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Microtetraspora malaysiensis sp. nov., isolated from Malaysian primary dipterocarp forest soil

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The taxonomic position of three actinomycete strains isolated from Malaysian soil was established by using a polyphasic approach. The isolates formed chains composed of four spores on the tip of sporophores branching from the aerial mycelium, and their chemotaxonomic properties were common to those of members of the family *Streptosporangiaceae*. These phenotypic properties as well as a phylogenetic analysis based on 16S rRNA gene sequences indicated that they should be classified in the genus *Microtetraspora*. The three isolates showed a unique pattern of cultural, physiological and biochemical properties that distinguished them from previously described species of the genus *Microtetraspora*. The isolates showed more than 72% DNA relatedness to each other, but only 58% or less relatedness to any previously described species. On the basis of the data presented, a new species of the genus *Microtetraspora*, *Microtetraspora malaysiensis*, is proposed. The type strain of the new species is strain H47-7^T (=JCM 11278^T=DSM 44579^T).

Key Words—Actinomycete; family *Streptosporangiaceae*; *Microtetraspora malaysiensis* sp. nov.; polyphasic taxonomy

Introduction

The genus *Microtetraspora* was proposed by Thiemann et al. (1968) for actinomycete strains on the basis of morphological properties, i.e. production of four spores in chains on aerial hyphae. The genus belongs to the family *Streptosporangiaceae* of the suborder *Streptosporangineae* within the order *Actinomycetales* (Stackebrandt et al., 1997). In the "Approved lists of bacterial names" (Skerman et al., 1980), four species were cited as members of the genus *Microtet-*

raspora. Subsequently, Miyadoh et al. (1989) transferred *M. viridis* to the genus *Actinomadura* as *A. viridis* from chemotaxonomic points of view. In 1990, Kroppenstedt et al. revised the genus *Microtetraspora* by transferring *Micropolyspora angiospora*, *Nocardio-opsis africana*, and thirteen *Actinomadura* species, which was represented by *Actinomadura pusilla*, to the genus *Microtetraspora*. Wang et al. (1996) suggested that the three species originally designated the genus *Microtetraspora*, i.e. *Microtetraspora glauca*, *Microtetraspora fusca* and *Microtetraspora niveoalba* forming spore chains composed of four spores, formed a coherent clade distant from the species of the *Microtetraspora pusilla* group on the basis of 16S rDNA sequence analysis, and Zhang et al. (1998) proposed emendation of the genus *Microtetraspora* and transfer

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of the *M. pusilla* group to the new genus *Nonomuraea*.

In the meanwhile, *Microtetraspora tyrrhenii* was proposed by Tomita et al. (1991) for a strain producing hook or spiral spore chains and the authors of the species stated that it should be placed in the *M. pusilla* group. Although nomenclatural change for the species has not been proposed because of unavailability of the type strain, it should be transferred to the genus *Nonomuraea* judging from the morphological characteristics.

During the course of seeking novel actinomycetes from tropical regions of Southeast Asia, we isolated three strains, H47-7^T, Y1-5-3 and Y25-12, showing typical morphological characteristics of the genus *Microtetraspora* from soil samples collected in Malaysia. In this paper we report taxonomic characterization and classification of these isolates and propose a new species *Microtetraspora malaysiensis* sp. nov. for the strains.

Materials and Methods

Bacterial strains. Strains H47-7^T, Y1-5-3 and Y25-12 were isolated from soil samples collected from just below the leaf litter of mainly *Shorea* spp. from primary lowland dipterocarp forest at Pasoh, Negeri Sembilan (strains H47-7^T, Y25-12) and steep hill dipterocarp forest at the Virgin Jungle Reserve, Gombak, Selangor (strain Y1-5-3) in Peninsular Malaysia. Both sites are maintained in original condition as conservation areas (Kochummen and LaFrankie, 1991; Manokaran and Kochummen, 1990). The isolate was maintained by the dilution-agar plating method using humic acid/vitamin (HV) agar (Hayakawa and Nonomura, 1987) and 0.02% yeast extract agar. The following type strains of the named species belonging to the genus *Microtetraspora* were used for the comparative studies: *M. glauca* JCM 3300^T, *M. fusca* JCM 3083^T and *M. niveoalba* JCM 3149^T.

