Planotetraspora kaengkrachanensis sp. nov. and *Planotetraspora phitsanulokensis* sp. nov., isolated from soil

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Two novel bacterial strains were isolated from tropical rain forest soil from Thailand. Strains A-T 0875^T and A-T 1383^T stained Gram-positive and were filamentous bacteria that developed cylindrical sporangia containing four oval- to rod-shaped spores at the ends of short sporangiophores on branched aerial mycelium. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid, glutamic acid and alanine as cell-wall amino acids; whole-cell hydrolysates contained rhamnose, madurose, glucose, galactose and 3-O-methylmannose as whole-cell sugars. The predominant menaquinone was MK-9(H₄). Mycolic acids were not detected. The diagnostic phospholipid was phosphatidylethanolamine. The predominant cellular fatty acids were iso-C_{16:0} and 10-methyl-C_{17:0}. For both strains, the G + C content of the genomic DNA was 71 mol%. Phenotypic and chemotaxonomic analyses showed that the characteristics of the two isolates were typical of members of the genus *Planotetraspora*. Furthermore, 16S rRNA gene sequence analysis also indicated that the strains belonged to the genus *Planotetraspora* but as representatives of two novel species. Following an evaluation of our phenotypic, chemotaxonomic and genotypic studies, two novel species are proposed, *Planotetraspora kaengkrachanensis* sp. nov. (type strain A-T 0875^T=BCC 24832^T=NBRC 104272^T) and *Planotetraspora phitsanulokensis* sp. nov. (type strain A-T 1383^T=BCC 26045^T=NBRC 104273^T).

The genus *Planotetraspora* was first proposed by Runmao *et al.* (1993) and classified in the family *Streptosporangiaceae* and, at the time of writing, it included three recognized species: *Planotetraspora mira* (Runmao *et al.*, 1993) as the type species, *Planotetraspora silvatica* (Tamura & Sakane, 2004) and *Planotetraspora thailandica* (Suriyachadkun *et al.*, 2009). Members of the genus are Gram-positive, non-acid-fast and aerobic. They develop cylindrical sporangia

containing four non-motile spores at the ends of short sporangiophores on the aerial mycelium. Substrate mycelium is non-fragmented. Vegetative mycelia are white to pale yellow and good growth is observed between 25 and 30 °C. These strains have *meso*-diaminopimelic acid (*meso*-A₂pm), glutamic acid and alanine in the cell wall. They contain madurose and rhamnose as whole-cell sugars. Mycolic acids are not detected. MK-9(H₄) is the major isoprenoid quinone and the phospholipid pattern is type PIV. The predominant cellular fatty acids are iso-C_{16:0} and 10-methyl-C_{17:0} corresponding to fatty acid pattern 3c of Kroppenstedt (1985). The DNA G + C content is 70–71 mol%. The genus *Planotetraspora* is closely related to the genera *Microbispora* (Nonomura & Ohara, 1957),

Abbreviation: *meso*-A₂pm, *meso*-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Planotetraspora kaengkrachanensis* A-T 0875^T and *Planotetraspora phitsanulokensis* A-T 1383^T are FJ426333 and FJ426332, respectively.

Microtetraspora (Thiemann *et al.*, 1968), *Herbidospora* (Kudo *et al.*, 1993) and *Acrocarpospora* (Tamura *et al.*, 2000). Members of these genera exhibit a high 16S rRNA gene sequence similarity, but members of the genus *Planotetraspora* can be distinguished from those of the other four genera on the basis of morphological and chemotaxonomic criteria.

During a project on the diversity of actinomycetes in Thailand, strains A-T 0875^T and A-T 1383^T were isolated from forest soils collected from Kaeng Krachan District in Prachuap Khiri Khan Province and Nakhon Thai District in Phitsanulok Province, respectively. The isolates were identified as two novel members of the genus *Planotetraspora* based on morphological and chemotaxonomic studies, phylogenetic analysis and DNA–DNA relatedness studies. Therefore, we consider that A-T 0875^T and A-T 1383^T represent two novel species, *Planotetraspora kaengkrachanensis* sp. nov. and *Planotetraspora phitsanulokensis* sp. nov., respectively.

