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ORIGINAL ARTICLE

Micromonospora spongicola sp. nov., an actinomycete isolated from a marine sponge in the Gulf of Thailand

Khomsan Supong[1], Chanwit Suriyachadkun[2], Pattama Pittayakhajonwut[2], Khanit Suwanborirux[3,4] and Chitti Thawai[1,5]

An actinomycete strain, S3-1^T, was isolated from marine sponge sample collected from the Gulf of Thailand. The strain is aerobic, Gram-positive and produced single spores at the tip of the substrate mycelium. Strain S3-1^T contained *meso*-diaminopimelic acid in the peptidoglycan, whole-cell sugars were arabinose, galactose, glucose, rhamnose, ribose and xylose. The polar lipid profile of strain S3-1^T consisted of phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, phosphoglycolipid and unknown polar lipids. Morphological and chemotaxonomic characteristics of the strain were identified as a member of the genus *Micromonospora*. Phylogenetic analysis based on 16S rRNA gene sequence analysis of the strain showed similarity to *Micromonospora nigra* DSM 43818^T (98.8%), *Micromonospora yangpuensis* FXJ6.011^T (98.7%) and *Micromonospora narathiwatensis* BTG4-1^T (98.6%). The DNA G + C content was 72.7 mol%. The phenotypic characteristics and DNA–DNA relatedness values supported that the classification of this strain as a novel species in the genus *Micromonospora*, for which the name *Micromonospora spongicola* sp. nov. (type strain S3-1^T = BCC 45595^T = NBRC 108779^T) is proposed.
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INTRODUCTION

The genus *Micromonospora* was proposed by Orskov,[1] which belongs to the family *Micromonosporaceae*.[2] The genus can be distinguished from the other member of its family based on the morphological,[3] chemotaxonomic and genotypic characteristics.[4] Micromonosporae form single non-motile spores on the substrate mycelium, which do not fragment and have absence of aerial mycelium. The genus *Micromonospora* is probably the second most prolific producer of antibiotics after *Streptomyces*[5] and the first antimicrobial activity by a member of this genus was isolated from *Micromonospora echinospora*.[6] The genus *Micromonospora* has gathered attention due to the secondary metabolites production with either structural diversity or significant biological activities; for example, Lomaiviticins,[7] Lupinacidin[8] and Maklamicin,[9] in addition, novel compounds exhibiting antitumor and/or antibacterial activities have been also isolated from marine *Micromonospora,* including Diazepinomicin[10] and anthracyclinones.[11] Members of *Micromonospora* are widely distributed in terrestrial, aquatic and marine environments,[3,12,13] but recently members of this genus have also been recovered from marine sponge.[14] In this study, we described the isolation and taxonomic characterization of a novel strain S3-1^T, which was isolated from marine sponge in the Gulf of Thailand.

MATERIALS AND METHODS

Strain S3-1^T was isolated from marine sponge collected from the Gulf of Thailand by scuba diving at a depth of 5 m in the Sichang Island in Chonburi province. The strain was isolated using modified starch-casein nitrate sea water agar.[15] The isolation plate was incubated at 30 °C for 21 days. Actinomycete isolate was purified on ISP 2 medium. Strain S3-1^T was grown on soil extract agar medium for 21 days at 30 °C and observed by light microscopy and scanning electron microscopy (model LEO/1455VP). Samples for scanning electron microscopy were prepared as described previously.[16]

Cultural characteristics were determined using 14 day-cultures grown at 30 °C on several standard agar media. The ISCC–NBS Color Charts standard sample no. 2106 was used for determining color designations.[17] Carbon utilization medium (ISP 9)[18] supplemented with a final concentration of 1% of the carbon source and 0.05% casamino acids was used to determine the carbon utilization of the strain. The decomposition of various compounds and acid production from carbon sources were examined using the basal medium recommended by Gordon *et al.*[19] To determine the tolerance of NaCl, pH and the effect of temperature for growth were determined 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[20] and Williams and Cross.[21]

Freeze-dried cells used for chemotaxonomic analyses were obtained from cultures grown in ISP 2 broth on rotary shaker (200 r.p.m.) at 30 °C for 7 days.

