Sphaerisporangium krabiense sp. nov., isolated from soil

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A Gram-staining-positive, filamentous bacterial strain, designated A-T 0308^T, was isolated from soil of a tropical mangrove forest in Thailand. Strain A-T 0308^T developed spherical sporangia containing non-motile spores on aerial mycelium. The novel strain contained *meso*-diaminopimelic acid, *N*-acetyl-type peptidoglycan and madurose, mannose, ribose, galactose and glucose as whole-cell sugars. The predominant menaquinones were MK-9(H₄) and MK-9(H₆); a small amount of MK-9(H₂) and MK-9 was also detected. Mycolic acids were not detected. The diagnostic phospholipids were phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside and phosphoglycolipid. The predominant cellular fatty acids were iso-C_{16:0} and 10-methylated C_{17:0}. The G + C content of the DNA was 72 mol%. Phenotypic and chemotaxonomic analyses showed that the novel isolate had characteristics typical of members of the genus *Sphaerisporangium*. 16S rRNA gene sequence analysis also indicated that the strain belongs to the genus *Sphaerisporangium* and that it represents a clade distinct from other members of the genus with sequence similarities ranging from 96.3 to 97.8% between the novel strain and its closest relatives. Based on the results of phenotypic, chemotaxonomic and phylogenetic studies, strain A-T 0308^T (=BCC 21702^T =NBRC 107571^T) represents a novel species of the genus *Sphaerisporangium*, for which the name *Sphaerisporangium krabiense* sp. nov. is proposed.

The genus *Sphaerisporangium* was first proposed by Ara & Kudo (2007) as a member of the family *Streptosporangiaceae*. At the time of writing, the genus included six species with validly published names: *Sphaerisporangium melleum* (the type species of the genus), *Sphaerisporangium rubeum*, *Sphaerisporangium cinnabarinum* and *Sphaerisporangium viridialbum* (Ara & Kudo, 2007) and *Sphaerisporangium flaviroseum* and *Sphaerisporangium album* (Cao *et al.*, 2009).

During the course of a project investigating the diversity of actinomycetes in Thailand, a Gram reaction-positive, non-acid-fast, aerobic bacterial strain, designated A-T 0308^T, was isolated from forest soil in Krabi Province. The novel isolate was identified as a member of the genus *Sphaerisporangium* based on the results of morphological, chemotaxonomic and phylogenetic analyses as well as DNA–DNA relatedness studies. Here, we report the taxonomic characterization and classification of strain A-T 0308^T and propose that it represents a novel species.

Strain A-T 0308^T was isolated from tropical mangrove forest soil collected in Krabi Province (8° 3' N 98° 47' E), Thailand, and was kept at 4 °C prior to isolation. The soil sample was dried at room temperature for 8 days and the method of Nonomura & Hayakawa (1988) was used for isolation. The organism was isolated on humic acid–vitamin agar (HV)

Abbreviations: *meso*-A₂pm, *meso*-diaminopimelic acid; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A-T 0308^T is HM037365.

Two supplementary figures and two supplementary tables are available with the online version of this paper.

supplemented with 25 mg nalidixic acid l⁻¹, 50 mg cycloheximide l⁻¹ and 1 mg terbinafine l⁻¹. The pure culture was preserved by freezing at -80 °C in 10 % (v/v) glycerol and also by liquid-drying.

Morphological characteristics were determined by growth on modified soil-extract agar, yeast extract–starch agar (Suriyachadkun *et al.*, 2009) and inorganic salts–starch agar (ISP4 medium; Shirling & Gottlieb, 1966) for 7–14 days at 28 °C followed by observation with a light microscope and a scanning electron microscope (model JSM-5410 LV; JEOL). Samples on modified soil-extract agar were prepared for scanning electron microscopy as described previously (Itoh *et al.*, 1989).

Strain A-T 0308^T had characteristics typical of members of the genus *Sphaerisporangium*. Cells developed spherical sporangia on aerial mycelium. Globose bodies were not observed. Spores were not motile as determined with a light microscope using cells grown on modified soil-extract agar for 3 weeks at 28 °C and then incubated at 28 °C for 30–60 min in 0.1 M potassium phosphate buffer (pH 7).