Strain A-T 0875^T was isolated from soil samples treated with phenol as recommended by Hayakawa *et al.* (1991) and strain A-T 1383^T was isolated from soil samples that were thermally treated (100 °C for 1 h). The strains were isolated on humic acid salts-vitamin (HV) agar supplemented with 25 mg nalidixic acid l⁻¹, 50 mg cycloheximide l⁻¹ and 1 mg terbinafine l⁻¹. Pure cultures were preserved by freezing at –80 °C in glycerol (15 %, v/v) and liquid-drying.

For determination of morphological characteristics, 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 was observed with a light microscope and a scanning electron microscope (model JSM-5410 LV; JEOL). Samples grown on modified soil extract agar for scanning electron microscopy were prepared as described previously (Itoh *et al.*, 1989).

Characteristics of strains A-T 0875^T and A-T 1383^T were typical of those of members of the genus *Planotetraspora*. They developed cylindrical sporangia (0.8–1.0 × 2.0–3.0 µm) containing four oval- to short rod-shaped spores (0.4–0.6 × 0.8–1.0 µm) at the ends of short sporangio-phores on the aerial mycelium. The spores were not motile, as determined by light microscopy of cells grown on HV agar for 10 days at 28 °C and then incubated at 28 °C for 30–60 min in 0.1 M potassium phosphate buffer (pH 7).

The phenotypic characteristics of the two novel strains were examined by using several standard methods. Cultural characteristics were tested using 14–21 day cultures grown at 28 °C on various agar media (Shirling & Gottlieb, 1966). The NBS-ISCC colour system was used to determine the names and designations of colony colours. The temperature, pH and NaCl tolerance for growth were determined on ISP2 medium. Acid production from carbon sources was examined using the medium recommended by Gordon

et al. (1974). Utilization of carbohydrates as sole carbon sources was tested by using ISP9 medium as a basal medium (Shirling & Gottlieb, 1966) supplemented with a final concentration of 1 % carbon source. Gelatin liquefaction, peptonization of milk, nitrate reduction and starch hydrolysis were determined by 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 a culture grown in glucose-yeast extract broth (Tamura *et al.*, 1994) on a rotary shaker at 30 °C for 7 days. Cell-wall peptidoglycan was prepared and hydrolysed according to the methods of Kawamoto *et al.* (1981) and the amino acid composition was determined by HPLC. The A₂pm isomer in the cell wall was determined by using the method of Stanek & Roberts (1974). The acyl group of the muramic acid in the peptidoglycan was determined by using the method of Uchida & Aida (1984). The reducing sugars from whole-cell hydrolysates were analysed by HPLC using a Sugar-Pak column (Waters) according to Mikami & Ishida (1983). Cell 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 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 [Cosmosil 5C₁₈ column (4.6 × 150 mm); Nacalai Tesque] with a mixture of methanol and 2-propanol (2 : 1, v/v) as elution solvent.

The chemotaxonomic characteristics of the two novel strains were similar to those of members of the genus *Planotetraspora*. Cell-wall amino acids contained *meso*-A₂pm, alanine and glutamic acid. These amino acids correspond to murein type A1γ as described by Schleifer & Kandler (1972). The two novel strains contained rhamnose, madurose, glucose, galactose and 3-O-methylmannose as whole-cell sugars, but not arabinose or xylose (whole-cell sugar pattern B of Lechevalier & Lechevalier, 1970). Phosphatidylethanolamine was detected, but phosphatidylglycerol, phosphatidylcholine and glucosamine-containing phospholipids were not detected. This pattern corresponds to phospholipid type PIV of Lechevalier *et al.* (1977). The cellular fatty acid compositions of strains A-T 0875^T and A-T 1383^T and the three recognized *Planotetraspora* species are shown in Table 2. Strain A-T 0875^T contained iso-C_{16:0}, 10-methyl-C_{17:0}, iso-C_{15:0} and C_{15:0}, and strain A-T 1383^T contained iso-C_{16:0}, 10-methyl-C_{17:0}, iso-C_{15:0} and anteiso-C_{17:0}, corresponding to fatty acid pattern 3c of Kroppenstedt (1985), but 10-methyl-C_{18:0} was not detected as a major component. Mycolic acids were absent. The major menaquinone found in the two novel strains was MK-9(H₄).

