Actinoplanes subglobosus sp. nov., isolated from mixed deciduous forest soil

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A novel filamentous bacterial strain, A-T 5400\textsuperscript{T}, which developed subglobose sporangia at the end of sporangiophores on substrate mycelia, was isolated from mixed deciduous forest soil collected in Thailand. The taxonomic position of this micro-organism was described using a polyphasic approach. The 16S rRNA gene sequence and phylogenetic analysis indicated that strain A-T 5400\textsuperscript{T} belonged to the genus *Actinoplanes* and was most closely related to *Actinoplanes hulinensis* NEAU-M9 (98.82 \% 16S rRNA gene sequence similarity) and *Actinoplanes philippinensis* NBRC 13878\textsuperscript{T} (98.75 \%). The DNA–DNA relatedness values that distinguished the novel strain from the closest species were below 70 \%. The cell-wall peptidoglycan contained meso-diaminopimelic acid. The whole-cell sugars were ribose, galactose, glucose and xylose. The predominant menaquinone was MK-9(H\textsubscript{4}). The diagnostic phospholipids were phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. The predominant cellular fatty acids were unsaturated fatty acids C\textsubscript{16:1}, branched fatty acids iso-C\textsubscript{16:0} and iso-C\textsubscript{15:0}. The G+C content of the genomic DNA was 71 mol\%. Following evidence from phenotypic, chemotaxonomic and genotypic studies, the new isolate is proposed to represent a novel species of the genus *Actinoplanes* named *Actinoplanes subglobosus* sp. nov. The type strain is A-T 5400\textsuperscript{T} (=BCC 42734\textsuperscript{T}=TBRC 5832\textsuperscript{T}=NBRC 109645\textsuperscript{T}).

The genus *Actinoplanes* was first described by Couch (1950) with *Actinoplanes philippinensis* as the type species. Members of this genus are characterized by the presence of glocbose, subglobose, cylindrical, lobate, flask-shaped, bell-shaped, or irregular sporangia that contain motile sporangiospores with a clump of polar or peritrichous flagella at the tip of sporangiophores on substrate mycelia (Goodfellow et al., 1990). Aerial mycelia are scant. Cells are Gram-positive, non-acid-fast and aerobic. The peptidoglycan of this genus contains meso-diaminopimelic acid, but hydroxydiaminopimelic acid is also present. The whole-cell sugars contain xylose as a marker. MK-9(H\textsubscript{4}) is the major isoprenoid quinone. The phospholipid pattern is of type II and usually includes phosphatidylethanolamine as the diagnostic phospholipid. Predominant cellular fatty acids are often present as iso-ante-iso-branched and monounsaturated fatty acids and/or cis-9, 10-octadecanoic acid (Tamura & Hatano, 2001). The DNA G+C content is 67–76 mol\% (Sazak et al., 2012). Many species of this genus produce bioactive compounds, such as friulimicins, a novel lipopeptide antibiotic from *Actinoplanes friulensis* (Aretz et al., 2000), and candiplanicin, a new antifungal from *Actinoplanes regularis* (Itoh et al., 1981).

During a study on the diversity of actinomycetes in Thailand, strain A-T 5400\textsuperscript{T} was isolated from mixed deciduous forest soil collected in Lamphun Province, Northern Thailand. The soil sample was taken from the organic layer on the soil surface and kept at 4 \degree C. The sample was dried at room temperature for 10 days then heat-dried at 100 \degree C for 1 h. The dried sample was treated using a method of Suzuki (2001) and plated on soil extract agar (Suriyachadkun et al., 2009) supplemented with 25 mg l\textsuperscript{-1} nalidixic acid, 50 mg l\textsuperscript{-1} cycloheximide and 1 mg l\textsuperscript{-1} terbinafine, then incubated at 28 \degree C for 21 days. The isolate was purified and maintained on ISP medium no. 2 as a working culture. The pure culture was preserved in glycerol (10 \%, v/v) at −80 \degree C and in liquid-dried form for long-term storage. Strains of the two closest species, *Actinoplanes hulinensis* NEAU-M9 and A.
philippinensis NBRC 13878T were used in this study for comparison of physiological, morphological and chemotaxonomic properties and DNA–DNA hybridization.

