Micromonospora aurantionigra sp. nov., Isolated from a Peat Swamp Forest in Thailand

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Actinomycete strain TT1-11^T, which produces single non-motile spores on substrate hyphae, was isolated from peat swamp forest soil in Thailand. The isolate showed morphological and chemotaxonomic properties consistent with its classification in the genus *Micromonospora*. Phylogenetic analysis using 16S rDNA sequences supported this assignment and it formed a subclade with *M. coerulea*. This isolate can be distinguished from *M. coerulea* by its physiological and biochemical properties, and it showed a less than 53% DNA relatedness to the type strains of all the validly named *Micromonospora* species. On the basis of phenotypic, genotypic and phylogenetic data, strain TT1-11^T (=JCM 12357^T =PCU 239^T =DSM 44815^T=TISTR 1232^T) is proposed as the type strain of a novel *Micromonospora* species, *Micromonospora aurantionigra* sp. nov.

INTRODUCTION

The genus *Micromonospora* was first described by Ørskov¹⁾ for an actinomycete that produces non-motile spores borne singly on sporophores branched from substrate hyphae. This genus is well established according to its morphological and chemotaxonomic characteristics^{2–4)} as well as through 16S rDNA-based phylogenetic analyses⁵⁾.

In the *Approved Lists of Bacterial Names*⁶⁾, 12 species and 7 subspecies were cited as members of the genus *Micromonospora*, and *Micromonospora olivasterospora*⁷⁾, *M. rosaria*⁸⁾ and *M. chersina*⁹⁾ were later validly proposed as new species. Lee *et al.*¹⁰⁾ transferred *Catellatospora matsumotoense* to the genus *Micromonospora* as *M. matsumotoense* on the basis of 16S rDNA analysis. Recently, reclassification at the species level of the genus *Micromonospora* was carried out by Kasai *et al.*¹¹⁾ on the basis of *gyrB* sequence analyses and DNA-DNA hybridization experiments. They reclassified the members of *Micromonospora* into the following 14 species: *M. aurantiaca*, *M. carbonacea*, *M. chalcea*, *M. chersina*, *M. coerulea*, *M. echinospora*, *M. gallica*, *M. halophytica*, *M. inositol*, *M. nigra*, *M. olivasterospora*, *M. pallida*, *M. purpureochromogenes* and *M. rosaria*. Consequently, at present, the genus *Micromonospora* is thought to accommodate 15 species, that is, the 14 species reclassified by Kasai *et al.* and *M. matsumotoense*. According to Kawamoto¹²⁾, the type strain of *M. gallica* (NTCT 4582^T) is no longer available.

In the course of investigations of novel actinomycetes from peat swamp forest soil in southern Thailand, we isolated strain TT1-11^T showing unique phenotypic charac-

teristics that differ from all known type strains of the genus *Micromonospora*. Based on polyphasic taxonomic study, this isolate was shown to be a novel species of the genus *Micromonospora*, for which the name *Micromonospora aurantionigra* sp. nov. is proposed.

MATERIALS AND METHODS

Microorganisms and culture conditions

Strain TT1-11^T was obtained from a soil sample collected in a peat swamp forest in Thatien, Pattalung, Thailand and isolated on starch-casein nitrate agar after incubation at 30°C for 21 days. The colonies of this strain were purified on yeast extract-malt extract agar (ISP medium no. 2) and maintained on the same medium as a working culture. The 14 type strains of all the validly described species of the genus *Micromonospora*, except for *M. gallica*, were obtained from the Japan Collection of Microorganisms (JCM).

Morphology

Strain TT1-11^T was grown on ISP medium no. 2 at 30°C for 14 days then observed by light and scanning electron microscopes (model JSM-5410 LV; JEOL, Ltd., Tokyo, Japan). The sample for scanning electron microscopy was prepared by cutting a block from an agar plate, then fixing it with 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 dried with a critical point dryer (model Samdri-780, Balzers CPD 020). It was then placed onto a stub bearing adhesive and sputter-coated with gold under a vacuum¹³⁾.