Genomic DNA was prepared using a slight modification of the method of Saito & Miura (1963). The 16S rRNA gene

Table 1. Characteristics differentiating members of the genus *Planotetraspora*

Species: 1, *P. kaengkrachanensis* sp. nov. A-T 0875^T (data from this study); 2, *P. phitsanulokensis* sp. nov. A-T 1383^T (data from this study); 3, *P. thailandica* BCC 21825^T (Suriyachadkun *et al.*, 2009); 4, *P. mira* NBRC 15435^T (Suriyachadkun *et al.*, 2009); 5, *P. silvatica* NBRC 100141^T (Suriyachadkun *et al.*, 2009). +, Positive; –, negative; ±, weakly positive.

Characteristic	1	2	3	4	5
Colony colour on ISP2 medium					
Degree of growth	Poor	Poor	Good	Good	Good
Aerial mycelium or spore	White	Colourless	Pale yellow	Pale yellow	White
Substrate mycelium	Pale yellow	Colourless	Pale yellow	Pale yellow	White to colourless
Colony colour on ISP3 medium					
Degree of growth	Poor	Moderate	Moderate	Poor	Moderate
Aerial mycelium or spore	Colourless	White	Colourless	Pale yellow	Yellowish white
Substrate mycelium	Colourless	White	Colourless	Pale yellow	Yellowish white
NaCl tolerance	≤ 1.5 %	≤ 1 %	≤ 1 %	≤ 1 %	≤ 3 %
Acid from:					
D-Galactose	+	–	–	+	+
D-Lactose	–	–	–	+	–
D-Mannitol	+	+	+	+	–
D-Mannose	+	+	+	+	–
Melibiose	–	–	+	–	–
Maltose	–	+	–	–	–
L-Rhamnose	–	–	+	+	–
D-Xylose	–	–	+	+	+
Utilization of:					
D-Fructose	–	–	+	–	–
D-Galactose	–	+	+	±	+
D-Glucose	–	+	±	+	–
D-Mannitol	+	+	+	+	–
Melibiose	±	–	+	±	+
Raffinose	–	–	+	–	+
L-Rhamnose	±	–	+	±	±
D-Xylose	–	–	+	+	–
Salicin	–	–	+	–	–
Skim milk peptonization	–	–	–	–	+
Skim milk coagulation	–	+	–	+	–

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). Amplified 16S rRNA genes were sequenced directly with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit on an ABI PRISM model 310 Genetic Analyzer (both from 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). The neighbour-joining method was used to construct a phylogenetic tree (Saitou & Nei, 1987). Comparison of the sequence data obtained was made on 1376 bases and the robustness for individual branches was

estimated by bootstrapping with 1000 replications (Felsenstein, 1985). Strain A-T 0875^T exhibited highest 16S rRNA gene sequence similarities of 98.7 % to strain A-T 1383^T, *P. thailandica* BCC 21825^T and *P. mira* NBRC 15435^T, and 98.1 % to *P. silvatica* TT 00-51^T, whereas strain A-T 1383^T exhibited highest 16S rRNA gene sequence similarities of 98.4, 97.9 and 98.2 % to *P. thailandica* BCC 21825^T, *P. mira* NBRC 15435^T and *P. silvatica* TT 00-51^T, respectively. A neighbour-joining tree showing the phylogenetic relationship between strain A-T 0875^T, A-T 1383^T and some members of the family *Streptosporangiaceae* revealed that these novel isolates should be classified in the genus *Planotetraspora* and that they were clearly separate from their closest relatives (Fig. 1).

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 followed by HPLC using a Shimadzu LC-6AD apparatus equipped with a Cosmosil 5C₁₈-AR column (4.6 × 150 mm; Nacalai Tesque).