[1]Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand; [2]National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park, Klong Luang, Pathumthani, Thailand; [3]Center for Bioactive Natural Products from Marine Organisms and Endophytic Fungi (BNPME), Bangkok, Thailand; [4]Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand and [5]Actinobacterial Research Unit, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand
Correspondence: Dr C Thawai, Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, 1 Chalongkrung road, Ladkrabang, Bangkok 10520, Thailand.
E-mail: ktchitti@kmitl.ac.th
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The isomer of diaminopimelic acid in the cell wall was determined by the method of Staneck and Roberts.[22] The acyl group of muramic acid in the peptidoglycan was determined by the method of Uchida and Aida.[23] The whole-cell hydrolysate sugars were analyzed by the cellulose TLC method of Komagata and Suzuki.[24] Polar lipids were extracted and identified by the method of Minnikin *et al.*[25] Fatty acid methyl ester analysis was performed by GLC according to the instructions of the Microbial Identification System (MIDI, version 6.0) (Sasser,[26] and Kämpfer and Kroppenstedt[27]) with the ACTIN1 MIDI database. Isoprenoid quinones were extracted by the method of Collins *et al.*[28] and were examined by reverse phase LC-MS employing UV detection and electrospray MS. The LC solvent system was methanol and 2-propanol (2:1, v/v).

Genomic DNA was isolated from cells grown in ISP 2 broth according to the method of Tamaoka.[29] The G + C content (mol%) of the genomic DNA was determined using the HPLC method of Tamaoka and Komagata.[30] An equimolar mixture of nucleotides for analysis of the DNA base composition (Yamasa Shoyu) was used as the quantitative standard. DNA–DNA hybridization was conducted in microdilution-well plates, as reported by Ezaki *et al.*[31] DNA–DNA relatedness (%) was determined by the colorimetric method.[32] PCR-mediated amplification of the 16S rRNA gene[33] and sequencing of the PCR products (Macrogen, Seoul, Korea) using universal primers.[34] The 16S rRNA gene sequence was aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases by using the CLUSTAL W programme version 1.81.[35] The alignment was manually verified and adjusted before the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor-joining[36] with genetic distances computed by using Kimura's 2-parameter model,[37] maximum parsimony[38] and maximum-likelihood[39] methods in the MEGA 5 software.[40] The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses[41] based on 1000 resamplings. The values for sequence similarity among all recognized *Micromonospora* species were first determined using the EzTaxon-e database.[42] 16S rRNA gene sequence similarities among closely related species were calculated manually after pairwise alignments obtained using the CLUSTAL_X program.[43]

RESULTS AND DISCUSSION

Strain S3-1^T formed well-developed and branched substrate mycelia but lacked aerial mycelia. Oval spores were borne singly from the substrate mycelium and the spore surface appeared smooth (Figure 1). The growth of the strain was good on ISP 2 medium and the color of colony of strain S3-1^T was light to strong orange but the colony on czapek's sucrose agar was colorless. Light to strong orange soluble pigment was produced in various media but not produced in czapek's sucrose agar (Table 1). The temperature and pH range for growth were 20–40 °C and pH 7–12, with optimum growth at 30 °C and pH 8, respectively. The maximum NaCl concentration for growth is 4% (w/v). Other physiological characteristics are given in Table 2 and in the species description.

