Verrucosispora andamanensis sp. nov., isolated from a marine sponge

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An actinomycete strain, SP03-05T, was isolated from a marine sponge sample (Xestospongia sp.) collected from Phuket Province of Thailand. The strain was aerobic, Gram-stain-positive and produced single spores at the tips of the substrate mycelium. Strain SP03-05T contained meso-diaminopimelic acid in the peptidoglycan; whole-cell sugars were arabinose, galactose, glucose, rhamnose, ribose and xylose. The polar lipid profile of strain SP03-05T consisted of phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and unknown polar lipids. Morphological and chemotaxonomic characteristics of the strain identified it as a member of the family Micromonosporaceae. Phylogenetic analysis based on 16S rRNA gene sequences showed similarity of the strain to Verrucosispora lutea YIM 013T (96.90 %), Verrucosispora sediminis MS426T (96.90 %), Verrucosispora gifhornensis DSM 44337T (96.80 %), Verrucosispora maris AB-18-032T (96.80 %) and Verrucosispora qiuiae RtIII47T (95.40 %). The DNA G+C content was 72.4 mol%. The phenotypic, genotypic and DNA–DNA hybridization results supported the classification of this strain as a representative of a novel species in the genus Verrucosispora, for which the name Verrucosispora andamanensis sp. nov. is proposed. The type strain is SP03-05T (BCC 45620T = NBRC 109075).

The genus Verrucosispora was established by Rheims et al. (1998), belongs to the family Micromonosporaceae and, at the time of writing, comprises only five described species, namely Verrucosispora gifhornensis (Rheims et al., 1998), Verrucosispora lutea (Liao et al., 2009), Verrucosispora sediminis (Dai et al., 2010), Verrucosispora qiuiae (Xi et al., 2011) and Verrucosispora maris (Goodfellow et al., 2012), which were isolated from peat bog, mangrove sediment, deep-sea sediment, mangrove swamp and marine sediment samples, respectively. Members of the genus Verrucosispora are described as aerobic, Gram-stain-positive, non-acid-fast actinomycetes that form a well-developed substrate mycelium, absent aerial mycelium or sporangia, and spores with smooth, warty or hairy surfaces which are borne singly, in pairs or in clusters. Biologically active compounds exhibiting antibacterial and/or anticancer activities have been isolated from marine members of the genus Verrucosispora, for example, abyssomicins (Bister et al., 2004; Riedlinger et al., 2004; Keller et al., 2007), gifhornenolones (Shirai et al., 2010), proximicins (Fiedler et al., 2008; Schneider et al., 2008) and Thiocoraline A.
(Wyche, et al., 2011). In this study, we describe the isolation and taxonomic characterization of a novel strain, SP03-05\textsuperscript{T}, which was isolated from a marine sponge (Xestospongia sp.) collected from the Andaman Sea of Thailand.

The strain was isolated using modified starch-casein nitrate seawater agar containing 10 g soluble starch, 1 g sodium caseinate, 0.5 g KH\textsubscript{2}PO\textsubscript{4}, 0.5 g MgSO\textsubscript{4} and 18 g agar in 1 litre of seawater, pH 8.3. The isolation plate was incubated at 30 °C for 21 days. The actinomycete isolate was purified on ISP 2 agar medium (Shirling & Gottlieb, 1966) supplemented with seawater. Strain SP03-05\textsuperscript{T} was grown on soil extract seawater agar (0.5 g CaSO\textsubscript{4}.2H\textsubscript{2}O, 0.25 g Ca(NO\textsubscript{3})\textsubscript{2}.4H\textsubscript{2}O, 0.05 g MgSO\textsubscript{4}.7H\textsubscript{2}O, 0.03 g K\textsubscript{2}SO\textsubscript{4}, 0.02 g KH\textsubscript{2}PO\textsubscript{4}, 0.1 g NaHCO\textsubscript{3}, 0.02 g CaCl\textsubscript{2}.2H\textsubscript{2}O, 0.1 g yeast extract, 0.1 g casamino acids, 0.2 g glucose, 100 mL soil extract, and 18 g agar in 1 L of sea water, pH 8.0-8.3) for 21 days at 30 °C and observed using light microscopy and scanning electron microscopy (model JSM-5410 LV; JEOL). Samples for scanning electron microscopy were prepared as described by Itoh et al. (1989).

Cultural characteristics were determined using 14-day-old cultures grown at 30 °C on ISP agar media, as described by Shirling & Gottlieb (1966). The NBS/IBCC colour system was used for determining colour designations (Kelly, 1964). The decomposition of various compounds and acid production from carbon sources were examined using the basal medium recommended by Gordon et al. (1974). Tolerance of NaCl and pH and the effect of temperature on growth were determined on ISP 2 agar. 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).

