Micromonospora eburnea sp. nov., isolated from a Thai peat swamp forest

Chitti Thawai,^{1,3} Somboon Tanasupawat,¹ Takashi Itoh,³
Khanit Suwanborirux,² Ken-ichiro Suzuki⁴ and Takuji Kudo³

Correspondence

Somboon Tanasupawat
Somboon.T@Chula.ac.th

^{1,2}Departments of Microbiology¹ and Pharmacognosy², Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

³Japan Collection of Microorganisms, RIKEN BioResource Center, Wako, Saitama 351-0198, Japan

⁴NITE Biological Resource Center, National Institute of Technology and Evaluation, Kisarazu, Chiba 292-0818, Japan

Two actinomycete strains, LK2-10^T and LK2-5, which produced single, non-motile spores, were isolated from peat swamp forest soil in Yala Province, Thailand. A polyphasic study was carried out to establish the taxonomic position of these strains. Morphological and chemotaxonomic characteristics of these strains coincided with those of the genus *Micromonospora*. Phylogenetic analysis using 16S rRNA gene sequences also indicated that these strains should be classified in the genus *Micromonospora* and clearly separated from their closest relative, *Micromonospora nigra* DSM 43818^T. Furthermore, a combination of DNA–DNA hybridization results and physiological and biochemical properties indicated that these strains were distinguished from all recognized *Micromonospora* species. These strains therefore represent a novel species, for which the name *Micromonospora eburnea* sp. nov. is proposed. The type strain is LK2-10^T (=JCM 12345^T=PCU 238^T=DSM 44814^T=TISTR 1531^T).

The genus *Micromonospora* was first described by Ørskov (1923) for actinomycete strains that produce spores singly borne on sporophores branched from substrate hyphae. Skerman *et al.* (1980) listed 12 species and seven subspecies as members of the genus *Micromonospora*; subsequently, *Micromonospora olivasterospora* (Kawamoto *et al.*, 1983), *Micromonospora rosaria* (Horan & Brodsky, 1986) and *Micromonospora chersina* (Tomita *et al.*, 1992) have been recognized as novel species. Lee *et al.* (1999) transferred *Catellatospora matsumotoense* to the genus *Micromonospora* as *Micromonospora matsumotoense* on the basis of 16S rRNA gene sequence analysis. The genus was well defined on both a chemotaxonomic and a phylogenetic basis (Kroppenstedt, 1985; Kawamoto, 1989; Koch *et al.*, 1996a, b; Lee *et al.*, 1999) as well as on morphological grounds. Reclassification of *Micromonospora* species was carried out by Kasai *et al.* (2000) on the basis of *gyrB* gene sequence analyses and DNA–DNA hybridization experiments. Consequently, the genus *Micromonospora* is

considered to accommodate 15 species: *Micromonospora aurantiaca*, *Micromonospora carbonacea*, *Micromonospora chalicea*, *M. chersina*, *Micromonospora coerulea*, *Micromonospora echinospora*, *Micromonospora gallica*, *Micromonospora halophytica*, *Micromonospora inositol*, *M. matsumotoense*, *Micromonospora nigra*, *M. olivasterospora*, *Micromonospora pallida*, *Micromonospora purpureochromogenes* and *M. rosaria*. According to Kawamoto (1989), the type strain (NCTC 4582^T) of *M. gallica* is no longer available.

During investigation of novel actinomycetes from peat swamp forest soil in southern Thailand, we isolated two strains, LK2-10^T and LK2-5, that showed typical morphological characteristics of the genus *Micromonospora* but with an unusual colony colour for this genus. Here we report on the taxonomic characterization and classification of these isolates and propose a novel species, *Micromonospora eburnea* sp. nov., for the strains.

Strains LK2-10^T and LK2-5 were isolated from soil samples collected from peat swamp forest at Lankwai in Yala Province, Thailand. Samples were taken from the top peat layer (surface of soil). First, 0.5 g soil was suspended in 4.5 ml sterile distilled water, and the suspension was treated using a wet-heat technique (70 °C for 15 min). Next, 0.5 ml of this suspension was transferred to another 4.5 ml sterile distilled water and this step was repeated to

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Abbreviation: A₂pm, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LK2-10^T and LK2-5 are AB107231 and AB159778.

set up a ten-fold dilution series to 10^{-3} . At the final dilution step, aliquots of 100 μ l were spread onto starch-casein nitrate agar (pH 7.2) supplemented with 37 μ g novobiocin ml^{-1} and 223 μ g mycostatin ml^{-1} . After 21 days of aerobic incubation at 30 °C, growth of strains LK2-10^T and LK2-5 was indicated by yellowish white colonies. The colonies of these strains were transferred onto yeast extract-malt extract agar (ISP2 medium).