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Cultural, physiological and biochemical characteristics

The phenotypic properties were examined using several standard methods; cultural characteristics were tested using 14-day cultures grown at 30°C on various agar media (Table 1). The *Jacal Color Card L2200* (Japan Color Research Institute) was used to determine the color designations and names. The decomposition of various compounds was examined using the basal medium recommended by Gordon *et al.*¹⁴⁾ The temperature range for growth and tolerance to NaCl was determined on ISP medium no. 2. Carbon source utilization was tested using ISP medium no. 9¹⁵⁾ supplemented with 1% (w/v) carbon sources and 0.05% (w/v) casamino acids. Gelatin liquefaction, milk peptonization, nitrate reduction and starch hydrolysis were determined as described by Arai¹⁶⁾, and Williams & Cross¹⁷⁾. Melanoid pigment production was examined in the slants of tyrosine and peptone-iron agars supplemented with 0.1% (w/v) yeast extract.

Chemotaxonomic characteristics

The 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 hydrolyzed according to the methods of Kawamoto *et al.*¹⁸⁾, and amino acid composition was analyzed with an automatic amino acid analyzer. The isomers of diaminopimelic acid (A₂pm) in the cell walls were determined according to the method of Stanek & Roberts¹⁹⁾, and the acyl group of muramic acid in the peptidoglycan was determined according to the method of Uchida & Aida²⁰⁾. The reducing sugars from the whole-cell hydrolysates were analyzed according to the HPLC method of Mikami & Ishida²¹⁾, and the phospholipids were extracted and analyzed according to the method of Minnikin *et al.*²²⁾ Fatty acid methyl ester analysis was performed by GLC according to the instructions of the Microbial Identification System (MIDI)^{23–24)}. Isoprenoid quinones were extracted according to the method of Collins *et al.*²⁵⁾ and analyzed with HPLC equipped with a Cosmosil 5C₁₈ column (4.6 by 150 mm; Nacalai Tesque, Kyoto, Japan). A mixture of methanol and 2-propanol (2:1, v/v) was used as the elution solvent.

Preparation of DNA, DNA base composition and DNA-DNA hybridization

Chromosomal DNA was extracted from cells grown in yeast extract-malt extract broth according to the method of Tamaoka²⁶⁾. The G+C content of the DNA was determined using the HPLC method of Tamaoka and Komagata²⁷⁾. An equimolar mixture of nucleotides for analysis of DNA base composition (Yamasa Shoyu, Choshi, Japan) was used as the quantitative standard. DNA-DNA relatedness was measured fluorometrically using the microplate hybridization method devised by Ezaki *et al.*²⁸⁾

Fig. 1. Scanning electron micrographs of strain TT1-11^T grown on yeast extract-malt extract agar at 30°C for 14 days. (A) Bar, 1 µm, (B) Bar, 2 µm.

Table 1. Cultural characteristics of strain TT1-11^T.

Medium	Growth	Color of colony	Soluble pigment
Yeast extract-malt extract agar (ISP medium no. 2)	Abundant	Brownish black to black	Brown
Oatmeal agar (ISP medium no. 3)	Abundant	Brownish black	None
Inorganic salts-starch agar (ISP medium no. 4)	Good	Orange to brownish black	None
Glycerol-asparagine agar (ISP medium no. 5)	Poor	Orange	None
Glucose-asparagine agar	Poor	Orange	None
Czapek's agar	Abundant	Orange	None
Nutrient agar	Moderate	Dark orange	None

PCR amplification of the 16S rDNA and sequence analysis

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.*²⁹⁾ The sequence was aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases using CLUSTAL W version 1.81. The alignment was manually verified and adjusted prior to the construction of a phylogenetic tree, which was constructed by using the neighbor-joining³⁰⁾ and maximum parsimony³¹⁾ methods in the MEGA program version 2.1³²⁾. The confidence values of the phylogenetic tree branches were determined using bootstrap analyses³³⁾ based on 1000 resamplings. The sequence similarity values among the close strains were calculated manually after pairwise alignments obtained using the CLUSTAL X program³⁴⁾. Gaps and ambiguous nucleotides were eliminated from the calculations.

Nucleotide sequence accession numbers

The DDBJ accession number for the 16S rDNA sequence of strain TT1-11^T is AB159779.

RESULTS

Morphology

Strain TT1-11^T produced well-developed and branched substrate hyphae on ISP medium no. 2 but no aerial hyphae. Spores were borne singly on the tips of sporophores produced on substrate hyphae, and were spherical in shape, rough on the surface (Fig. 1), and non-motile.

Cultural characteristics

As shown in Table 1, strain TT1-11^T grew well on ISP medium no. 2, oatmeal agar (ISP medium no. 3) and Czapek's agar. The color of the vegetative mycelium was vivid orange, turning to brownish black or black after sporulation. A brown diffusible pigment was produced in ISP medium no. 2 after incubation at 30°C for 21 days.

