Acrocarpospora phusangensis sp. nov., isolated from a temperate peat swamp forest soil

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A novel actinomycete, strain PS33-18T, that formed club-shaped and spherical structures borne on the tip of the aerial mycelia was isolated from a temperate peat swamp forest soil in Phu-Sang National Park, Phayao Province, Thailand. The isolate contained glutamic acid, alanine and meso-diaminopimelic acid in the cell-wall peptidoglycan. The whole-cell sugars of strain PS33-18T were glucose, madurose, mannose, rhamnose and ribose. The characteristic phospholipids were phosphatidylethanolamine, phosphatidylmethylthanolamine, hydroxy-phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and ninhydrin-positive phosphoglycolipids. The predominant menaquinone was MK-9(H4). The major cellular fatty acids were C17:1\("\text{v8c}\), iso-C16:0 and C16:0. The G+C content of the genomic DNA of strain PS33-18T was 71.0 mol%. Phylogenetic analysis using 16S rRNA gene sequences revealed that strain PS33-18T should be classified in the genus Acrocarpospora.

The level of similarity between this strain and the closely related species Acrocarpospora macrocephala NBRC 16266T was 98.3 %, Acrocarpospora pleiomorpha NBRC 102641T was 97.6 %, Acrocarpospora corrugata NBRC 13972T was 97.6 %, and Planotetraspora kaengkrachanensis NBRC 104272T was 97.3 %. DNA–DNA hybridization results and physiological and biochemical properties indicated that strain PS33-18T could be distinguished readily from its closest phylogenetic relatives. On the basis of these phenotypic and genotypic data, this strain represents a novel species, for which the name Acrocarpospora phusangensis sp. nov. is proposed. The type strain is PS33-18T (=BCC 46906T =NBRC 108782T).

The genus Acrocarpospora Tamura et al. (2000) belongs to the family Streptosporangiaceae in the order Actinomycetales (Zhi et al., 2009). This genus forms club-shaped and spherical structures on aerial mycelia. At the time of writing, the genus Acrocarpospora contained three species: Acrocarpospora pleiomorpha, Acrocarpospora macrocephala (Tamura et al., 2000) and Acrocarpospora corrugata (basonym Streptosporangium corrugatum; Williams & Sharples, 1976). Members of the genus have been isolated from diverse sources such as soil (Tamura et al., 2000) and beach sand (Williams & Sharples, 1976). Members of the genus Acrocarpospora present meso-diaminopimelic acid in the cell wall and madurose as a characteristic sugar in whole-cell hydrolysates. The diagnostic phospholipid is phosphatidylethanolamine. The predominant menaquinones are MK-9(H2) and MK-9(H4).

During investigation of novel actinomycetes from temperate peat swamp forest soil in the northern area of Thailand, we isolated a strain, designated PS33-18T, showing morphological and chemotaxonomic characteristics typical of members of the genus Acrocarpospora but which was distinguishable genotypically and phenotypically from all
recognized Acrocarpospora species. Here, we describe the polyphasic characterization of strain PS33-18T and describe it as representing a novel species of the genus Acrocarpospora.

Strain PS33-18T was isolated from a Thai temperate peat swamp forest soil sample collected from the Phu-Sang National Park in Phayao Province, Thailand. The soil sample was air-dried at room temperature for 14 days. The dried soil sample was treated with dry heat in a hot air oven at 100 °C for 1 h and then serially diluted in sterile distilled water as recommended by Thawai et al. (2005). The strain was isolated on modified soil extract agar (Suriyachadkun et al., 2009) supplemented with (per litre) 25 mg nalidixic acid, 50 mg cycloheximide and 1 mg terbinafin. The pure culture was preserved by freezing at −80 °C and freeze-drying.

Morphological characteristics of strain PS33-18T grown on modified soil extract agar were observed by light and scanning electron microscopy (model JSM-5410 LV; JEOL). The samples for scanning electron microscopy were prepared as described previously (Thawai et al., 2005).

Phenotypic characteristics were examined by using several standard methods. Cultural characteristics were tested using 14-day cultures grown at 30 °C on various agar media. The ISCC–NBS Colour Charts standard sample no. 2106 was used for determining colour designations (Kelly, 1964). Hydrolysis of various compounds and acid production from carbon sources were examined using the basal medium recommended by Gordon et al. (1974). Temperature tolerances were determined on yeast extract-malt extract agar [International Streptomyces Project (ISP) 2 medium; Shirling & Gottlieb, 1966] for 14 days. pH and NaCl tolerances were determined on ISP 2 medium at 30 °C for 14 days. Carbon source utilization was tested by using ISP 9 medium (Shirling & Gottlieb, 1966) supplemented with a final concentration of 1% of the carbon sources and 0.05% Casamino acids. 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). The results are indicated in detail in the species description and Table 1.