Morphological properties of strains LK2-10^T and LK2-5 grown on ISP2 medium were observed by light and scanning electron microscopy (model JSM-5410 LV; JEOL). Samples for scanning electron microscopy were prepared by cutting a block from an agar plate and then fixing it in 1% OsO₄ in 0.1 M phosphate buffer (pH 7.2) at room temperature for 4 h. The samples were dehydrated in a graded series of ethanol and then were dried with a critical-point dryer (model Samdri-780, Balzers CPD 020). The samples were placed onto a stub bearing adhesive and sputter-coated with gold under vacuum.

Phenotypic properties were examined by using several standard methods; culture characteristics were tested using 14-day cultures grown at 30 °C on various agar media (Table 1). The Jacal Colour Card L2200 (Japan Colour Research Institute) was used for determining colour designations and names. Decomposition of various compounds was examined using the basal medium recommended by Gordon *et al.* (1974). Temperature and NaCl tolerance were determined on ISP2 medium. Carbon source utilization was tested using ISP9 medium (Shirling & Gottlieb, 1966) supplemented with a final concentration of 1% of the tested carbon sources and 0.05% Casamino acid. Gelatin liquefaction, peptonization of milk, nitrite production, cellulose decomposition and starch hydrolysis were determined by cultivation on various media as described by Arai (1975) and Williams & Cross (1971). Melanin and hydrogen sulphide production were examined on slants

of tyrosine agar and peptone iron agar supplemented with 0.1% (w/v) yeast extract, respectively.

Freeze-dried cells used for chemotaxonomic analyses were obtained from cultures grown in yeast extract-malt extract broth (containing 0.4% glucose, 0.4% yeast extract and 1% malt extract; pH 7.3) on a rotary shaker at 30 °C. Cell wall peptidoglycan was prepared and hydrolysed by the methods of Kawamoto *et al.* (1981), and the amino acid composition was analysed with an automatic amino acid analyser. The isomers of diaminopimelic acid (A₂pm) in cell walls were determined by the method of Stanek & Roberts (1974). The acyl group of muramic acid in peptidoglycan was determined by the method of Uchida & Aida (1984). The reducing sugars from whole cell hydrolysates were analysed by the HPLC method of Mikami & Ishida (1983). Phospholipids in cells were extracted and analysed by the method of Minnikin *et al.* (1984). Fatty acid methyl ester analysis was performed by GLC according to the instructions of the Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Isoprenoid quinones were extracted by the method of Collins *et al.* (1977) and were analysed by HPLC equipped with a 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 ISP2 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 DNA base composition (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

Table 1. Culture characteristics of strains LK2-10^T and LK2-5

Medium	LK2-10 ^T			LK2-5		
	Growth	Colour of colony	Soluble pigment	Growth	Colour of colony	Soluble pigment
Yeast extract-malt extract agar (ISP2 medium)	Good	Greyish black	Pale yellow	Abundant	Greyish black	Pale yellow
Oatmeal agar (ISP3 medium)	Abundant	Dull orange	Pale yellow	Abundant	Dull orange	Pale yellow
Inorganic salts-starch agar (ISP4 medium)	Poor	Colourless	–	Poor	Colourless	–
Glycerol-asparagine agar (ISP5 medium)	Poor	Yellowish white	–	Poor	Yellowish white	–
Peptone-yeast extract iron agar (ISP6 medium)	Good	Dark yellowish brown	–	Good	Greyish black	–
Tyrosine agar (ISP7 medium)	Poor	Yellowish white	–	Moderate	Yellowish white	–
Glucose-asparagine agar	Poor	Yellowish white	–	Moderate	Yellowish white	–
Czapek's agar	Poor	Yellowish white	–	Moderate	Yellowish white	–
Nutrient agar	Moderate	Dark yellowish brown	Pale yellow	Good	Dark yellowish brown	Pale yellow

were carried out as described by Nakajima *et al.* (1999). The 16S rRNA gene sequence was multiply aligned with selected sequences obtained from the GenBank/EMBL/DBJ databases by using CLUSTAL W version 1.81. Alignment was manually verified 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 the MEGA program version 2.1. Confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. 16S rRNA gene sequence similarities among closely related species were calculated manually after pairwise alignments obtained using the CLUSTAL X program (Thompson *et al.*, 1997). Gaps and ambiguous nucleotides were eliminated from the calculations.

The morphological and chemical properties of strains LK2-10^T and LK2-5 are consistent with their classification in the genus *Micromonospora* (Kawamoto, 1989). Strains LK2-10^T and LK2-5 produced well-developed and branched substrate hyphae on ISP2 medium, but no aerial hyphae. Spores were borne singly on the substrate hyphae, and were 0.45 µm in diameter. Spores had a rough and nodular surface and were non-motile. The colour of the substrate mycelium was yellowish white to dull orange (Table 1). Pale-yellow soluble pigment is produced in ISP2 and ISP3 media and in nutrient agar.