Phenotypic characteristics of the novel strain were examined using several standard methods. Cultural characteristics were tested using cells grown at 28 °C on various agar media for 14–21 days (Shirling & Gottlieb, 1966). The NBS-ISCO Colour System was used to determine names and designations of colony colours. Growth at different temperatures, pH levels and NaCl concentrations was determined on ISP2 medium. Utilization of carbohydrates as sole carbon sources was tested by using ISP-9 medium as a basal medium (Shirling & Gottlieb, 1966) supplemented with 1 % (final concentration) of each of the carbon sources. Hydrolysis of high molecular mass compounds was determined using the media and method of Gordon *et al.* (1974). Gelatin liquefaction, peptonization of milk, nitrate reduction and starch hydrolysis were determined through cultivation on various media as described by Arai (1975) and Williams & Cross (1971). Catalase and oxidase activities were determined using the method of Greenwood & Pickett (1979). The results of these tests are given in the species description and Table 1.

Freeze-dried cells obtained from a culture grown in glucose–yeast extract broth (Tamura *et al.*, 1994) on a rotary shaker at 30 °C for 7 days were used for chemotaxonomic analyses. The cell-wall peptidoglycan was prepared and hydrolysed by using the methods of Kawamoto *et al.* (1981), and the amino acid composition was analysed by TLC (Lechevalier & Lechevalier, 1980). An isomer of diaminopimelic acid (A₂pm) was detected in the cell wall by using the method of Stanek & Roberts (1974). An acyl group of muramic acid was detected in the peptidoglycan by using the method of Uchida & Aida (1984). Sugars were analysed from whole-cell hydrolysates using cellulose TLC, following the method of Komagata & Suzuki (1987). Phospholipids were extracted and analysed by using the method of Minnikin *et al.* (1984). Fatty acid methyl ester analysis was performed by GLC according to the instructions of the ACTINO database of the

Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Isoprenoid quinones were extracted according to the method of Collins *et al.* (1977) and analysed by HPLC using Cosmosil 5C₁₈ columns (4.6 × 150 mm; Nacalai Tesque) with a mixture of methanol and 2-propanol (2 : 1, v/v) as the elution solvent.

Chemotaxonomic characteristics of the novel strain were similar to those of the members of the genus *Sphaerisporangium*. The cell-wall amino acids contained *meso*-diaminopimelic acid (*meso*-A₂pm). The novel strain contained madurose, mannose, ribose, galactose and glucose as whole-cell sugars but not rhamnose, arabinose or xylose (whole-cell sugar pattern B of Lechevalier & Lechevalier, 1970). Diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and phosphoglycolipid were detected but phosphatidylcholine and glucosamine-containing phospholipids were not detected (Supplementary Fig. S1, available in IJSEM Online). This pattern corresponds to phospholipid type PIV of Lechevalier *et al.* (1977). The major cellular fatty acids of strain A-T 0308^T were iso-C_{16:0} (69.28 %) and 10-methylated C_{17:0} (9.19 %), corresponding to fatty acid pattern 3c of Kroppenstedt (1985). Detailed cellular fatty acid compositions of strain A-T 0308^T and type strains of the six recognized species of the genus *Sphaerisporangium* are shown in Supplementary Table S1. Mycolic acids were absent from strain A-T 0308^T. Menaquinones found in the novel isolate were MK-9(H₄) (66.47 %), MK-9(H₆) (20.86 %), MK-9(H₂) (9.62 %) and MK-9(H₀) (3.05 %).

Genomic DNA was prepared according to the method of Saito & Miura (1963). The 16S rRNA gene was amplified by PCR as described previously (Yamada *et al.*, 2000; Katsura *et al.*, 2001; Kawasaki *et al.*, 1993). Two primers, 20F and 1500R (Suriyachadkun *et al.*, 2009), were used for preliminary phylogenetic analysis. Positions in the rRNA gene fragments were based on the *Escherichia coli* numbering system (accession number V00348; Brosius *et al.*, 1981). The amplified 16S rRNA gene was sequenced directly with an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit using an ABI PRISM model 310 Genetic Analyzer (Applied Biosystems). The following primers were used for sequencing: 20F, 520F, 920F, 520R, 920R and 1500R (Yukphan *et al.*, 2004). Multiple alignments of the sequences were carried out with the software BioEdit Sequence Alignment Editor (version 7.0.0). Distance matrices for the aligned sequences were calculated by using the two-parameter method of Kimura (1980). Phylogenetic trees were reconstructed from 1302 bases by using the neighbour-joining (NJ; Saitou & Nei, 1987) and maximum-parsimony (MP; Felsenstein, 1983) methods in MEGA v4.0 and the maximum-likelihood (ML) method in PHYLIP v3.6 (Felsenstein, 1981). The robustness of individual branches was estimated by bootstrapping with 1000 replications (Felsenstein, 1985). Pair-wise sequence similarities were calculated for 16S rRNA gene sequences (1341 nt). Strain A-T 0308^T exhibited 16S rRNA gene sequence similarities of 97.8, 97.3, 97.3, 97.3, 96.9 and 96.3 % to *S. melleum* 3-28(8)^T, *S. album* YIM 48782^T, *S.*