Biochemical and physiological characteristics

Strain TT1-11^T utilized D-glucose, D-melibiose, D-raffinose, salicin, lactose, D-galactose, cellobiose, D-ribose, L-arabinose, D-fructose and D-xylose but not L-rhamnose, glycerol and D-mannitol. Strain TT1-11^T was positive for starch hydrolysis and gelatin liquefaction, weakly positive for milk peptonization, and negative for nitrate reduction, xanthine, hypoxanthine, tyrosine and adenine decomposition, and the formation of melanoid pigments. Good growth was observed between 25 and 30°C. No growth was observed above 40°C. The pH range for growth was 5 to 10 and the NaCl concentration range for growth was 0 to 2%.

Chemotaxonomic characteristics

The chemotaxonomic characteristics of strain TT1-11^T coincided with those of members of the genus *Micromonospora*. The purified cell wall of strain TT1-11^T contained glutamic acid, glycine, alanine and A₂pm in a ratio of 1.0:0.8:0.5:1.1, and the isomer of A₂pm was *meso* accompanied by its 3-hydroxy derivative (3-OH-A₂pm). The glycolate test was positive, hence the strain has N-glycolyl muramic acid in the peptidoglycan. The whole-cell hydrolysates contained glucose, xylose, arabinose, galactose, mannose and ribose, but no rhamnose. Strain TT1-11^T was therefore characterized by cell wall type II of Lechevalier & Lechevalier²⁾, peptidoglycan type A1γ' of Schleifer & Kandler³⁵⁾ and whole-cell sugar pattern D of Lechevalier & Lechevalier²⁾. Diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositolmannosides and phosphatidylethanolamine were detected but phosphatidylcholine and glucosamine-containing phospholipids were not. (type II of Lechevalier *et al.*³⁾). The cellular fatty acid composition is shown in Table 2. The major fatty acids of strain TT1-11^T were iso-C_{16:0}, iso-C_{15:0} and small

Table 2. Cellular fatty acid composition of strain TT1-11^T.

Fatty acid	Percentage of total
Saturated fatty acids	
C _{12:0}	0.3
C _{15:0}	0.5
C _{16:0}	0.7
C _{17:0}	1.4
C _{18:0}	1.5
C _{19:0}	0.3
Unsaturated fatty acids	
2OH-C _{16:1}	0.6
C _{17:1} ω8c	1.5
C _{18:1} ω9c	1.1
Branched fatty acids	
i-C _{10:0}	0.3
i-C _{12:0}	0.8
i-C _{13:0}	0.3
i-C _{14:0}	3.5
i-C _{15:0}	11.5
a-C _{15:0}	4.6
i-C _{15:1}	1.0
i-C _{16:0}	39.1
i-C _{16:1}	9.5
3OH-i-C _{16:0}	0.2
i-C _{17:0}	4.7
a-C _{17:0}	5.8
i-C _{18:0}	1.9
i-C _{17:1} ω9c	2.7
a-C _{17:1} ω9c	1.4
10-Methyl fatty acids	
10-Me C _{17:0}	2.4
10-Me C _{18:0} (TBSA)	0.9
Summed feature 3 ^a	0.3
Summed feature 6 ^a	0.1

^a Summed features 3 contains C16:1ω7c and/or C15:0 iso 2-OH; summed features 6 contains C19:1ω11c and/or C19:1ω9c.

amount of iso- $C_{17:0}$, anteiso- $C_{17:0}$, anteiso- $C_{15:0}$ were also present. This pattern corresponds to fatty acid type 3b of Kroppenstedt⁴⁾. Mycolic acids were absent. The major menaquinones were MK-10(H_4) (55%), MK-9(H_4) (16%), MK-10(H_6) (11%), and small amounts of MK-9(H_6), MK-10(H_2), MK-10(H_8) and MK-9(H_2) were also present. The G+C content of the DNA was 72.8 mol%.