Morphology. The isolates were grown on yeast extract-starch agar and oatmeal-nitrate agar for 14 days at 28°C, and observed with light and scanning electron microscopes. Samples for scanning electron microscopy were prepared as described previously (Itoh et al., 1989). Cultural characteristics were tested by using 14-day cultures grown at 28°C on selected agar media (Table 1). The "Color Harmony Manual" (Jacobson et al., 1958) was used for determining color designations of colonies.

Cultural, physiological and biochemical characteris-

tics. Decomposition of various compounds was examined by using the basal medium recommended by Gordon et al. (1974). Utilization of carbohydrates as sole carbon sources was tested by using neutralized yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI, USA) as a basal medium according to the method of Stevenson (1967). Utilization of organic acids was examined on the defined medium of Gordon et al. (1974) supplemented with the vitamin B complex solution used in HV agar (Hayakawa and Nonomura, 1987). The requirements for vitamins (each at a concentration of 0.5 µg ml⁻¹) were examined as described previously (Kudo et al., 1993). Tolerance to sodium chloride and growth temperature were determined on yeast extract-starch agar.

Chemotaxonomy. Freeze-dried cells for chemotaxonomic analyses were obtained from cultures grown in yeast extract-glucose broth (containing 10 g yeast extract and 10 g D-glucose in 1,000 ml distilled water; pH 7.2) on a rotary shaker at 28°C. Cell wall peptidoglycan was prepared and hydrolyzed by the methods of Kawamoto et al. (1981), and the amino acid composition was analyzed with an automatic amino acid analyzer. The isomers of diaminopimelic acid (A₂pm) in cell walls were determined by the method of Stanek and Roberts (1974), and reducing sugars from whole-cell hydrolysates were analyzed by the HPLC method of Mikami and Ishida (1983). The acyl group of muramic acid in peptidoglycan was determined by the method of Uchida and Aida (1984). Phospholipids in cells were extracted and identified by the method of Minnikin et al. (1984). Methyl esters of cellular fatty acids were prepared by the direct transmethylation method with methanolic hydrochloride (Suzuki and Komagata, 1983) and were analyzed using the MIDI system (Sherlock Microbial Identification System; MIDI, Hewlett Packard, Palo Alto, CA, USA). Isoprenoid quinones were extracted by the method of Collins et al. (1977) and were analyzed by HPLC equipped with a Cosmosil 5C₁₈ column (4.6 by 150 mm; Nacalai Tesque, Kyoto, Japan). The elution solvent was a mixture of methanol and 2-propanol (2:1, v/v).

16S rDNA sequence analysis. Genomic DNA extraction, PCR-mediated amplification of 16S rDNA, sequencing of the PCR products and determination of the phylogenetic position were carried out as described previously (Nakajima et al., 1999).

Nucleotide sequence accession numbers. The accession numbers of the 16S rDNA sequences of the

Fig. 1. (A) Light micrograph of strain H47-7^T grown on dilute (1/5) yeast extract-starch agar for 14 days at 28°C. (B) Scanning electron micrograph of strain H47-7^T grown on oatmeal-nitrate agar for 7 days at 28°C. Bar, 5 µm.

isolates determined in this study and submitted to the DDBJ are as follows: AB062383 (H47-7^T=JCM 11278^T), AB062384 (Y1-5-3=JCM 11279) and AB062385 (Y25-12=JCM 11280).

Preparation of DNA, DNA base composition and DNA-DNA hybridization. DNA was extracted from cells grown in yeast extract-glucose broth by using the method of Saito and Miura (1963) with minor modification described previously (Kudo et al., 1998). The G+C contents of the DNA were determined by the HPLC method of Tamaoka and Komagata (1984). 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 by using the microplate hybridization method devised by Ezaki et al. (1989).

Results and Discussion

Strains H47-7^T, Y1-5-3 and Y25-12 had chemical and morphological properties consistent with their classification in the genus *Microtetraspora* (Thieman et al., 1968). The substrate hyphae of the isolates were well developed, branched and non-fragmented. The aerial hyphae bore spore chains, typically containing four spores on the short sporophores (Fig. 1). Occasionally they produced chains containing only two or three spores and rarely chains containing five spores. Spores were smooth, oval to short cylindrical and non-motile. Neither sporangium-like bodies nor any other

special structures were observed. The color of the substrate mycelium tended to be cream to yellow tint and that of the aerial mycelium was white (Table 1). No diffusible pigments were produced.

