ORIGINAL ARTICLE

Sphaerisorangium siamense sp. nov., an actinomycete isolated from rubber-tree rhizospheric soil

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A Gram-positive aerobic actinomycete, designated SR14.14^T, isolated from the rhizospheric soil of rubber tree was determined taxonomically using a polyphasic approach. The organism contained *meso*-diaminopimelic acid and the *N*-acetyl type of peptidoglycan. The predominant menaquinones were MK-9, MK-9(H₂) and MK-9(H₄). Madurose was detected in the whole-cell hydrolysates. Mycolic acids were not presented. Major phospholipids were diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol mannoside. Major cellular fatty acid was iso-C_{16:0} and the G+C content was 71.9 mol%. Phylogenetic analysis based on 16S rRNA gene sequence suggested that the isolate belongs to the genus *Sphaerisorangium*. The sequence similarity value between the strain SR14.14^T and its closely related species, *Sphaerisorangium album*, was 97.8%. DNA–DNA hybridization values between them were well below 70%. Based on genotypic and phenotypic data, strain SR14.14^T represents a novel species in the genus *Sphaerisorangium*, for which the name *Sphaerisorangium siamense* sp. nov. is proposed. The type strain is SR14.14^T (=BCC 41491^T=NRRL B-24805^T=NBRC 107570^T).

The Journal of Antibiotics (2011) 64, 293–296; doi:10.1038/ja.2011.17; published online 16 March 2011

Keywords: actinomycete; rhizosphere soil; rubber-tree; *Sphaerisorangium*

INTRODUCTION

The genus *Sphaerisorangium* was proposed by Ara and Kudo¹ with *Sphaerisorangium melleum* as type species. This genus was emended by Cao *et al.*² for the strain containing MK-9(H₆), MK-9(H₄), MK-9(H₂) and MK-9 as predominant menaquinones and the DNA G+C contents of 67–72 mol%. The genus formed a phyletic line within the 16S rRNA genes tree of the *Streptosporangiaceae*, the family of which included the following genera: *Acrocarpospora*, *Herbidospira*, *Microbispora*, *Microtetrastora*, *Nonomuraea*, *Planobispora*, *Planomonospora*, *Planotetrastora*, *Sphaerisorangium*, *Streptosporangium* and *Thermopolyspora*.^{1,3,4} At the time of writing, the genus *Sphaerisorangium* encompassed six species. *Sphaerisorangium album* and *S. flaviroseum* were isolated from forest soil samples in China.² *Sphaerisorangium cinnabarinum*, *S. melleum* and *S. rubeum* were isolated from sandy soil samples in Bangladesh.¹ *Sphaerisorangium viridialbum* was transferred from *Streptosporangium viridialbum*.^{1,5}

During our investigation of actinomycetes from rhizospheric soil of rubber tree collected from Phatthalung province, Thailand, the strain SR14.14^T was isolated. A polyphasic study was designed to establish the taxonomic status of this strain. Resultant data based on this

polyphasic study showed that this isolate represented a novel species of the genus *Sphaerisorangium*.

MATERIALS AND METHODS

Strain SR14.14^T was isolated from the rhizospheric soil of rubber tree using the spread plate method on starch casein agar,⁶ supplemented with ketokonazole (100 µg ml⁻¹) and nalidixic acid (25 µg ml⁻¹). The plates were incubated at 28 °C for 2 weeks. The strain was purified and maintained on glucose yeast extract (GYE) agar (containing glucose 1.0% (w/v), yeast extract 1.0% (w/v) and agar 1.5% (w/v)) at room temperature. Suspensions of spores or mycelium were stored at –20 °C in 20% glycerol and lyophilized for long-term preservation. *Sphaerisorangium album* DSM 45172^T was used in this study for comparison purposes.

Cultural characteristics were determined using 14-days culture on ISP media 2, 3, 4 and 5 of Shirling and Gottlieb⁷ (Difco, Detroit, MI, USA) oatmeal-nitrate agar (JCM medium 52; Difco) and yeast-starch agar (JCM medium 42; Difco) at 27 °C. The color of mycelium and soluble pigment were determined by comparison with the color of chips in the Color Harmony Manual.⁸ The morphological characteristics were observed by light microscopy and scanning electron microscopy (JEOL-JSM 5600 LV, Japan) of 14-day-old cultures grown on ISP medium 3. Motility was observed with a light microscope

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Received 28 December 2010; revised 11 February 2011; accepted 13 February 2011; published online 16 March 2011

using cells grown on ISP medium 2 for 14 days and incubated in soil-extracted solution (5 g garden soil in 25 ml distilled water, mixed well and filtered through a 0.22- μ m Millipore membrane (Millipore, Darmstadt, Germany)) for 30 min.