Strains LK2-10^T and LK2-5 had identical chemotaxonomic characteristics, and these were similar to those of members of the genus *Micromonospora*. Cell wall hydrolysates of the two strains contained glutamic acid, glycine, alanine and A₂pm in a molar ratio of 1.0:0.9:0.5:1.1 (calculated by defining the amount of glutamic acid as 1.0), and the isomer of A₂pm was *meso*, indicating that these strains have wall chemotype II of Lechevalier & Lechevalier (1970) and peptidoglycan type A1γ of Schleifer & Kandler (1972). The acyl type of cell wall muramic acid was glycolyl. The strains contained glucose, xylose, arabinose, galactose,

mannose and ribose as whole-cell sugars, but rhamnose was not detected (whole-cell sugar pattern D of Lechevalier & Lechevalier, 1970). Characteristic phospholipids were diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositolmannosides and phosphatidylethanolamine, but not phosphatidylcholine. This pattern corresponds to phospholipid type II of Lechevalier *et al.* (1977). The major cellular fatty acids of strains LK2-10^T and LK2-5 were iso-C_{15:0}, iso-C_{16:0}, iso-C_{17:0}, anteiso-C_{15:0}, C_{17:0} and anteiso-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), MK-10(H₄) and MK-9(H₆), and minor amounts of MK-9(H₈), MK-10(H₆), MK-10(H₈) and MK-9(H₂) were also present. The DNA G+C contents of strains LK2-10^T and LK2-5 were 71.5 and 71.2 mol%.

Almost complete 16S rRNA gene sequences (1477 nt) were determined for strains LK2-10^T and LK2-5; a 1421 nt fragment was used for phylogenetic analysis and compared against 16S rRNA gene sequences of members of the family *Micromonosporaceae*. Phylogenetic analysis based on this large dataset revealed that strains LK2-10^T and LK2-5 were placed within the clade of the genus *Micromonospora* (data not shown). When the sequences of strains LK2-10^T and LK2-5 were compared with corresponding 16S rRNA gene sequences of all type strains of recognized *Micromonospora* species, selected sequences of the genus *Actinoplanes* and the 16S rRNA gene sequence of *Streptomyces ambofaciens* (as an outgroup), they formed a clade with *M. nigra* DSM 43818^T (Fig. 1). 16S rRNA gene sequence similarity values between strain LK2-10^T and all of the type strains of the genus *Micromonospora* ranged from 98.1% (with *M. matsumotoense* IMSNU 22003^T) to 99.1% (with *M. nigra* DSM 43818^T) and strain LK2-10^T showed 99.9% sequence similarity to strain LK2-5; these similarities represent 28, 14 and one nucleotide differences, respectively. On the basis of morphological, chemotaxonomic and phylogenetic data, the isolates should be classified in the genus *Micromonospora*.

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on almost-complete 16S rRNA gene sequences showing relationships among strains LK2-10^T, LK2-5, the 14 recognized *Micromonospora* species and representatives of the genus *Actinoplanes* (A.). *Streptomyces ambofaciens* is 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 >50% are indicated. Bar, 0.01 substitutions per nucleotide position.

Table 2. Differential physiological and biochemical characteristics of strains LK2-10^T and LK2-5 and type strains of the closest *Micromonospora* species

Strains: 1, LK2-10^T; 2, LK2-5; 3, *M. nigra* JCM 8973^T; 4, *M. halophytica* JCM 3125^T; 5, *M. aurantiaca* JCM 10878^T. +, Positive; –, negative; w, weakly positive. All strains were positive for nitrate reduction, peptonization of milk, utilization of D-glucose, D-melibiose, D-galactose, cellobiose and D-xylose and growth at 40 °C and in 4% NaCl and negative for decomposition of adenine, hypoxanthine and xanthine and utilization of D-ribose.

Characteristic	1	2	3	4	5
Gelatin liquefaction	+	+	–	w	+
Starch hydrolysis	w	w	+	+	–
Tyrosine decomposition	–	–	–	–	+
Growth at 45 °C	+	w	–	–	–
Utilization of:					
D-Mannitol	w	w	–	–	–
L-Rhamnose	+	+	–	w	–
D-Raffinose	+	+	+	+	–
Glycerol	+	+	–	–	–
Salicin	+	+	+	+	–
Lactose	+	+	+	+	–
L-Arabinose	–	–	w	+	w
D-Fructose	–	–	w	+	+

The optimal temperature for growth of strains LK2-10^T and LK2-5 was 25–30 °C. No growth was observed above 45 °C. The maximum NaCl concentration for growth was 4 %, as with *M. nigra* JCM 8973^T, *M. aurantiaca* JCM 10878^T and