Table 1. Characteristics differentiating strain A-T 0308^T and the six recognized species of the genus *Sphaerisporangium*

Strains: 1, *S. krabiense* A-T 0308^T; 2, *S. melleum* 3-28(8)^T; 3, *S. rubeum* 3D-72(35)^T; 4, *S. cinnabarinum* DSM 44094^T; 5, *S. viridialbum* DSM 43801^T; 6, *S. flaviroseum* YIM 48771^T; 7, *S. album* YIM 48782^T. +, Positive; −, negative; w, weakly positive. All data are from this study unless otherwise indicated.

Characteristics	1	2	3	4	5	6	7
Colonies on ISP 2 medium							
Aerial mycelium or spore	Greyish pink	−	−	−	−	−	Pinkish white
Substrate mycelium	Vivid red	Honey gold	Coral red	Bamboo	Light tan	Deep yellow pink	Pale grey
Growth at 37 °C	+	+	+	+	−	−	+
DNA G + C content (mol%)*	72	71	70.4	70	72	67.1	71
Major menaquinones*	MK-9(H ₄), MK-9(H ₆)	MK-9(H ₄), MK-9(H ₆)	MK-9(H ₆), MK-9(H ₄)	MK-9(H ₄), MK-9(H ₆)	MK-9(H ₄), MK-9(H ₂)	MK-9(H ₄), MK- 9(H ₂), MK-9	MK-9(H ₄), MK-9(H ₂), MK-9
Milk coagulation and peptonization	+	+	−	−	−	−	−
Nitrate reduction	+	+	+	w	−	−	−
Oxidase activity	+	−	−	−	−	−	+
Hydrolysis of starch	−	+	+	+	+	−	+
NaCl tolerance (%)	3.5	1	1.5	2	1.5	1	2
Utilization of:							
L-Arabinose	w	−	w	w	w	+	w
D-Fructose	−	w	+	−	−	+	w
D-Galactose	w	+	w	w	w	+	+
D-Glucose	w	+	+	+	w	+	+
Lactose	+	+	+	w	w	+	+
D-Mannitol	w	w	+	+	+	w	+
Melibiose	+	+	−	+	+	+	+
Raffinose	w	w	w	w	w	−	+
D-Ribose	−	w	−	+	w	+	+
L-Rhamnose	+	+	−	+	w	+	+
D-Xylose	w	+	+	+	w	+	+
Salicin	w	w	w	w	w	−	w
L-Proline	+	−	+	+	+	w	+
L-Serine	w	−	+	+	w	w	+
L-Tryptophan	−	−	−	w	w	−	−
Xanthine	w	−	w	+	w	−	−
L-Valine	+	−	−	+	+	+	+

*Data for columns 2–7 were taken from Cao *et al.* (2009).

cinnabarinum DSM 44094^T, *S. viridialbum* DSM 43801^T, *S. flaviroseum* YIM 48771^T and *S. rubeum* 3D-72(35)^T, respectively. The phylogenetic tree (Fig. 1) reconstructed using the NJ, MP and ML methods, showing the phylogenetic relationship between strain A-T 0308^T and the members of the family *Streptosporangiaceae*, revealed that this strain should be classified as a member of the genus *Sphaerisporangium* and showed that it was clearly separated from its closest relatives. The neighbour-joining tree (Supplementary Fig. S2a), maximum-parsimony tree (Supplementary Fig. S2b), and maximum-likelihood tree (Supplementary Fig. S2c) showed a similar tree topology.