Genomic DNA for determination of G+C content analysis and 16S rRNA gene sequence analysis was prepared according to the method of Saito & Miura (1963) from a culture grown in yeast-extract-glucose broth on a rotary shaker at 200 r.p.m., 30 °C for 7 days. The G+C content of the genomic DNA of strain A-T 51038 was determined using the method of Tamaoka & Komagata (1984) after treatment with P1 nuclease and alkaline phosphatase. HPLC was carried out using a Shimadzu LC-6AD apparatus equipped with a Cosmosil 5C18-AR column (4.6×150 mm; Nacalai Tesque). The phylogenetic position of strain A-T 5400T was determined by 16S rRNA gene sequence analysis. The 16S rRNA gene was amplified by PCR as described previously (Katsura et al., 2001; Yamada et al., 2000). The PCR product for the 16S rRNA gene was sequenced by Macrogen Inc. using universal primers. BLAST analysis was used to compare the nearly complete 16S rRNA gene sequences of strain A-T 5400T with sequences of representatives of the genus Actinoplanes retrieved from the nucleotide databases (EzTaxon-e server; Kim et al., 2012). The 16S rRNA gene sequence similarity was calculated using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). Multiple alignments were carried out with CLUSTAL W (Thompson et al., 1994) of the software BioEdit Sequence Alignment Editor (version 7.0.0). Phylogenetic trees were generated by neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Felsenstein, 1983) and maximum-likelihood (Felsenstein, 1981) methods using the MEGA6 software package. The evolutionary distance matrix using the Tamura–Nei model was calculated for neighbour-joining and maximum-likelihood method. The maximum-parsimony search method used Subtree-Pruning-Regrafting (SPR). The robustness for individual branches was estimated by bootstrapping with 1000 replications (Felsenstein, 1985). DNA–DNA hybridization was carried out using a photobiotin-labelling method in microdilution wells as described by Ezaki et al. (1989). DNA–DNA relatedness (%) was determined using the colourimetric method (Verlander, 1992).

Pairwise sequence similarities of strain A-T 5400T were calculated using the 16S rRNA gene sequence of 1443 bases. This strain exhibited the highest 16S rRNA gene sequence similarities of 98.82 % and 98.75 % with ‘A. hulinensis’ NEAU-M9 and A. philippinensis NBRC 13878T, respectively. Phylogenetic trees reconstructed by neighbour-joining (Fig. 1), maximum parsimony (Fig. S1a, available in the online Supplementary Material) and maximum likelihood (Fig. S1b) methods showed similar tree topologies. The phylogenetic relationship between strain A-T 5400T and members of the genus Actinoplanes revealed that the novel strain formed a cluster with A. philippinensis NBRC 13878T. The G+C content of genomic DNA from strain A-T 5400T was 71 mol%. DNA–DNA relatedness levels of strain A-T 5400T were 34–44 % compared with ‘A. hulinensis’ NEAU-M9 and 33–45 % compared with A. philippinensis NBRC 13878T, which are significantly below 70 %, the cut-off point recommended by Wayne et al. (1987) for the delineation of separate bacterial species. Results of 16S rRNA gene sequence analysis and DNA–DNA relatedness were sufficient to categorize strain A-T 5400T as a distinct species from previously described species of the genus Actinoplanes.