The chemotaxonomic features of our isolates were similar to each other and those of members of the family *Streptosporangiaceae*. The cell wall peptidoglycan of the isolates contained glutamic acid, alanine and A₂pm in a molar ratio of 1.0:1.6:1.1 (calculated by defining the amount of glutamic acid as 1.0), and the isomer of A₂pm was *meso*. These data indicate that their cell wall type of Lechevalier and Lechevalier (1970) and peptidoglycan type of Schleifer and Kandler (1972) are type III and type A1γ, respectively. The *N*-acyl group of muramic acid in the peptidoglycan was the acetyl type. They contained glucose, mannose, ribose and madurose (3-*O*-methyl-D-galactose) in whole-cell hydrolysates, but galactose, arabinose, xylose and rhamnose were not [whole-cell sugar pattern B of Lechevalier and Lechevalier (1970)]. Predominant menaquinones from the test strains were MK-9(III, VIII-H₄) and MK-9(H₂). Major fatty acids found in whole-cell preparations were iso-C_{16:0}, 10-methyl C_{17:0}, C_{17:1} (ω6c) (Table 2). The phospholipid pattern was type PIV of Lechevalier et al. (1977) containing phosphatidylethanolamine, hydroxyphosphatidylethanolamine, and ninhydrin-positive glycopospholipids, but not phosphatidylcholine. The G+C contents of DNA of the isolates were 69 to 70 mol% (Table 4).

The almost complete 16S rDNA sequences (1,444 nucleotides) of our isolates, strains H47-7^T, Y1-5-3,

Table 1. Cultural characteristics^a of strains H47-7^T, Y1-5-3 and Y25-12.

Media	H47-7 ^T		Y1-5-3		Y25-12	
	Growth and color	Aerial mycelium and color	Growth and color	Aerial mycelium and color	Growth and color	Aerial mycelium and color
Hickey-Tresner agar	Moderate (Old gold, 2 ne)	Moderate (Pearl, 2 ba)	Moderate (Old gold, 2 ne)	Moderate (White, a)	Poor (Amber, 3 pe)	Poor (White, a)
Inorganic salts-starch agar	Poor (Yellow tint, 1 ba)	None	Poor	None	Poor (Cream, 1 1/2 ca)	Poor (White, a)
Oatmeal agar	Moderate (Cream, 1 1/2)	Poor (White, a)	Moderate (Cream, 1 1/2 ca)	Poor (White, a)	Poor (Cream, 1 1/2 ca)	Poor (White, a)
Oatmeal-nitrate agar	Moderate (White, a)	Moderate (White, a)	Moderate (Yellow tint, 1 ba)	Moderate (White, a)	Poor (Yellow tint, 1 ba)	Poor (White, a)
Yeast extract-malt extract agar	Good (Honey gold, 2 ic)	Good (White, a)	Good (Old gold, 2 ne)	Good (Shell pink, 5 ba)	Good (Mustard gold, 2 pg)	Good (White, a)
Yeast extract-starch agar	Moderate (Cream, 1 1/2 ca)	Moderate (Oyster white, b)	Moderate (Light maize, 2 ea)	Moderate (White, a)	Moderate (Lt maize, 2 ea)	Moderate (White, a)
Dilute (1/5) yeast extract-starch agar	Moderate (Pearl, 2 ba)	Moderate (Oyster white, b)	Moderate (Cream, 1 1/2 ca)	Moderate (White, a)	Moderate (Cream, 1 1/2 ca)	Moderate (White, a)

^a Color numbers and names were taken from the "Color Harmony Manual" (Jacobson et al., 1958).

Table 2. Cellular fatty acid profiles^a of strains H47-7^T, Y1-5-3, Y25-12 and the type strains of previously described species of the genus *Microtetraspora*.