Phylogenetic analysis

An almost complete 16S rDNA sequence comprising of 1,482 nucleotides was determined for strain TT1-11^T and its 1,426 nucleotides were used for phylogenetic analysis. The strain showed more than 98.0% similarity to members of the genus *Micromonospora*. Of the type strains of the 14 validly named *Micromonospora* species, *M. coerulea* (98.7%) showed the highest 16S rDNA similarity to strain TT1-11^T. The sequence of strain TT1-11^T was compared with the corresponding 16S rDNA sequences of all the type strains validly described as *Micromonospora* species, except for *M. gallica*, as well as with selected members of the genus *Actinoplanes* and *Streptomyces ambofaciens* (as an outgroup). As a result, the isolate formed a clade with *M. coerulea* but this was not supported by a high

Table 3. DNA base composition and DNA-DNA relatedness between strain TT1-11^T and type strains of validly described *Micromonospora* species.

Strain	G+C content (mol%)	Percentage DNA complementarity with labeled DNA from strain TT1-11 ^T
TT1-11 ^T	72.8	100
<i>M. chersina</i> JCM 9459 ^T	72.9	48
<i>M. coerulea</i> JCM 3175 ^T	71.7	37
<i>M. purpureochromogenes</i> JCM 3156 ^T	73.0	43
<i>M. echinospora</i> JCM 3073 ^T	71.7	33
<i>M. carbonacea</i> JCM 3139 ^T	73.3	32
<i>M. chalicea</i> JCM 3031 ^T	71.9	41
<i>M. inositola</i> JCM 6239 ^T	71.4	38
<i>M. olivasterospora</i> JCM 7348 ^T	71.9	35
<i>M. nigra</i> JCM 8973 ^T	71.7	37
<i>M. halophytica</i> JCM 3125 ^T	72.5	53
<i>M. aurantiaca</i> JCM 10878 ^T	71.6	40
<i>M. rosaria</i> JCM 3159 ^T	72.9	34
<i>M. matsumotoense</i> JCM 9104 ^T	71.0	32
<i>M. pallida</i> JCM 3133 ^T	71.1	35

Fig. 2. Neighbour-joining tree³⁰⁾ based on almost-complete 16S rDNA sequences showing relationships among strain TT1-11^T, the 14 validly described *Micromonospora* species and selected type strains of the genus *Actinoplanes*. *Streptomyces ambofaciens* is used as the outgroup. Asterisks indicate branches of the tree that were also found in the tree constructed using the maximum-parsimony method³¹⁾. 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.

Table 4. Differential physiological and biochemical characteristics of strain TT1-11^T and the type strains of the genus *Micromonospora* having hydrogenated MK-10 as predominant isoprenoid quinones.

Characteristic ^a	Strain ^b									
	1	2	3	4	5	6	7	8	9	10
Nitrate reduction	–	–	–	–	+	–	–	–	–	+
Peptonization of milk	w	+	+	–	w	+	+	+	+	+
Starch hydrolysis	+	+	+	w	–	+	+	+	+	–
Decomposition of:										
Tyrosine	–	–	–	–	–	–	–	–	–	+
Growth at:										
10°C	–	+	–	–	+	–	–	+	–	–
12°C	+	+	–	+	+	–	–	+	–	+
Maximum NaCl tolerance	2%	1.5%	5%	1.5%	1.5%	3%	3%	3%	2%	4%
Utilization of:										
D-Mannitol	–	–	–	–	–	–	–	–	+	–
D-Ribose	+	–	–	–	–	–	w	w	w	–
L-Rhamnose	–	–	–	–	–	+	–	–	+	–
D-Melibiose	+	+	+	+	+	w	–	–	+	+
D-Raffinose	+	+	+	w	w	–	–	+	–	w
Glycerol	–	–	–	–	–	–	–	–	–	–
Salicin	+	w	–	–	–	–	–	–	–	–
Lactose	+	+	+	+	w	w	–	+	+	–
D-Galactose	+	+	+	+	+	+	–	+	+	+
L-Arabinose	+	–	w	–	–	+	+	+	w	w
Cellobiose	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	w	w	+	+	–	+	–	+
D-Xylose	+	+	+	+	+	+	+	+	+	+

^a +, Positive; –, negative; w, weakly positive.

^b 1, TT1-11^T; 2, *M. coerulea* JCM 3175^T; 3, *M. chalicea* JCM 3031^T; 4, *M. inositol*a JCM 6239^T; 5, *M. purpureochromogenes* JCM 3156^T; 6, *M. olivasterospora* JCM 7348^T; 7, *M. echinospora* JCM 3073^T; 8, *M. matsumotoense* JCM 9401^T; 9, *M. rosaria* JCM 3159^T; 10, *M. aurantiaca* JCM 10878^T.

bootstrap value (Fig 2).