Freeze-dried cells used for chemotaxonomic analyses were obtained from cultures grown in yeast extract-glucose broth on a rotary shaker (200 r.p.m.) at 30 °C for 4 days. The cell-wall peptidoglycan was prepared and hydrolysed by the methods of Kawamoto et al. (1981), and the amino acid composition was analysed by TLC (Lechevalier & Lechevalier, 1980). The acyl group of muramic acid in the peptidoglycan was determined by the method of Uchida & Aida (1984). The reducing sugars from whole-cell hydrolysates were analysed by the cellulose TLC method of Komagata & Suzuki (1987). Phospholipids in cells were extracted and analysed according to 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 RTSBA6 MIDI database. The presence of mycolic acids was investigated by using the method of Minnikin et al. (1975). Isoprenoid quinones were extracted according to Collins et al. (1977) and were

Table 1. Differential characteristics of strain PS33-18T and the type strains of recognized Acrocarpospora species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>Whole-cell sugars</td>
<td>Glu, Rha, Rib, Mad, Man</td>
<td>Glu, Gal, Rha, Mad, Xyl†</td>
<td>Glu, Gal, Mad, Xyl†</td>
<td>Gal, Mad†</td>
</tr>
<tr>
<td>Pattern of phospholipids</td>
<td>DPG, PG, PE, PME, OH-PE, PI, PIMs, NPG, 2 unknown phospholipids, 6 lipids</td>
<td>PE, NPG*</td>
<td>PE, NPG*</td>
<td>DPG, PE, OH-PE, PI†</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Maximum NaCl tolerance (%, w/v)</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1-Arabinose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>D-Galactose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>D-Fructose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Carbon utilization:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Arabinose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

*Data for the reference species were taken Tamura et al. (2000). All other phenotypic data were determined in this study. Glu, glucose; Gal, galactose; Rha, rhamnose; Rib, ribose; Mad, madurose; Man, mannose; Xyl, xylose; DPG, diposphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PME, phosphatidylmethylethanolamine; OH-PE, hydroxy-phosphatidylethanolamine; PI, phosphatidylinositol; PIMs, phosphatidylinositol mannosides; NPG, ninhydrin-positive phosphoglycolipids.

†Data for the reference species were taken from Stackebrandt et al. (1994).
analysed by HPLC with a Cosmosil 5C18 column (4.6 x 150 mm; Nacalai Tesque). The elution solvent was a mixture of methanol and 2-propanol (2:1, v/v).

Chromosomal DNA was isolated from cells grown in yeast extract-glucose 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 (Yamasho Shoyu) was used as the quantitative standard. DNA–DNA hybridization was conducted in micro-dilution-well plates, as reported by Ezaki et al. (1989). DNA–DNA relatedness was determined by using the colorimetric method (Verlander, 1992). The 16S rRNA gene was amplified as described by Suriyachadtun et al. (2009). The PCR products were sequenced (Macrogen) using universal primers 27F (5′-GTTTGATCCTGCTTGGCAGC-3′), 350F (5′-TACGGGAGGCAGCACG-3′), 780F (5′-GATTAGATACCTGGTAGTAG-3′), 1100F (5′-GCAAAGCAGCCAGAACCC-3′), 350R (5′-CTCGTCCTCCGTAG-3′), 780R (5′-TACCCAGGTTATCTAATCC-3′) and 1492R (5′-GGTTAATCTGGTGACGT-3′) (Lane, 1991). The 16S rRNA gene sequence was multiply aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases by using the CLUSTAL W program version 1.81 (Thompson et al., 1994). The alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) with genetic distances computed by using 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 the branches in the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Levels of 16S rRNA gene sequence similarity among all recognized Acrocarpospora species were first determined using the EzTaxon-e database (Kim et al., 2012). 16S rRNA gene sequence similarities among closely related species were calculated manually after pairwise alignments obtained using the CLUSTAL X program (Thompson et al., 1997).

Strain PS33-18T grew well on ISP 3, ISP 6 and nutrient agar, moderately on ISP 2, and weakly on ISP 4, ISP 5, ISP 7, Czapek’s sucrose agar and glucose-asparagine agar. The strain formed well-developed and branched substrate hyphae on ISP 2, ISP 3, ISP 6 and nutrient agar. The colour of substrate hyphae on these media was brilliant yellow to pale greenish yellow. White aerial hyphae were formed on ISP 2, ISP 3, ISP 4, ISP 5, ISP 7 and glucose-asparagine agar. Soluble pigments were not produced on any of the media tested. Strain PS33-18T formed club-shaped and spherical structures borne on the tip of the aerial mycelia on modified soil extract agar at 30 °C for 30 days (Fig. 1).