Strain S3-1^T contained *meso*-diaminopimelic acid as the diagnostic peptidoglycan diamino acid. The acyl type of the cell wall peptidoglycan was determined to be the glycolyl type. Whole-cell hydrolysates contained arabinose, galactose, glucose, rhamnose (trace), ribose and xylose that were detected as whole-cell sugars in this strain. Analysis of polar lipids revealed that the strain contained phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, phosphoglycolipid and unknown polar lipids. The major fatty acids (>5%) detected in strain S3-1^T was iso-$C_{16:0}$ (38.1%), anteiso-$C_{17:0}$ (19.2%), $C_{18:1}$ ω9c (9.5%), iso-$C_{17:0}$ (7.2%) and $C_{18:0}$ (7.2%) (Table 3). The predominant menaquinones were MK-10(H_4) (65.2%) and MK-10(H_6) (34.8%). The G + C content was 72.7 mol%.

The almost-complete 16S rRNA gene sequence (1457 nucleotides) was obtained for strain S3-1^T and compared with those deposited in the public databases. The results indicated that this strain belongs to the genus *Micromonospora* and showed the highest similarity value was observed with *Micromonospora nigra* DSM 43818^T (98.8%) followed by *Micromonospora yangpuensis* FXJ6.011^T (98.7%) and *Micromonospora narathiwatensis* BTG4-1^T (98.6%). The phylogenetic tree constructed with 16S rRNA gene sequences data of all members of the genus *Micromonospora* also indicated that strain S3-1^T formed a cluster with *Micromonospora nigra* DSM 43818^T and *Micromonospora yangpuensis* FXJ6.011^T using the neighbor-joining method (Figure 2). DNA–DNA hybridization studies performed between strain S3-1^T and the related strains *Micromonospora nigra* NBRC 16103^T, *Micromonospora yangpuensis* NBRC 107727^T and *Micromonospora narathiwatensis* BTG4-1^T were 25.0 ± 0.7%, 19.4 ± 1.5% and 14.8 ± 0.9%, respectively.

The morphological and chemotaxonomic analyzes indicated that strain S3-1^T was identified as a member of the genus *Micromonospora*. However, the 16S rRNA gene sequence similarity values are low (98.6–98.8%) and DNA–DNA relatedness value between strain S3-1^T

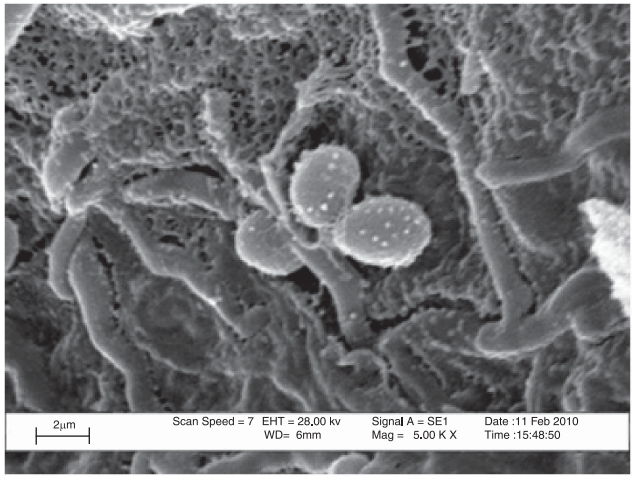

Figure 1 Scanning electron micrograph of strain S3-1^T grown on ISP 2 agar medium for 21 days at 30 °C. Bar, 2 μm.

Table 1 Cultural characteristics of strain S3-1^T on various media incubated at 30 °C after 14 days

Media	*Growth*	*Color of colony*	*Soluble pigment*
Yeast extract-malt extract agar (ISP 2)	Good	Strong orange	Moderate orange
Oatmeal agar (ISP 3)	Poor	Strong orange	Moderate orange
Inorganic salt-starch agar (ISP 4)	Moderate	Light orange	Light orange
Glycerol-asparagine agar (ISP 5)	Moderate	Light orange	Light orange
Peptone-yeast extract iron agar (ISP 6)	Poor	Light orange	Light orange
Tyrosine agar (ISP 7)	Poor	Moderate reddish orange	Moderate reddish orange
Czapek's sucrose agar	Poor	Colorless	-
Glucose-asparagine agar	Moderate	Strong orange	Strong orange
Nutrient agar	Moderate	Moderate orange	Moderate orange