Fatty acid	Isolate			<i>M. glauca</i> JCM 3300 ^T	<i>M. fusca</i> JCM 3183 ^T	<i>M. niveoalba</i> JCM 3149 ^T
	H47-7 ^T	Y1-5-3	Y25-12			
Saturated fatty acids						
C _{13:0}	0.1		0.2	0.2		
C _{14:0}	0.5	0.2	1.1	0.7	0.1	0.3
C _{15:0}	2.9	1.0	2.6	3.8	0.9	0.5
2OH-C _{15:0}	0.4	0.3	0.3	0.5	0.3	
C _{16:0}	2.3	0.6	2.0	5.5	0.3	1.9
2OH-C _{16:0}	0.2	0.2	0.3	0.5	0.1	
C _{17:0}	2.8	0.3	0.7	14.9	0.3	0.5
2OH-C _{17:0}				0.3	0.3	
C _{18:0}			2.8	4.2		
C _{19:0}				0.2		
Unsaturated fatty acids						
C _{15:1} (ω6c)	0.3	0.2		0.1		
C _{16:1} (ω9c)	0.5		0.3	0.3		
2OH-C _{16:1}					0.2	0.2
C _{17:1} (ω6c)	10.2	10.2	5.9	3.6	12.4	9.2
C _{17:1} (ω8c)	1.7	0.5	0.5	3.9	0.8	0.3
C _{17:1} (ω9c)	1.4		0.4	1.0		
C _{18:1} (ω7c)	0.3		0.2	0.4		
C _{18:1} (ω9c)	0.5	0.2	0.4	1.2	0.1	
Branched fatty acids						
i-C _{14:0}	2.9	2.4	3.3	1.0	0.6	1.0
i-C _{15:0}	4.1	3.3	8.7	3.3	3.0	2.7
a-C _{15:0}	0.7	0.2	0.3	0.5	0.1	0.1
2OH-i-C _{16:1}	3.8	2.4	2.1	0.2	1.0	0.5
i-C _{16:0}	44.0	58.1	43.9	28.1	56.4	65.6
i-C _{17:0}	0.6	0.3	0.4	1.8	0.5	0.3
a-C _{17:0}	0.9	0.4	0.6	1.7	0.4	0.9
2OH-i-C _{18:1}	0.3	0.3	0.2	0.1	0.3	
i-C _{18:0}	0.8	0.8	0.4	4.3	3.6	3.4
10-Methyl fatty acids						
C _{16:0}	1.7	2.7	7.1	1.5	1.9	3.3
C _{17:0}	14.5	13.6	12.7	13.0	14.6	6.6
C _{18:0}	0.8	1.3	1.9	2.2	1.5	2.8
C _{19:0}				0.1		
Summed feature 3 ^b	0.8	0.6	0.9	1.0	0.4	

^a Values are percentages of total cellular fatty acids.^b Summed features represent groups of one or two fatty acids which could not be separated by GLC with the MIDI system. Summed feature 3 contained one or more of the following fatty acids: 2OH-i-C_{15:0} and/or C_{16:1} (ω7c).

Y25-12, were compared with those of selected members of the family *Streptosporangiacea* (Fig. 2). The data set used for construction of the phylogenetic tree contained 1347 nucleotide positions of each sequence as a result of elimination of gaps and ambiguous nu-

cleotides from the sequences between positions 57 and 1491 (*Escherichia coli* position number [Brosius et al., 1978]). As shown in Fig. 2, the three isolates had almost identical 16S rDNA sequences and formed a monophyletic clade with members of the genus *Mi-*

Fig. 2. Neighbor-joining tree based on nearly complete 16S rDNA sequences of three isolates and selected organisms belonging to the family *Streptosporangiaceae*.

The numbers at the branches indicate the percentage bootstrap values of 1,000 replicates (greater than 50%). The numbers in parentheses are accession numbers of the DDBJ/EMBL/GenBank databases. Abbreviations of culture collections: ATCC, American Type Culture Collection, Manassas, VA, USA; DSM, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; IFO, Institute for Fermentation, Osaka, Osaka, Japan; JCM, Japan Collection of Microorganisms, RIKEN (Institute of Physical and Chemical Research), Wako, Saitama, Japan; NRRL, Agricultural Research Service Culture Collection, United States Department of Agriculture, Peoria, IL, USA.

crotetraspora. This phylogenetic relationship was supported by the 100% bootstrap value. On the basis of the morphological, chemotaxonomic and phylogenetic data, the isolates should be classified in the genus *Microtetraspora*.

The optimal temperature for growth of the isolates was 25–30°C, and growth did not occur at more than 40°C. No growth occurred in the presence of 3% NaCl. Table 3 summarizes the results of biochemical tests of our isolates and strains used for comparison. Our iso-

lates showed almost identical biochemical profiles, which serve to distinguish them from all of the validly described *Microtetraspora* species. In particular, the non-utilization of L-arabinose and arbutin were effective for discrimination between the isolates and previously described species.

Table 4 shows results of DNA-DNA hybridization experiments among our isolates and the type strains of all of the validly described species of the genus *Microtetraspora*. The levels of DNA relatedness among

Table 3. Differential physiological characteristics of the isolates and the type strains of previously described species of the genus *Microtetrastora*.