Chemotaxonomic characteristics of strain PS33-18T were similar to those of members of the genus Acrocarpospora. The cell walls of strain PS33-18T contained meso-diaminopimelic acid, glutamic acid and alanine. The acyl type of the cell-wall peptidoglycan was acetyl. The reducing sugars in whole-cell hydrolysates were glucose, madurose, mannose, rhamnose and ribose. The predominant menaquinone was MK-9(H4) (77.2 %); substantial amounts of MK-9(H2) (12.1 %) and MK-9(H6) (10.7 %) were present. The major cellular fatty acids (≥5 %) were C17:1ω8c (15.5 %), iso-C16:0 (12.9 %), C16:0 (12.6 %), C17:0 (7.6 %), anteiso-C15:0 (7.1 %), iso-C14:0 (6.7 %), C16:1ω7c (5.8 %), 10-methyl-C17:0 (5.5 %) and iso-C15:0 (5.0 %) (Table S1). Mycolic acids were absent. The diagnostic phospholipids were diphasphatidylglycerol, phosphatidylglycerol, phosphatidylyethanolamine, phosphatidylymethylethanolamine, hydroxy-phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides, ninhydrin-positive phosphoglycolipids, two unidentified phospholipids and six unidentified lipids (Fig. S1). The G+C content of the genomic DNA was 71.0 mol%.

The almost-complete 16S rRNA gene sequence was obtained for strain PS33-18T (1453 nt). 16S rRNA gene sequence analysis showed that strain PS33-18T exhibited a close relationship with members of the family Strep-tosporangiaceae and the strain was placed in a monophyletic cluster with the genus Acrocarpospora (Fig. 2). The relationship between these strains was supported by the results from the neighbour-joining, maximum-parsimony and maximum-likelihood methods. 16S rRNA gene

Fig. 1. Light micrograph (a) and scanning electron micrograph (b) of cells of strain PS33-18T grown on modified soil extract agar for 4 weeks at 30 °C. Bars, 30 μm (a) and 10 μm (b).
between strain PS33-18T and the type strains of all recognized *Acrocarpospora* species. Based on the morphological, chemotaxonomic and phylogenetic data, this strain should be classified in the genus *Acrocarpospora*.

The characteristics shown in Table 1 clearly indicate that strain PS33-18T possesses distinct chemotypic and phenotypic characteristics that separate it from its closest relatives, *A. macrocephala* NBRC 16266T, *A. pleiomorpha* NBRC 16267T and *A. corrugata* NBRC 13972T. In particular, strain PS33-18T contained ribose and mannose in cell-wall hydrolysates but not galactose, whereas the type strains of the three recognized *Acrocarpospora* species had galactose in their cell-wall hydrolysates but not ribose or mannose. The phospholipid profile of strain PS33-18T was different from that of *Acrocarpospora* species in terms of phosphatidylmethyllethanolamine and phosphatidylinositol mannosides.

Furthermore, the ability to reduce nitrate to nitrite, maximum NaCl for growth, utilization of L-arabinose, D-fructose and sucrose, and production of acid from L-arabinose, D-galactose and sucrose are effective in discriminating between strain PS33-18T and the closely related species. The DNA–DNA relatedness values between strain PS33-18T and *A. macrocephala* NBRC 16266T, *A. pleiomorpha* NBRC 16267T and *A. corrugata* NBRC 13972T ranged from 17.1 ± 1.5 to 30.6 ± 0.9 %, values 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).

Based on the phenotypic, chemotaxonomic and genotypic data presented, strain PS33-18T can be distinguished from all recognized *Acrocarpospora* species, which supports its classification as representing a novel species of the genus *Acrocarpospora*, for which the name *Acrocarpospora phusangensis* sp. nov. is proposed.

**Description of Acrocarpospora phusangensis sp. nov.**

*Acrocarpospora phusangensis* (phu.sang.en’sis. N.L. fem. adj. *phasisangensis* pertaining to Phu-Sang National Park, where the type strain was isolated).

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**Fig. 2.** Neighbour-joining tree (Saitou & Nei, 1987) based on almost-complete 16S rRNA gene sequences (1453 nt) showing the relationships between strain PS33-18T, the type strains of recognized *Acrocarpospora* species and members of the genera *Herbidospora*, *Planotetraspora*, *Microtetraspora*, *Planobispora* and *Streptosporangium*. *Streptomyces ambofaciens* NBRC 12836T was used as an outgroup. Asterisks (*) indicate branches of the tree that were also found using the maximum-parsimony and maximum-likelihood methods. The numbers on the branches indicate the percentage bootstrap values of 1000 replicates; only values ≥ 50 % are indicated. Bar, 0.01 substitutions per nucleotide position.
Gram-stain-positive, mesophilic actinomycete that forms club-shaped and spherical structures borne on the tip of aerial mycelia. No soluble pigment is produced in test culture media. Nitrate is not reduced to nitrite. Utilizes cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannitol, melibiose, raffinose, sucrose, lactose, L-arabinose and L-rhamnose as sole carbon sources, but not D-xylene or glycerol. Acid production from cellobiose, D-fructose, D-galactose, D-mannitol, D-ribose, L-arabinose and L-rhamnose. Peptonization of milk and hydrolysis of starch are positive. Gelatin liquefaction and coagulation of milk are negative. Optimal temperature for growth is 25–30 °C. No growth at 37 °C. The maximum NaCl concentration for growth is 1 % (w/v). The pH range for growth is 7–11. The cell-wall peptidoglycan contains meso-diaminopimelic acid, glutamic acid and alanine. The acyl type of the cell wall is acetyl. The predominant menaquinone is MK-9(H4). The cell-wall peptidoglycan contains