Morphological characteristics of strain A-T 5400T were observed using a light microscope (model CX 31; Olympus) with a 40× long working distance objective lens (model LUPLFLN40XRC; Olympus) and a scanning electron microscope (model JSM-5410 LV; JEOL) after cultivation on soil extract agar (Suriyachadkun et al., 2009) at 30 °C for 14–21 days. Cultures for scanning electron microscopy were prepared as described previously (Itoh et al., 1989). For observations of spore motility, the strain was grown on soil extract agar (30 °C, 30 days), sporangia flooded with 0.1 M potassium phosphate buffer (pH 7) at 30 °C for 30 min and observed with a light microscope. Cultural characteristics of strain A-T 5400T compared with ‘A. hulinensis’ NEAU-M9 and A. philippinensis NBRC 13878T were determined on various media described by Shirling & Gottlieb (1966), ISP2, ISP3, ISP4, ISP5, ISP7 and yeast extract-starch agar, following incubation at 28 °C for 14 and 21 days. The colony colour was determined using ISCC-NBS colour charts (Kelly, 1964). Physiological characteristics were examined using several standard methods. Temperature, pH and NaCl tolerances were determined using ISP2 as a basal medium after incubation for 2 weeks. The temperature range for growth was determined at 20, 25, 30, 37 and 45 °C. The pH range for growth was determined at pH 4.0–11.0 (intervals of 1.0 pH unit) at 30 °C. NaCl tolerance was determined with 0, 1, 2, 3, 4 and 5 % (w/v) NaCl at 30 °C. Utilization of carbohydrates as sole carbon sources was tested using ISP9 medium as a basal medium (Shirling & Gottlieb, 1966) supplemented with a final concentration of 1 % of the carbon sources. Hydrolysis of various compounds was examined using a basal medium recommended by Gordon et al. (1974). Tests in the API ZYM commercial system (bioMérieux) were performed according to the manufacturer’s instructions. 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).

Freeze-dried cells for chemotaxonomic analysis were obtained from a culture growing in glucose-yeast extract broth (Tamura et al., 1994) on a rotary shaker at 200 r.p.m., 30 °C for 7 days. The isomer of diaminopimelic acid in the cell wall was determined using the method of Stanek & Roberts (1974). The acyl type of peptidoglycan was determined using the method of Uchida & Aida (1984). The acyl type of peptidoglycan was determined using the method of Komagata & Suzuki (1987). Polar lipids in whole cells were extracted and analysed by two-dimensional TLC according to the method of Minnikin et al. (1984). Cellular fatty acid methyl esters were
Fig. 1. Phylogenetic relationships derived from 16S rRNA gene sequences between strain A-T 5400T and other species of the genus *Actinoplanes*. The phylogenetic tree was reconstructed by using the neighbour-joining method. *Dactylosporangium aurantiacum* DSM 43157T was used as an out-group. Asterisks represent clades that were also recovered with the maximum-

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*Actinoplanes capillaceus* K95-5561T (AB013495)

*Actinoplanes campanulatus* NBRC 12511T (AB036995)

*Actinoplanes hulinensis* NEAU-M9 (U007323)

*Actinoplanes philippinensis* NBRC 13878T (D85474)

*Actinoplanes subglobosus* A-T 5400T (KM396265)

*Actinoplanes sichuanensis* 03-723T (EU531458)

*Actinoplanes auranticolor* NBRC 12245T (U58526)

*Actinoplanes lobatus* NBRC 12513T (AB037006)

*Actinoplanes xinjiangensis* 03-8772T (EU531457)

*Actinoplanes derwentensis* NBRC 14935T (AB036999)

*Actinoplanes rectilineatus* NBRC 13941T (AB037010)

*Actinoplanes couchii* GW8-1761T (AM400230)

*Actinoplanes italicus* JCM 3165T (AB048217)

*Actinoplanes utahensis* NBRC 13244T (AB037012)

*Actinoplanes luteus* A-T 5190T (KM396264)

*Actinoplanes palleronii* NBRC 14916T (AB037009)

*Actinoplanes cyaneus* DSM 46137T (X93186)

*Actinoplanes liguriensis* DSM 43865T (AJ865471)

*Actinoplanes lutulentus* NEAU-GRX6T (KC134255)

*Actinoplanes siamensis* A-T 6646T (JX283293)

*Actinoplanes sibiricus* NEAU-M9 (U007323)

*Actinoplanes ianthinogenes* NBRC 13996T (AB047495)

*Actinoplanes teichomyceticus* NBRC 13999T (AB047513)

*Actinoplanes regularis* DSM 43151T (X93188)

*Actinoplanes abujensis* A4029T (HQ157185)

*Actinoplanes inaCC A522 (LC027115)

*Actinoplanes brasiliensis* DSM 43805T (X93185)

*Actinoplanes deccanensis* NBRC 13994T (AB036998)

*Actinoplanes atraurantiacus* strain Y16T (HQ839788)

*Actinoplanes digitatis* NBRC 12512T (AB037000)

*Actinoplanes nipponensis* FH 2241T (AJ871310)

*Actinoplanes lichenis* LDG1-22T (LC033899)