The utilization of a variety of substrates as sole carbon sources was tested using the basal inorganic nitrogen medium⁹ supplemented with a final concentration of 1% (v/v) of the filter sterile-tested carbon sources. Catalase and oxidase activities were determined with 3% (v/v) hydrogen peroxide solution and 1% tetramethyl *p*-phenylenediamine dihydrochloride solution, respectively. Growth at various pH values and the tolerance of NaCl were examined on ISP medium 2. The temperature range for growth was determined on ISP medium 2 using a temperature gradient incubator (Tokyo Kagaku Sangyo, Tokyo, Japan) between 5 and 50 °C. Enzyme activity profiles were carried out using the API ZYM (bioMérieux) test kits. Melanin pigment was examined on ISP medium 7.⁷ The production of hydrogen sulfide was detected using lead acetate strips. Hydrolysis of casein, gelatin and nitrate reduction was examined by following the methods of Gordon and Mihm.¹⁰

Biomass used for chemotaxonomic analyses was obtained from cultures grown in shake flasks of ISP medium 2 for 10 days at 27 °C. The cells were harvested by centrifugation and washed three times with distilled water before freeze-drying. Standard procedures were used to determine the isomers of diaminopimelic acid.^{11,12} The acyl type of the cell wall was analyzed according to the method of Uchida and Aida.¹³ Whole-cell sugars were analyzed according to the method of Becker *et al.*¹¹ Polar lipids were examined using two-dimensional TLC and identified by the method of Minnikin *et al.*¹⁴ Menaquinones were extracted and purified by the method of Collins *et al.*,¹⁵ and isoprene units were subsequently analyzed by LC/MS (JMS-T100LP, JEOL) with a PEGASIL ODS column (20 $\times$ 50 mm; Senshu, Tokyo, Japan) using methanol/2-propanol (7:3). Mycolic acids were detected by TLC according to the method of Tomiyasu.¹⁶ The G+C content (mol%) of the DNA was determined by HPLC according to the method of Tamaoka and Komagata.¹⁷ The cellular fatty acid composition was analyzed by TechnoSuruga (Shizuoka, Japan) according to the instructions of the Microbial Identification System (MIDI, version 4.5) using a gas chromatograph (model HP6890; Hewlett Packard, Palo Alto, CA, USA) and identified with the ACTIN1 database. DNA–DNA relatedness was measured fluorometrically using the microplate hybridization method.¹⁸

The phylogenetic position of the isolate SR14.14^T was determined based on 16S rRNA gene sequence. Genomic DNA was isolated and purified from biomass following the method of Kieser *et al.*¹⁹ The 16S rRNA gene was amplified as described by Duangmal *et al.*²⁰ The PCR products were sequenced (First Base, Seri Kembangan, Malaysia) using universal primers.²¹ The resultant 16S rRNA gene sequence was aligned with the corresponding sequences of representatives of the genus *Sphaerisporangium* and related genera, retrieved from the GenBank databases, using the CLUSTAL X²² and PHYLIT programs (<http://plaza.snu.ac.kr/~jchun/phydit/>). Phylogenetic trees were inferred by the least-squares,²³ maximum-parsimony²⁴ and neighbor-joining²⁵ tree-making algorithms from the PHYLIP suite of programs²⁶ and TREECON software.²⁷ The resultant phylogenetic trees were viewed by TREEVIEW program.²⁸

RESULTS AND DISCUSSION

Strain SR14.14^T exhibited phenotypic properties typical of members of the genus *Sphaerisporangium*. The strain was Gram-positive, non-acid–alcohol-fast, and produced branched and non-fragmented substrate mycelia. Spherical spore vesicles were born on aerial mycelia with non-motile spores. Observations using scanning electron microscopy indicated that the sizes of spherical spore vesicles were between 4–5 μ m (Figure 1). Good growth of pale pink to pale orange substrate mycelia was observed on ISP medium 2, oatmeal-nitrate agar and yeast-starch agar. The strain formed white aerial hyphae with moderate sporulation on ISP medium 2, ISP medium 3, oatmeal-nitrate agar and yeast-starch agar. Light yellow soluble pigment was produced on ISP medium 3 and oatmeal-nitrate agar. Poor growth was observed on ISP media 4 and 5. Melanin pigment was not observed.