Table 2 Differential characteristics of strain S3-1^T and type strains of the related *Micromonospora* species

Characteristics	*1*	*2*	*3*	*4*
Hydrolysis of starch	+	+	+	−
Gelatin liquefaction	+	−	+	+
Growth at 45 ºC	−	+	−	−
Growth at pH 6	−	+	+	+
Utilization of				
Inositol	+	−	+	+
D-fructose	+	+	+	−
Sorbose	+	+	−	+
D-xylose	+	−	+	+
Salicin	+	+	+	−
Xylitol	−	−	+	−
L-arabitol	+	−	+	+
L-rhamnose	+	+	−	−
Glycerol	+	−	+	+
Sorbitol	+	−	+	+
Lactose	+	+	−	−
Acid production from				
D-cellobiose	+	+	+	−
D-fructose	+	+	+	−
D-galactose	+	−	+	−
D-melibiose	+	+	+	−
D-raffinose	−	+	−	−
D-xylose	+	−	+	−
L-arabinose	−	−	+	+
D-sucrose	−	−	+	−
Lactose	+	−	+	−
Sorbose	+	+	+	−
Salicin	+	+	+	−

Strains: 1, S3-1^T; 2, *M. nigra* NBRC 16103^T; 3, *M. yangpuensis* NBRC 107727^T; 4, *M. narathiwatensis* BTG4-1^T. All phenotypic data was determined in this study. +, Positive; −, Negative.

Table 3 Cellular fatty acid compositions (%) of strain S3-1^T and type strains of the related *Micromonospora* species

Fatty acids	*1*	*2*	*3*	*4*
Saturated fatty acids				
$C_{12:0}$	ND	ND	0.3	0.4
$C_{14:0}$	ND	1.4	1.1	0.7
$C_{16:0}$	2.4	2.9	11.5	1.1
$C_{17:0}$	1.3	1.1	4.1	6.2
$C_{18:0}$	7.2	2.2	3.9	0.6
$C_{19:0}$	0.3	ND	0.4	0.5
Unsaturated fatty acids				
$C_{16:1}$ ω7c	1.6	1.3	2.8	0.3
$C_{17:1}$ ω5c	0.2	ND	ND	ND
$C_{17:1}$ ω8c	2.0	2.5	12.2	11.0
i-$C_{17:1}$ ω9c	0.5	7.7	1.3	ND
a-$C_{17:1}$ ω9c	1.3	0.7	0.3	ND
$C_{18:1}$ ω7c	0.3	0.7	1.1	0.4
$C_{18:1}$ ω9c	9.5	15.8	18.1	1.4
Branched fatty acids				
i-$C_{14:0}$	ND	2.4	0.5	0.7
i-$C_{15:0}$	3.0	5.6	6.3	41.8
i-$C_{15:1}$	ND	0.4	ND	0.8
a-$C_{15:0}$	0.3	1.3	0.8	4.6
i-$C_{16:0}$	38.1	39.3	26.8	9.3
i-$C_{16:1}$	1.5	2.5	0.5	0.7
i-$C_{17:0}$	7.2	3.3	3.2	3.8
3-OH -i-$C_{17:0}$	0.2	ND	ND	ND
a-$C_{17:0}$	19.2	3.3	2.8	3.9
i-$C_{18:0}$	2.4	1.2	0.6	ND
10-Methylated				
$C_{17:0}$	ND	ND	ND	3.0
$C_{18:0}$	ND	1.7	1.1	0.5

Abbreviation: ND, not detected.
Strains: 1, S3-1^T; 2, *M. nigra* NBRC 16103^T; 3, *M. yangpuensis* NBRC107727^T; 4, *M. narathiwatensis* BTG4-1^T. All cellular fatty acid composition data was determined in this study.