Freeze-dried cells for chemotaxonomic analyses were obtained from cultures grown in ISP 2 broth on rotary shaker (200 r.p.m.) at 30 °C for 4 days. Cell-wall peptidoglycan was prepared and hydrolysed as described by Kawamoto et al. (1981). The isomer of diaminopimelic acid in the cell wall was determined by the method of Stanec & Roberts (1974). The acyl group of muramic acid in the peptidoglycan was determined by the method of Uchida & Aida (1984). The whole-cell hydrolysate sugars were analysed by the cellulose TLC method of Komagata & Suzuki (1988). Phospholipids in cells were extracted and analysed by the method of Minnikin et al. (1984). Fatty acid methyl ester analysis was performed by GLC according to the instructions of the Microbial Identification System (MIDI) Sherlock version 6.0 (Sasser, 1990; Kämpfer & Kroppenstedt, 1996) with the ACTIN1 MIDI database. Isoprenoid quinones were extracted by the method of Collins et al. (1977) and were analysed by reversed-phase HPLC with the solvent system methanol:2-propanol (2:1, v/v) at a flow rate of 0.6 ml min\textsuperscript{-1}.

Genomic DNA was isolated from cells grown in ISP 2 broth according to the method of Tamaoka (1994). The G+C content of the DNA was determined using the HPLC method of Tamaoka & Komagata (1984). An equimolar mixture of nucleotides for analysis of the DNA base composition (Yamas Shou, Choshi, Japan) was used as the quantitative standard. DNA–DNA hybridization was conducted in microdilution-well plates, as reported by Ezaki et al. (1989). DNA–DNA relatedness (%) was determined by using the colorimetric method (Verlander, 1992). PCR-mediated amplification of the 16S rRNA gene was carried out as described by Suriyachadkun et al. (2009) and the PCR products were sequenced (Macrogen) using universal primers (Lane, 1991). The 16S rRNA gene sequence was aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases using the CLUSTAL W programme version 1.81 (Thompson et al., 1994). The alignment was manually verified and adjusted prior to the reconstruction of a phylogenetic tree. The phylogenetic tree was reconstructed by using the neighbour-joining method (Saitou & Nei, 1987) with genetic distances computed by Kimura’s two-parameter model (Kimura, 1980), and the maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods in the MEGA 5 software (Tamura et al., 2011). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The values for sequence similarity among all recognized species of the genus Verrucosispora were first determined using the EzTaxon server (Kim et al., 2012). 16S rRNA gene sequence similarities among closely related species were calculated using the EzTaxon-EzBioCloud server (Kim et al., 2017).

Table 1. Differential characteristics of strain SP03-05\textsuperscript{T} and type strains of species of the genus Verrucosispora

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tbody>
<tr>
<td>Nitrate reduction</td>
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<td>+</td>
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<td>−</td>
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<td>+</td>
<td>−</td>
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<td>Growth at 40 °C</td>
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<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<td>6–12</td>
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<td>Acid production from:</td>
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<tr>
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<td>+</td>
<td>+</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>−</td>
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<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Sorbose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Strains: 1, SP03-05\textsuperscript{T}; 2, V. lutea NBRC 106530\textsuperscript{T}; 3, V. sediminis NBRC 107745\textsuperscript{T}; 4, V. gilhornensis DSM 44337\textsuperscript{T}; 5, V. maris DSM 45365\textsuperscript{T} and 6, V. qiuiae NBRC 106684\textsuperscript{T}. All phenotypic data were determined in this study. +, Positive; −, Negative.
Strain SP03-05\textsuperscript{T} formed well-developed and branched substrate mycelium but not aerial mycelium. Spherical spores were borne singly from the substrate mycelium, and the spore surfaces appeared smooth (Fig. S1, available in IJSEM Online). The strain grew well on ISP 2 and ISP 4 media and the colony colour was vivid orange to strong orange, but soluble pigment was not produced on any agar media tested (Table S3). The temperature and pH range for growth were 20–40 °C and pH 6–12, with optimum growth at 30 °C and pH 8. The physiological characteristics are given in Table 1 and in the species description.

The strain contained meso-diaminopimelic acid in the cell wall. The acyl type of the cell wall in the peptidoglycan was determined to be the glycolyl type. Whole-cell sugars were detected as arabinose, galactose, glucose, rhamnose, ribose and xylose. The polar lipid profile comprised phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and four unidentified

