, the differences in cultural, physiological and biochemical characteristics, including DNA–DNA relatedness, support the hypothesis that CYP1-1Bᵀ represents a novel species of the genus *Actinomadura* for which the name *Actinomadura montaniterrae* sp. nov. is proposed.

**Description of *Actinomadura montaniterrae* sp. nov.**

(mon.ta.ni.ter.‘rae. L. adj. montanus of a mountain, L. n. terra soil; N.L. gen. n. montaniterrae of mountain soil, where the type strain was isolated). Cells are aerobic, Gram-reaction-positive and form an extensively branched non-fragmenting substrate mycelium. The oval to ellipsoid spores are formed along the sporogenous hyphae and its branches. Spores exhibit rough surfaces after cultivation at 30 °C for 30 days. The colonial appearance and substrate mycelium color are dark grayish yellow after growth on ISP 2 for 14 days. The aerial mass is very pale green. Grows well on all test media (ISP2 to ISP7 and nutrient agar). Grayish yellow pigment is observed on ISP2 medium. The cell wall peptidoglycan comprises meso-diaminopimelic acid. The acyl-type of muramic acid is acetyl. Temperature and pH range for growth are 20–45 °C and pH 4.5–8, respectively. The maximum NaCl tolerance is 5 % (w/v). For carbon sources utilizes D-glucose, D-mannitol, L-rhamnose, lactose, L-arabinose, D-fructose, cellobose, sucrose and D-xylene. Starch hydrolysis is positive, but peptonization of milk, gelatin liquefaction and nitrate reduction are negative. The diagnostic diaminoc acid of the peptidoglycan is meso-diaminopimelic acid. Whole-cell sugars are galactose, glucose, madurose and ribose. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol mannosides. The predominant fatty acids consist of 10-methyl C₁₆:0, C₁₈:0 and C₁₈:1ω9c. The predominant menaquinones are MK-9(H₈), MK-9(H₆) and MK-9(H₄).

The type strain is CYP1-1Bᵀ (=JCM16995ᵀ=KCTC 39784ᵀ=PCU 349ᵀ=STISTR 2400ᵀ), isolated from mountain soil collected from Chaiyaphum Province, Thailand. The DNA G+C content of the type strain is 74.3 mol%.

**Acknowledgements**

This study was supported by the Thailand Research Fund for the 2006 Royal Golden Jubilee PhD Program as a scholarship to A. S. and a Grant for International Research Integration: Research Pyramid, Ratchadaphiseksomphot Endowment Fund (GCURP_58_01_33_01), Chulalongkorn University.
References