and the closed strains were below 25.0%, less than the value of 70% cutoff point recommended for the assignment of bacterial strains to the same genomic species.[44] In addition, the strain S3-1^T is distinguished from related strains by differences in the utilization of inositol, D-fructose, sorbose, D-xylose, salicin, xylitol, L-arabitol, L-rhamnose, glycerol, sorbitol and lactose; acid production from D-cellobiose, D-fructose, D-galactose, D-melibiose, D-raffinose, D-xylose, L-arabinose, D-sucrose, lactose, sorbose and salicin. The 10-methyl-$C_{18:0}$, $C_{14:0}$ and iso-$C_{14:0}$ were not detected in strain S3-1^T with differentiating properties from these related species. These results supported that strain S3-1^T represents a novel species in the genus *Micromonospora*, for which the name *Micromonospora spongicola* sp. nov. is proposed.

Description of *Micromonospora spongicola* sp. nov.

Micromonospora spongicola (spon.gi.co.la. L. n. *spongos-i* sponge; L. suff. *-cola* (from L. n. *incola*) inhabitant; N. L. n. (nominative in apposition) *spongicola* inhabitant of a sponge.

Aerobic, Gram-positive, which form oval spores on substrate mycelium. Aerial mycelium is absent. The color of the substrate mycelium on ISP 2, 3 and glucose-asparagine medium agar is strong orange. Moderate orange soluble pigment is produced on ISP 2, 3, 7 and nutrient medium agar. The maximum temperature for growth is 40 °C. The pH range for growth is 7.0–12.0. The maximum NaCl tolerance is 4% (w/v). L-arabitol, D-fructose, glycerol, inositol, lactose, D-xylose, L-rhamnose, salicin, sorbitol and sorbose are utilized as sole carbon sources but not D-mannitol, D-raffinose, D-ribose, D-mannose and xylitol. In addition, strain S3-1^T produced acid from D-cellobiose, D-fructose, D-galactose, lactose, D-melibiose, salicin, sorbose and D-xylose. Starch hydrolysis, gelatin liquefaction and milk peptonization are positive. Nitrate is not reduced. The diagnostic diamino acid of the peptidoglycan is *meso*-diaminopimelic acid. Whole-cell sugars are arabinose, galactose, glucose, rhamnose, ribose and xylose. Major polar lipids are phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, phosphoglycolipid and unknown polar lipids. The fatty acid pattern consists of iso-$C_{16:0}$, anteiso-$C_{17:0}$, $C_{18:1}$ ω9c, iso-$C_{17:0}$ and $C_{18:0}$. The predominant menaquinones are MK-10(H_4) and MK-10(H_6). The DNA G + C content of the type