*Actinoplanes friuliensis* DSM 43157T (X93181)

*Actinoplanes humidus* NBRC 14915T (AB037008)

*Actinoplanes consettensis* JCM 7624T (AB048214)

*Actinoplanes globisporus* JCM 3186T (AB048219)

*Actinoplanes rishiriensis* R50-RCA114T (AB641831)

*Actinoplanes phaeosporus* NEAU-A-2T (KF600711)

*Actinoplanes teichomyceticus* NEAU-M9 (U007323)

*Actinoplanes japonicus* A-T 5400T (KM396264)

*Actinoplanes missouriensis* NBRC 13243T (AB037002)

*Actinoplanes ianthinogenes* NBRC 13999T (AB047513)

*Actinoplanes regularis* DSM 43151T (X93188)

*Actinoplanes subglobosus* A-T 5400T (KM396264)

*Actinoplanes aurantiacus* DSM 43157T (X93191)
prepared and analysed following the instructions of TSBA6 method of the Microbial Identification System (MIDI, Sherlock version 6.2B) (Kämpfer & Kroppestedt, 1996; Sasser, 1990). The presence of mycolic acids in the cell wall was analysed by TLC according to the method of Tomiyasu (1982). Isoprenoid quinones were extracted and purified using the method of Collins et al. (1977) and determined using reverse-phase HPLC [Cosmosil 5C18 column (4.6×150 mm); Nacalai Tesque] with a mixture of methanol and 2-propanol (2:1, v/v) as elution solvent (Wu et al., 1989).

Strain A-T 5400T had characteristics typical of the genus Actinoplanes. It developed large subglobose sporangia (10–15 µm in diameter) containing motile sporangiospores at the tip of sporangiophores on substrate mycelia (Fig. 2). The spores were motile. Fragmentation of substrate mycelium was not observed. Phenotypic characteristics of strain A-T 5400T compared with ‘A. hulinensis’ NEAU-M9 and A. philippinensis NBRC 13878T are indicated in detail in Table 1 and the species description. Strain A-T 5400T showed good growth on ISP medium no. 2, 4 and 5, and yeast extract-starch agar. Moderate growth was observed on ISP3. Substrate mycelium was pale yellow to strong orange yellow. Soluble pigment was not observed on the media used.

The isomer of diaminopimelic acid of strain A-T 5400T was meso-diaminopimelic acid. The acyl type of the cell-wall muramic acid was glycolylmuramic acid. Whole-cell hydrolysates contained ribose, galactose, glucose and xylose [whole-cell sugar pattern D of Lechevalier & Lechevalier (1970)]. Menaquinoles were MK-9(H4) (3 %), MK-9(H6) (93 %), and MK-9(H8) (4 %). Phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, two unidentified glycolipids and two unidentified phospholipids were detected, while phosphatidylcholine was not detected (Fig. S2). The unsaturated fatty acids C16:1 (15.5 %), branched fatty acids iso-C16:0 (24.8 %), and iso-C15:0 (10.6 %) were major fatty acids (>10 % each) and saturated fatty acids C14:0 (6.9 %), C16:0 (5.9 %) and unsaturated fatty acids C18:1 (8.2 %) were found in moderate amounts. Mycolic acids were absent.

The strain A-T 5400T developed subglobose sporangia, while the closest species, ‘A. hulinensis’ NEAU-M9 and A. philippinensis NBRC 13878T developed bell-shaped and globose sporangia, respectively. Strain A-T 5400T and ‘A. hulinensis’ NEAU-M9 did not produce soluble pigment, while A. philippinensis NBRC 13878T produced soluble pigment (Table 1). Low DNA–DNA relatedness values were observed between strain A-T 5400T and type strains of the closest species. The phenotypic (Table 1) and genotypic data show that strain A-T 5400T can be distinguished from previously described species of the genus Actinoplanes. It is therefore concluded that this strain be classified as representative a novel species of the genus Actinoplanes, for which the name Actinoplanes subglobosus sp. nov. is proposed.

**Description of Actinoplanes subglobosus sp. nov.**

Actinoplanes subglobosus (sub.glo.bo’sus. L. pref. sub nearly; L. adj. globosus globose, spheric; N.L. masc. adj. subglobosus referring to the nearly globose or subglobose sporangia that are developed on substrate mycelium).