The G + C content of the genomic DNA was determined by the method of Tamaoka & Komagata (1984). After treatment with P1 nuclease and alkaline phosphatase,

HPLC was performed using a Shimadzu LC-6AD apparatus equipped with a Cosmosil 5C₁₈-AR column (4.6 × 150 mm; Nacalai Tesque). DNA–DNA hybridizations were carried out in microdilution wells using the photobiotin-labelling method as described by Ezaki *et al.* (1989). DNA–DNA relatedness (%) was determined using the colorimetric method (Verlander, 1992). The DNA G + C content of strain A-T 0308^T was 72 mol%. The strain exhibited DNA–DNA relatedness levels with the type strains *S. melleum* 3-28(8)^T, *S. rubeum* 3D-72(35)^T, *S. cinnabarinum* DSM 44094^T, *S. viridialbum* DSM 43801^T, *S. flaviroseum* YIM 48771^T and *S. album* YIM 48782^T that were significantly below 70 % (Supplementary Table S2), the cut-off point recommended by Wayne *et al.* (1987) for the delineation of bacterial species. The results of 16S rRNA gene sequence analysis and DNA–DNA relatedness studies were sufficient to categorize

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences showing the relationship between strain A-T 0308^T and closely related members of the family Streptosporangiaceae. The phylogenetic tree was reconstructed using the NJ, MP and ML methods. *Actinomedura madurae* DSM 43067^T was used as an out group. Bootstrap values >50%, based on 1000 replications, are indicated at nodes (NJ/MP/ML). Bar, 0.005 substitutions per nucleotide position.

strain A-T 0308^T as distinct from previously described species of the genus *Sphaerisorangium*.

The phenotypic characteristics of strain A-T 0308^T were compared with recognized species of the genus *Sphaerisorangium* (Table 1). The novel strain could grow in 3.5% (w/v) NaCl, whereas recognized species of the genus *Sphaerisorangium* could only grow in <2% (w/v) NaCl. The novel strain also contained a larger amount (>60%) of the fatty acid iso-C_{16:0} than the other characterized species and did not contain iso-C_{15:0}, which was present in all the other strains used for comparison (Supplementary Table S1). These phenotypic and genotypic data indicated that strain A-T 0308^T merits classification as a novel species of the genus *Sphaerisorangium*, for which the name *Sphaerisorangium krabiense* sp. nov. is proposed.

Description of *Sphaerisorangium krabiense* sp. nov.

Sphaerisorangium krabiense (kra.bi.en'se. N.L. neut. adj. *krabiense* of Krabi Province, Thailand, where the type strain was isolated).

Cells are Gram-reaction-positive, mesophilic, non-motile actinomycetes that form pink substrate mycelia and pink-grey aerial mycelia on ISP2 medium. Spherical sporangia containing non-motile spores are formed on aerial mycelia. Substrate mycelium is non-fragmented. No soluble pigment is detected on ISP2, 3, 4 and 5 media. Optimal temperature for growth is between 25 and 37 °C. Maximum NaCl concentration for growth is 3.5%. Catalase- and oxidase-positive. Utilizes L-arabinose, cellobiose, D-galactose, D-glucose, lactose, D-mannitol, melibiose, raffinose, L-rhamnose, salicin and D-xylose as sole carbon and energy sources but does not utilize D-fructose, glycerol or D-ribose. Hydrolyses casein, hypoxanthine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-tyrosine, L-valine and xanthin but not adenine, L-glycine or L-tryptophan. Positive for nitrate reduction, milk coagulation and peptonization but negative for hydrolysis of starch and gelatin liquefaction. Contains meso-A₂pm as the cell-wall amino acid and N-acetyl-type peptidoglycan. Mycolic acids are not detected. The predominant menaquinones are MK-9(H₄) and MK-9(H₆); small amounts of MK-9(H₂) and MK-9 are also detected. The whole-cell sugars are madurose, mannose, ribose, galactose

and glucose. The diagnostic phospholipids are phosphatidylethanolamine, diphosphatidylglycerol, phosphatidyl-inositol, phosphatidylinositol mannoside and phosphoglycolipid, corresponding to phospholipid pattern type PIV. The major fatty acids are iso-C_{16:0} and 10-methylated C_{17:0}.

The type strain, A-T 0308^T (=BCC 21702^T=NBRC 107571^T), was isolated from soil of a tropical mangrove forest in Thailand. The DNA G+C content of the type strain is 72 mol%.

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