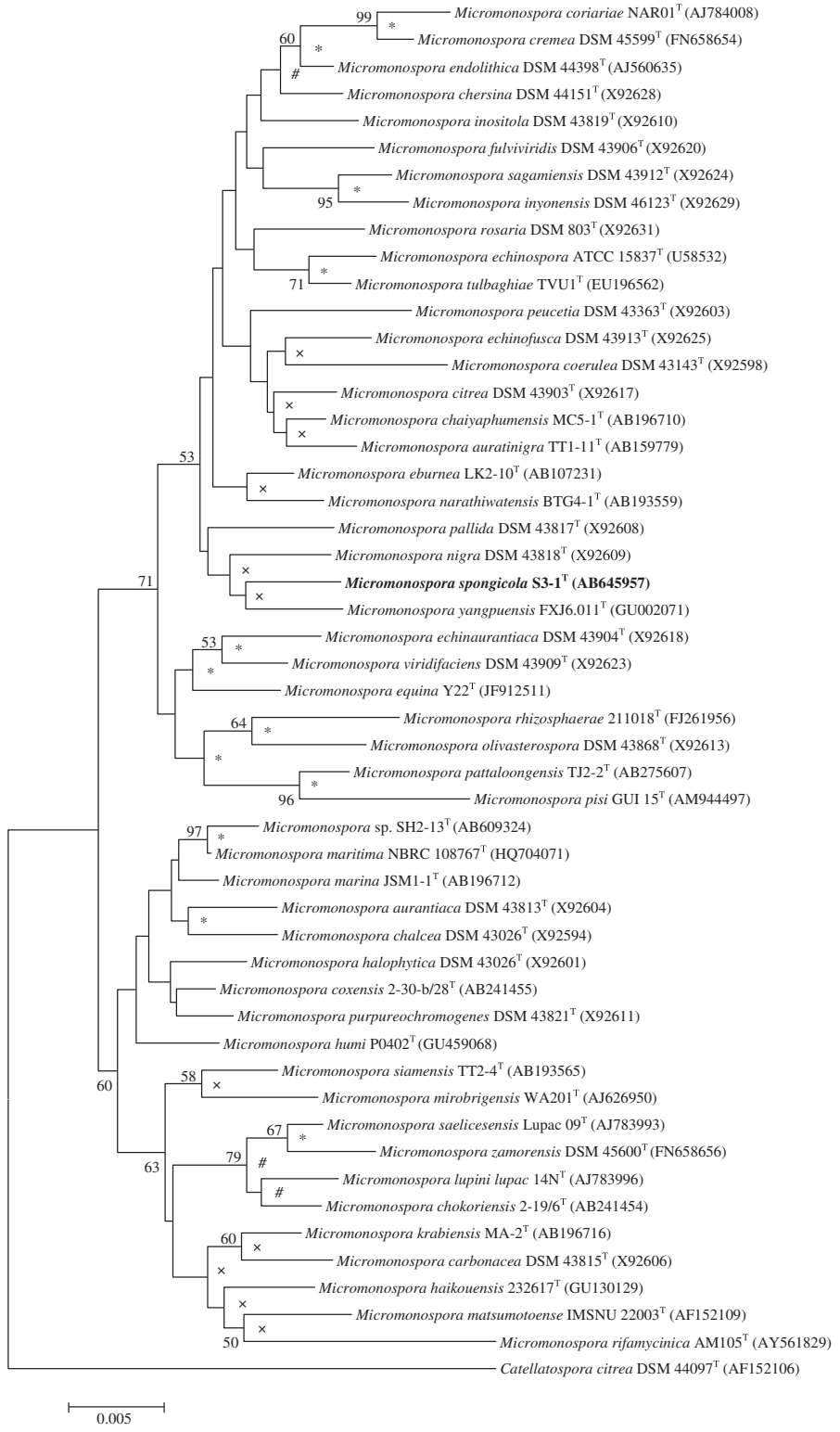

Figure 2 Phylogenetic dendrogram obtained by neighbour-joining tree based on almost-complete 16S rRNA gene sequences showing the position of strain S3-1^T among related species in the *Micromonospora* species. *Catellatospora citrea* DSM 44097^T was used as an outgroup. Asterisks (*) indicating the branches of the tree that were also found using the neighbour-joining, maximum-parsimony and maximum-likelihood methods; hashes (#) indicating the branches of the tree that were found in the neighbour-joining and maximum-parsimony methods; and crosses (x) indicating the branches of the tree that were found in the neighbor-joining and maximum-likelihood methods. The numbers on the branches indicate the percentage bootstrap values of 1000 replicates; only values >50% are indicated. Bar, 0.005 substitutions per nucleotide position.

strain is 72.7 mol%. The type strain is S3-1^T = BCC 45595^T = NBRC 108779^T, isolated from the marine sponge collected from the Gulf of Thailand.

Accession code

The DDBJ accession number for the 16S rRNA gene sequence of strain S3-1^T is AB645957.

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