Cells are aerobic, Gram-positive and non-acid-fast. Substrate mycelium is light orange on ISP2 and ISP4 and vivid orange on ISP5. Large subglobose sporangia, 10–15 µm in diameter, containing motile sporangiospores formed at the tip of sporangiophores on substrate mycelia. Growth occurs at 20–37 °C (optimum 25–30 °C), pH 6–10 (optimum pH 8–9) and in a maximum NaCl concentration of 1 % (w/v). Utilizes L-arabinose, cellobiose, D-galactose, D-glucose, glycerol, D-fructose, inositol, lactose, D-mannose, D-mannitol, raffinose, L-rhamnose, salicin, sucrose, trehalose and D-xylose, but not D-arabitol, melibiose, sorbose, D-ribose, D-sorbitol or D-xylitol. Hydrolysis of starch, xanthine and hypoxanthine are positive. Nitrate reduction and coagulation of milk are negative. Positive for activities of alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase,
Table 1. Characteristics differentiating strain A-T 5400T, ‘A. hulinensis’ NEAU-M9 and A. philippinensis NBRC 13878T

Strains: 1, Actinoplanes subglobosus sp. nov. A-T 5400T; 2, ‘A. hulinensis’ NEAU-M9; 3, A. philippinensis NBRC 13878T. All strains were positive for utilization of L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, inositol, lactose, D-mannose, D-mannitol, L-rhamnose and sucrose, and negative for utilization of D-arabitol, melibiose, sorbose and D-xylitol. +, Positive; –, negative.

<table>
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<tr>
<th>Characteristic</th>
<th>1</th>
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<tr>
<td>Colonial properties</td>
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<tr>
<td>ISP2 (substrate mycelium)</td>
<td>Light orange</td>
<td>Deep reddish-orange</td>
<td>Light orange</td>
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<tr>
<td>ISP4 (substrate mycelium)</td>
<td>Light orange yellow</td>
<td>Deep yellowish-brown</td>
<td>Strong yellowish-brown</td>
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<tr>
<td>ISP5 (substrate mycelium)</td>
<td>Vivid orange</td>
<td>Dark orange yellow</td>
<td>Moderate orange</td>
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<td>Soluble pigment</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Shape of sporangia</td>
<td>Subglobose</td>
<td>Bell-shaped</td>
<td>Globose</td>
</tr>
<tr>
<td>Major menaquinones</td>
<td>MK-9(H4)</td>
<td>MK-9(H4), MK-9(H6)</td>
<td>MK-9(H4)</td>
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<tr>
<td>Coagulation of milk</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Growth at 37 °C</td>
<td>–</td>
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<td>Utilization of:</td>
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<tr>
<td>Glycerol</td>
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<td>Raffinose</td>
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<td>D-Ribose</td>
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<td>Salicin</td>
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<tr>
<td>D-sorbitol</td>
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<td>D-Xylose</td>
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<td>Enzyme activity</td>
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<tr>
<td>Esterase Lipase (C 8)</td>
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<tr>
<td>Valve arylamidase</td>
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<tr>
<td>α-Chymotrypsin</td>
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<tr>
<td>Acid phosphatase</td>
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<td>+</td>
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<tr>
<td>N-Acetyl-β-glucosaminidase</td>
<td>–</td>
<td>+</td>
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α-glucosidase, β-glucosidase and catalase. Cell-wall amino acids contain meso-diaminopimelic acid. Acyl type of the cell-wall muramic acid is glycolylmuramic acid. The characteristic whole-cell sugars are ribose, galactose, glucose and xylose. The predominant menaquinone is MK-9(H4). The diagnostic phospholipids are phosphatidylethanolamine and diphosphatidylglycerol as major components; lacks phosphatidylcholine and aminoglycosyl lipids. The major fatty acids are unsaturated fatty acids C15:1ω6c and branched fatty acids iso-C15:0 and iso-C16:0.

The type strain is A-T 54090T (=BCC 42734T=TBRC 5832T=NBRC 109645T), isolated from mixed deciduous forest soil. The G+C content of the DNA of the type strain is 71 mol%.

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