M. halophytica JCM 3125^T. Strains LK2-10^T and LK2-5 showed almost identical physiological and biochemical properties to *M. nigra* JCM 8973^T, *M. aurantiaca* JCM 10878^T and *M. halophytica* JCM 3125^T, but could be distinguished on the basis of growth at 45 °C and utilization of D-mannitol, glycerol, L-arabinose and D-fructose. Differential characteristics between the new isolates and closely related type strains of *Micromonospora* species are shown in Table 2. The colour of substrate mycelium of the isolates was yellowish white, which distinguishes them from all other type strains of *Micromonospora* species, which generally have an orange colour. Levels of DNA–DNA relatedness between strains LK2-10^T and LK2-5 ranged from 89 to 92 %, whereas relatedness between the isolates and type strains of all recognized *Micromonospora* species except *M. gallica* ranged from 21 to 58 % (Table 3).

These phenotypic and genotypic data indicated that strains LK2-10^T and LK2-5 belong to the same species and merit classification as a novel species of the genus *Micromonospora*, for which we propose the name *Micromonospora eburnea* sp. nov.

Description of *Micromonospora eburnea* sp. nov.

Micromonospora eburnea (eb.ur'ne.a. L. fem. adj. *eburnea* ivory, referring to colour of colonies).

Aerobic, Gram-positive, mesophilic, non-motile actinomycete that forms well-developed and branched substrate hyphae. Colonies are yellowish white and turn greyish black after sporulation in ISP2 medium. Single spores are formed on substrate hyphae. Aerial mycelium is absent. The spore

Table 3. DNA base composition and DNA–DNA relatedness among the isolates and type strains of *Micromonospora* species

Strain	G+C content (mol%)	DNA–DNA binding (%) with labelled DNA from:	
		LK2-10 ^T	LK2-5
LK2-10 ^T	71.5	100	92
LK2-5	71.2	89	100
<i>M. chersina</i> JCM 9459 ^T	72.9	36	51
<i>M. coerulea</i> JCM 3175 ^T	71.7	26	40
<i>M. purpureochromogenes</i> JCM 3156 ^T	73.0	28	42
<i>M. echinospora</i> JCM 3073 ^T	71.7	21	36
<i>M. carbonacea</i> JCM 3139 ^T	73.3	27	54
<i>M. chalcone</i> JCM 3031 ^T	71.9	27	41
<i>M. inositol</i> JCM 6239 ^T	71.4	29	45
<i>M. olivasterospora</i> JCM 7348 ^T	71.9	24	38
<i>M. nigra</i> JCM 8973 ^T	71.7	26	42
<i>M. halophytica</i> JCM 3125 ^T	72.5	36	58
<i>M. aurantiaca</i> JCM 10878 ^T	71.6	28	41
<i>M. rosaria</i> JCM 3159 ^T	72.9	24	40
<i>M. matsumotoense</i> JCM 9104 ^T	71.0	26	39
<i>M. pallida</i> JCM 3133 ^T	71.1	28	45

surface appears rough and nodular. Spores are non-motile. Pale yellow soluble pigment is produced in ISP2 and ISP3 media and in nutrient agar. Nitrate is reduced to nitrite. Utilizes D-glucose, L-rhamnose, D-melibiose, D-raffinose, glycerol, salicin, lactose, cellobiose and D-xylose; weakly utilizes D-mannitol and D-galactose but not L-arabinose, D-fructose or D-ribose. Peptonization of milk, hydrolysis of starch and gelatin liquefaction are positive, but formation of melanin and H₂S production are negative. Optimal temperature for growth is 25–30 °C. No growth occurs above 45 °C. The maximum NaCl concentration for growth is 4 %. Cell wall contains glutamic acid, glycine, alanine and meso-A₂pm in the molar ratio 1:0.9:0.5:1.1. Acyl type of the cell wall is glycolyl. The predominant menaquinone is MK-9(H₄). Characteristic whole-cell sugars are xylose and arabinose. The phospholipid profile comprises diphosphatidylglycerol, phosphatidylinositolmannosides, phosphatidylinositol and phosphatidylethanolamine, but not phosphatidylcholine. The major fatty acids of the type strain are iso-C_{15:0} (24.1 %), iso-C_{16:0} (17.9 %), iso-C_{17:0} (7.7 %), anteiso-C_{15:0} (8.5 %), C_{17:0} (7.9 %) and anteiso-C_{17:0} (10.1 %). The G+C content of the DNA is 71.5 mol%. Habitat is soil.

The type strain is LK2-10^T (=JCM 12345^T=PCU 238^T=DSM 44814^T=TISTR 1531^T).

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