DNA-DNA relatedness

A DNA-DNA hybridization experiment was carried out between our isolate and the 14 type strains of the validly described *Micromonospora* species. The levels of DNA-DNA relatedness between strain TT1-11^T and the other type strains ranged from 32 to 53% (Table 3).

DISCUSSION

Strain TT1-11^T showed typical morphological and chemotaxonomic characteristics of the genus *Micromonospora* such as the formation of spores singly borne on substrate hyphae, cell wall type II³⁵, presence of *N*-glycolyl muramic acid in the peptidoglycan, whole-cell sugar pattern D², phospholipid type II³ and cellular fatty acid type 3b⁴. The phylogenetic data based on the 16S rDNA sequences clearly supported the assignment of this strain to the genus *Micromonospora*. The genus *Micromonospora* currently consists of 15 validly described species, which (except for *M. gallica*) can be divided into three groups based on the predominant menaquinone components, namely, MK-9, MK-10 and MK-12 groups¹². Species containing hydrogenated MK-9 as the major menaquinones (MK-9 group) are *M. carbonacea*, *M. halo-*

phytica, *M. nigra*¹²) and *M. chersina*⁹), while the MK-12 group is composed of one species, *M. pallida*¹²). Strain TT1-11^T belongs to the MK-10 group together with *M. chalicea*, *M. inositol*a, *M. coerulea*, *M. purpureochromogenes*, *M. olivasterospora*, *M. echinospora*¹²), *M. matsumotoense*¹⁰), *M. rosaria* and *M. aurantiaca*. Table 4 shows the differential physiological and biochemical characteristics of this new isolate and the type strains belonging to the MK-10 group. Strain TT1-11^T showed a different physiological and biochemical pattern compared to the other type strains belonging to the MK-10 group. In particular, the utilization of salicin effectively discriminates strain TT1-11^T from the other type strains of this group, while the utilization of L-arabinose and D-ribose differed between strain TT1-11^T and the phylogenetically closest species, *M. coerulea*. In addition, DNA relatedness values between strain TT1-11^T and the 14 type strains of the other validly described species were only 53% or less (Table 3). On the basis of these phenotypic and genotypic characteristics, it is suggested that strain TT1-11^T should be recognized as a novel species of the genus *Micromonospora* under the proposed name *Micromonospora aurantionigra* sp. nov.

Description of *Micromonospora aurantionigra* sp. nov.

Micromonospora aurantionigra (au.ran.ti.o.ni'gra. L. n. *aurum* gold; L. adj. *niger* black; N.L. fem. adj. *aurantionigra* pertaining to the color of colony). Aerobic, Gram-positive, mesophilic, non-motile actinomycete that forms well-developed and branched substrate hyphae. Colonies are vivid orange in ISP medium 2, turning to brownish black or black after sporulation. Single spores are formed on the substrate hyphae. Aerial mycelium is absent. The spores are non-motile and their surface appears rough. Brown soluble pigment is produced in ISP medium no. 2. Utilizes D-glucose, D-melibiose, D-raffinose, salicin, lactose, D-galactose, cellobiose, D-ribose, L-arabinose, D-fructose and D-xylose, but not L-rhamnose, glycerol and D-mannitol. Hydrolysis of starch and gelatin liquefaction are positive, milk peptonization is weakly positive, and nitrate reduction, xanthine, hypoxanthine, tyrosine and adenine decomposition, and the production of melanin and hydrogensulfide are negative. Good growth is observed between 25 and 30°C. No growth occurs above 40°C. The maximum NaCl concentration for growth is 2%. Cell wall contains glutamic acid, glycine, alanine and *meso*- and 3-OH-diaminopimic acid. The acyl type of the cell wall is glycolyl. The characteristic whole-cell sugars are xylose and arabinose. The phospholipid profile contains diphosphatidylglycerol, phosphatidylinositolmannosides, phosphatidylinositol and phosphatidylethanolamine, but not phosphatidylcholine. The major cellular fatty acids are iso-C_{16:0} and iso-C_{15:0}, and a small amount of iso-C_{17:0}, anteiso-C_{17:0} and anteiso-C_{15:0} are also present. Mycolic acids are absent. The predominant menaquinones are MK-10(H₄), MK-9(H₄), and MK-10(H₆). The G+C content of the DNA is 72.8 mol%. Habitat is soil. The type strain is strain TT1-11^T (=JCM 12357^T =PCU 239^T =DSM 44815^T =TISTR 1532^T).

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