The results from chemical analysis indicated that strain SR14.14^T has chemotaxonomic markers typical of the genus *Sphaerisporangium*.

Figure 1 Scanning electron micrograph of spherical spore vesicles of strain SR14.14^T grown on ISP medium 3 for 14 days at 28 °C. Bar, 2 μ m.

The strain contained *meso*-diaminopimelic acid in the peptidoglycan. The predominant menaquinones were MK-9, MK-9(H₂) and MK-9(H₄). Galactose, glucose, madurose, mannose and ribose were detected as the major sugars in the whole-cell hydrolysates. Polar lipid analysis showed that the organism contained diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol mannoside as major phospholipids. Small amounts of phosphatidylinositol and phosphoglycolipid were also detected. Similar observations of phosphatidylinositol and phosphoglycolipid were reported by Cao *et al.*² for *S. album* and *S. flaviroseum*. The cellular fatty acid profile was represented by the predominance of iso-C_{16:0} (47.9%). Fatty acids found in smaller proportions included 10methyl-C_{17:0} (14.7%), 10methyl-C_{16:0} (8.2%), iso G-C_{16:1} (4.0%), C_{16:0} (3.6%), 10methyl-C_{18:0} (3.2%), C_{18:0} (2.5%), C_{17:0} (2.3%), anteiso-C_{17:0} (2.3%), C_{15:0} (2.0%), iso-C_{15:0} (1.8%), iso-C_{18:0} (1.7%), cis 9-C_{16:1} (1.1%), iso-C_{14:0} (1.0%), iso-C_{17:0} (1.0%), anteiso-C_{15:0} (0.6%) and cis9-C_{17:1} (0.6%). No mycolic acids were detected. The G+C content was 71.9 mol%.

The almost-complete 16S rRNA gene sequence (1419 nt) of strain SR14.14^T was compared against related sequences in the GenBank database. The results indicated that this isolate belongs to the genus *Sphaerisporangium*. The strain SR14.14^T showed the highest level of 16S rRNA gene similarity with the type strain of *S. album* (97.8%, a value corresponding to 29 nucleotide differences from 1328 locations). The 16S rRNA gene sequence similarity value of strain SR14.14^T with the type strains of *S. melleum*, *S. viridialbum* and *S. cinnabarinum* were 97.8, 97.6 and 97.2%, respectively.

It is evident from the phylogenetic tree with members of the genus *Sphaerisporangium* and type strains representing species of the family Streptosporangiaceae that strain SR14.14^T is closely related to *S. album*, with a bootstrap value of 75% by neighbor-joining analysis (Figure 2). Similar tree topographies were obtained when the least-squares and maximum-parsimony methods were applied. The isolate showed low bootstrap values with other members of the genus *Sphaerisporangium*. DNA–DNA relatedness studies were carried out between strain SR14.14^T and *S. album* DSM 45172^T by reciprocal hybridizations. The DNA–DNA reassociation values were in the range of 32–39%, which confirmed that the strain SR14.14^T represented a separate genomic species based on whole-genomic DNA relatedness of less than the 70% cut-off point.²⁹

Figure 2 Neighbor-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the position of the strain SR14.14^T in the genus *Sphaerisorangium*. Asterisks indicate branches that were also found using the least-squares²³ and maximum-parsimony²⁴ tree-making algorithms. Numbers at the nodes indicate the percentage bootstrap support from an analysis of 1000 resampled datasets (only values greater than 50% are shown). Bar, 0.02 substitutions per site.

Strain SR14.14^T was positive for catalase and oxidase. The temperature range for growth was 16–40 °C and good growth was observed at 26–35 °C. No growth was observed above 40 °C. The strain was able to grow at pH 5.0–9.0 and 0–2% NaCl. The strain was readily differentiated from its closest relative, *S. album* DSM 45172^T, on the basis of physiological properties (Table 1), in particular aesculin, arbutin and xylan hydrolysis, which were positive in *S. album* but negative in strain SR14.14^T. The utilization of arabinose, D(–)fructose, myo-inositol, D(–)mannitol, melibiose, L(–)rhamnose and sucrose was negative in SR14.14^T, properties that were positive in *S. album*. Moreover, the strain SR14.14^T produced a light yellow pigment on ISP medium 3 and oatmeal-nitrate agar after incubation at 27 °C for 2 weeks, but none was detected in *S. album*.