Characteristic	Isolate			<i>M. glauca</i> JCM 3300 ^T	<i>M. fusca</i> JCM 3083 ^T	<i>M. niveoalba</i> JCM 3149 ^T
	H47-7 ^T	Y1-5-3	Y25-12			
Utilization ^a of:						
L-Arabinose	–	–	–	++	++	++
Arbutin	±	–	–	++	++	++
D-Fructose	++	++	++	++	–	++
D-Galactose	–	–	–	++	–	–
Glycerol	–	–	–	+	–	++
D-Mannitol	+	++	++	++	–	++
D-Mannose	–	–	+	+	+	+
L-Rhamnose	++	++	++	++	–	++
D-Trehalose	–	++	–	++	++	++
Xylitol	–	–	–	–	–	+
Utilization ^b of:						
Citrate	+	+	+	+	+	–
Fumarate	–	–	–	+	–	+
Malate	–	–	–	+	–	+
Decomposition ^b of:						
Hypoxanthine	–	–	–	+	–	+
Urea	–	–	–	–	+	+
Xylan	–	–	–	+	–	–
Requirement ^b for:						
Biotin	–	+	–	+	–	–
Thiamine	+	+	+	+	+	+

^a Growth: ++, good; +, moderate; ±, poor; –, none.^b +, positive; –, negative.Table 4. Levels of DNA-DNA relatedness among the isolates and known strains of the genus *Microtetrastora*.

Strain	G+C content (mol%)	Percentage DNA complementarity with labeled DNA from:					
		H47-7 ^T	Y1-5-3	Y25-12	JCM 3300 ^T	JCM 3183 ^T	JCM 3149 ^T
Isolate							
H47-7 ^T	69	100	98	79	33	55	34
Y1-5-3	69	82	100	75	28	50	30
Y25-12	70	72	82	100	25	47	30
<i>M. glauca</i> JCM 3300 ^T	69	22	29	24	100	21	25
<i>M. fusca</i> JCM 3183 ^T	70	47	58	43	22	100	32
<i>M. niveoalba</i> JCM 3149 ^T	71	30	38	31	25	33	100

strains H47-7^T, Y1-5-3 and Y25-12 ranged from 72 to 98%, while the levels of DNA relatedness between our isolates and the type strains of previously described species of the genus *Microtetrastora* ranged from 21 to 58%.

It is evident from the genotypic and phenotypic data that strains H47-7^T, Y1-5-3 and Y25-12 form a new taxonomic position in the genus *Microtetrastora*. It is therefore proposed that these strains be recognized as a new species *Microtetrastora malaysiensis* sp. nov.

Description of *Microtetraspora malaysiensis* sp. nov.

Microtetraspora malaysiensis sp. nov. (mal.ay.si.en'-sis. M.L. adj. *malaysiensis* pertaining to Malaysia, the source of the soil from which the type strain was isolated).

Aerobic, Gram-positive, non-motile actinomycete that forms an extensively branched substrate mycelium in cream to yellow tint and white aerial mycelium. No soluble pigment is produced. Straight chains of four spores are formed on sporophores branching from aerial mycelia. Spores are oval to short cylindrical and have smooth surfaces. Decomposes casein, esculin, testosterone, L-tyrosine and DNA, but not adenine, elastin, guanine, hypoxanthine, keratin, urea, xanthine and xylan. As sole carbon sources for energy, utilizes D-cellobiose, D-fructose, D-glucose, D-lactose, maltose, D-mannitol, L-rhamnose, D-ribose, salicin, starch and D-xylose, but not adonitol, L-arabinose, D-arabitol, arbutin, dulcitol, *D*-erythritol, D-galactose, glycerol, *myo*-inositol, inulin, D-mannose, D-melezitose, D-melibiose, α -methyl-D-glucoside, D-raffinose, D-sorbitol, L-sorbose, sucrose, D-trehalose or xylitol. Utilizes citrate but not benzoate, fumarate, lactate, malate, mucate, oxalate, succinate, or tartrate. No growth occurs in the presence of 3% NaCl. Requires thiamine for growth but requirement for biotin is varied. Mesophilic. Cell walls contain meso-A₂pm. Madurose (3-O-methyl-D-galactose) is the characteristic whole-cell sugar. The predominant menaquinones are MK-9(III, VIII-H₄) and MK-9(H₂). The DNA G+C content of the type strain is 69 to 70 mol%. Habitat is soil. The type strain is strain H47-7^T (=JCM 11278^T=DSM 44579^T).

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