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**Fig. 1.** Neighbour-joining tree based on almost-complete 16S rRNA gene sequences comparing strain SP03-05\textsuperscript{T} to species of the genus *Verrucosispora* and representatives of the family *Micromonosporaceae*. *Streptomyces albus* DSM 40313\textsuperscript{T} was used as an outgroup. Branches of the tree found by using the neighbour-joining, maximum-parsimony and maximum-likelihood methods (*), neighbour-joining and maximum-parsimony methods (#) and neighbour-joining and maximum-likelihood (×) methods are indicated. Numbers on branches indicate percentage bootstrap values of 1000 replicates; only values >50% are indicated. Bar, 0.01 substitutions per nucleotide position.
membrane of strain SP03-05T is a significant characteristic. Furthermore, the absence of phosphatidylglycerol in the cell polar lipids. (Fig. S2). Significant cellular fatty acids were iso-C_{15:0} (25.9 %), anteiso-C_{15:0} (10.8 %), C_{17:0} (9.0 %), iso-C_{16:0} (8.8 %), iso-C_{17:0} (7.3 %), C_{18:0} (7.2 %), anteiso-C_{17:0} (5.5 %), C_{17:1} \delta8c (5.1 %), C_{18:1} \delta9c (4.2 %) and C_{16:0} (4.0 %) (Table S1). The predominant menaquinones were MK-9(H_4) (86.6 %), MK-9(H_6) (7.6 %) and MK-9(H_2) (5.8 %). The G+C content of the DNA was 72.4 mol%.

An almost-complete 16S rRNA gene sequence (1401 nt) was obtained for strain SP03-05^T and was compared with those deposited in the public databases. The highest levels of 16S rRNA gene sequence similarity were with V. lutea YIM 013^T (96.90 %), V. sediminis MS426^T (96.90 %), V. giffhornensis DSM 44337^T (96.80 %), V. maris AB-18-032^T (96.80 %) and V. quiae RtlII47^T (95.40 %). The neighbour-joining tree showed that strain SP03-05^T formed a cluster with V. lutea YIM 013^T, V. sediminis MS426^T, V. giffhornensis DSM 44337^T and V. maris AB-18-032^T (Fig. 1). A low level of DNA–DNA relatedness was found between strain SP03-05^T and all species of the genus Verrucosispora, ranging from 10.1 ± 0.2–25.4 ± 0.4 (mean ± SD of 5 determinations), which is well below the 70 % cut-off point recommended for the assignment of bacterial strains to the same genomic species (Wayne et al., 1987) (Table S2). Furthermore, the morphological, chemotaxonomic and genotypic analyses indicated that strain SP03-05^T should be classified in the genus Verrucosispora. The characteristics shown in Table 1 indicated that strain SP03-05^T had some different physiological and biochemical characteristics compared to all recognized species of the genus Verrucosispora, distinguished on the basis of acid production from cellobiose, D-fructose, D-galactose and melibiose, nitrate reduction, growth at 40 °C, coagulation of milk and gelatin liquefaction. In addition, the fatty acid profile of strain SP03-05^T contained the unsaturated fatty acid C_{20:1} \delta7c that was not detected in the other strains. Furthermore, the absence of phosphatidylglycerol in the cell membrane of strain SP03-05^T is a significant characteristic that differentiated between strain SP03-05^T and its closest relative. Therefore, strain SP03-05^T represents a novel species of the genus Verrucosispora, for which the name Verrucosispora andamanensis sp. nov. is proposed.

**Description of Verrucosispora andamanensis sp. nov.**

Verrucosispora andamanensis (an.da.man.en’sis. N.L. fem. adj. andamanensis referring to the Andaman Sea, Thailand, the source of the type strain).

Aerobic, Gram-stain-positive. Form spherical spores on substrate mycelium but aerial mycelium is not formed. The cell wall peptidoglycan comprises meso-diaminopimelic acid. The acyl type of the muramyl residue is glycolyl. Grows well on ISP 2 and ISP 4 media agar; substrate mycelium produced is vivid orange to strong orange. No soluble pigment is produced on ISP medium agar. The growth temperature range is 20–40 °C, at pH 6–12. The maximum NaCl concentration for growth is 4 % (optimum 3 %). Produces acid from cellobiose, D-fructose, D-galactose, sucrose, lactose, salicin and sorbose. Starch hydrolysis and milk peptonization are positive. Nitrate, coagulation of milk and gelatin liquefaction are negative. 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, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and unknown polar lipids. The major fatty acid pattern (>4 %) consists of iso-C_{15:0}, anteiso-C_{15:0}, C_{17:0}, iso-C_{16:0}, iso-C_{17:0}, C_{18:0}, anteiso-C_{17:0}, C_{17:1} \delta8c, C_{18:1} \delta9c and C_{16:0}. The predominant menaquinones are MK-9(H_4), MK-9(H_6) and MK-9(H_2).

The type strain is SP03-05^T (=BCC 45620^T=NBRC 109075^T), isolated from a marine sponge (Xestospongia sp.) collected from the Andaman Sea of Thailand. The DNA G+C content of the type strain is 72.4 mol%.

**Acknowledgements**

This study was financially supported by the research fund from the Thailand Graduate Institute of Science and Technology, National Science and Technology Development Agency (NSTDA) (TGIST-01-52-003). This study was supported in part by the Actinobacterial Research Unit and Microbial Resource Management Unit, Scientific Instrument Center, Faculty of Science, King Mongkut’s Institute of Technology Ladkrabang, Thailand.

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