It is evident on the basis of genotypic and phenotypic data that strain SR14.14^T can be distinguished from members of the genus *Sphaerisorangium*. We concluded that strain SR14.14^T represented a novel species of *Sphaerisorangium*, for which the name *Sphaerisorangium siamense* sp. nov. is proposed.

Description of *Sphaerisorangium siamense* sp. nov.

Sphaerisorangium siamense: si.a.men'se. NL neut adj. siamense, of or belonging to Siam, the old name of Thailand, the source of the soil from which the type strain was isolated.

Gram-positive, non-acid-alcohol-fast, non-motile actinomycete. Pale pink to pale orange substrate mycelium is produced on ISP medium 2, oatmeal-nitrate agar and yeast-starch agar with white aerial mycelia. Light-yellowish soluble pigments are produced in ISP medium 3 and oatmeal-nitrate agar. Spherical spore vesicles are borne on aerial mycelia. The pH range for growth is pH 5.0–9.0. Cells can grow in the presence of 0–2% NaCl. Catalase, oxidase and H₂S production is positive. Allantoinase and nitrate reduction is negative. Utilization of raffinose, D(+)cellobiose, L(–)fucose, D(+)galactose,

Table 1 Differential characteristics of strain SR14.14^T and *Sphaerisorangium album* DSM 45172^T

Characteristics	SR14.14 ^T	<i>S. album</i> ^T
<i>On ISP medium 3</i>		
Growth	Moderate	Good
Soluble pigment color	Light yellow	Not produced
<i>On oatmeal-nitrate agar</i>		
Growth	Good	Good
Soluble pigment color	Light yellow	Not produced
<i>Utilization of</i>		
Arabinose	–	+
D(+)Cellobiose	+	–
D(–)Fructose	–	(+)
myo-Inositol	–	+
D(–)Mannitol	–	+
Melibiose	–	+
L(–)Rhamnose	–	+
Sucrose	–	+
<i>Degradation</i>		
Aesculin	–	+
Arbutin	–	+
Xylan	–	+

+, positive; –, negative; (+), weak growth.

D(+)glucose, lactose, maltose, D(+)mannose and D(–)ribose is positive. Utilization of adonitol, arabinose, D(–)fructose, myo-inositol, D(–)mannitol, melibiose, L(–)rhamnose, D(–)sorbitol, sucrose, xylitol and D(+)xylose is negative. Casein, hypoxanthine, L-tyrosine and starch are degraded. Aesculin, allantoin, arbutin, cellulose, guanine, xanthine and xylan are not degraded. Acid phosphatase, alkaline phosphatase, aminopeptidase, esterase C4, α-glucosidase, β-glucosidase, leucine, lipase C8, phosphoamidase, trypsin and valine aminopeptidase are detected using the API ZYM enzyme assay; chymotrypsin, cystine aminopeptidase, α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, lipase C14, α-mannosidase and N-acetyl-β-glucosaminidase are negative. The diagnostic diamino acid of the peptidoglycan is meso-diaminopimelic acid. Whole-cell sugars are galactose, glucose, madurose, mannose and ribose. The glycan moiety of the murein is acetylated. Major phospholipids are diphosphatidylglycerol, phosphatidylethanolamine and phosphatidyl-inositol mannoside. The predominant menaquinones are MK-9, MK-9(H₂) and MK-9(H₄). Mycolic acids are not detected. The major fatty acid in cellular fatty acids profile is iso-C_{16:0}. The G+C content of the type strain DNA is 71.9 mol%.

The type strain, strain SR14.14^T (=BCC 41491^T=NRRL B-24805^T=NBRC 107570^T), was isolated from the rhizospheric soil of rubber tree, collected from Phatthalung province, Thailand.

Accession code

The GenBank accession number for the 16S rRNA gene sequence of strain SR14.14^T is HM043727.

ACKNOWLEDGEMENTS

We thank Professor JP Euzéby for his kind advice on naming the species. This study was supported by the Thailand Research Fund, Commission on Higher Education and Kasetsart University Research and Development Institute.

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