Table 2. Cellular fatty acid composition of the five *Planotetraspora* species

Strains: 1, *P. kaengkrachanensis* sp. nov. A-T 0875^T (data from this study); 2, *P. phitsanulokensis* sp. nov. A-T 1383^T (data from this study); 3, *P. thailandica* BCC 21825^T (Suriyachadkun *et al.*, 2009); 4, *P. mira* NBRC 15435^T (Suriyachadkun *et al.*, 2009); 5, *P. silvatica* NBRC 100141^T (Suriyachadkun *et al.*, 2009). Values are percentages of total fatty acids. —, Not detected.

Fatty acid	1	2	3	4	5
Saturated					
C _{13:0}	<1	<1	<1	<1	<1
C _{14:0}	<1	<1	1.6	2.3	1.1
C _{15:0}	10.6	4.8	7.3	9.5	9.9
C _{16:0}	3.4	3.3	5.0	7.3	5.6
C _{17:0}	8.8	6.0	4.0	8.7	7.2
C _{18:0}	<1	1.1	<1	<1	<1
Unsaturated					
C _{15:1}	—	—	<1	—	—
C _{16:1}	1.0	1.2	2.0	1.8	2.4
C _{17:1}	6.0	3.7	3.0	4.6	6.3
C _{18:1}	<1	<1	<1	<1	<1
Branched					
i-C _{14:0}	2.3	1.0	3.2	1.6	1.4
i-C _{15:0}	12.3	15.4	7.8	15.5	15.2
ai-C _{15:0}	4.4	7.8	4.0	7.7	11.8
i-C _{16:0}	22.0	18.7	32.7	15.4	10.8
i-C _{16:1}	—	—	<1	—	—
i-C _{17:0}	1.6	4.1	<1	1.4	1.4
ai-C _{17:0}	3.7	10.8	4.9	5.0	9.2
i-C _{18:0}	<1	<1	<1	—	—
10-Methylated					
C _{16:0}	1.9	2.7	6.2	3.7	2.8
C _{17:0}	18.0	15.5	13.7	12.4	12.2
C _{18:0}	<1	1.4	<1	1.1	<1

DNA–DNA hybridization was carried out using the photobiotin-labelling method with microdilution wells as described by Ezaki *et al.* (1989). DNA–DNA relatedness (%) was determined using the colorimetric method (Verlander, 1992). The G+C contents of the genomic DNA from strains A-T 0875^T and A-T 1383^T were 71.0 and 70.9 mol%, respectively. Strain A-T 0875^T exhibited DNA–DNA relatedness levels of 32.06 % (SD=2.05) with strain A-T 1383^T, 22.71 % (SD=1.03) with *P. thailandica* BCC 21825^T, 37.71 % (SD=1.18) with *P. mira* NBRC 15435^T and 32.87 % (SD=0.46) with *P. silvatica* NBRC 100141^T. Strain A-T 1383^T exhibited DNA–DNA relatedness levels of 17.85 % (SD=1.13) with *P. thailandica* BCC 21825^T, 33.17 % (SD=1.08) with *P. mira* NBRC 15435^T and 31.14 % (SD=1.06) with *P. silvatica* NBRC 100141^T (mean values, all repeated three times). 16S rRNA gene sequence analysis and DNA–DNA relatedness data were sufficient to categorize strains A-T 0875^T and A-T 1383^T as distinct from previously described *Planotetraspora* species.

These phenotypic (Table 1) and genotypic data indicated that strains A-T 0875^T and A-T 1383^T merit classification as representatives of two novel species of the genus *Planotetraspora*, for which the names *Planotetraspora kaengkrachanensis* sp. nov. and *Planotetraspora phitsanulokensis* sp. nov., respectively, are proposed.

Description of *Planotetraspora kaengkrachanensis* sp. nov.

Planotetraspora kaengkrachanensis (kaeng.kra.chan.en'sis. N.L. fem. adj. *kaengkrachanensis* of Kaeng Krachan District, located in Thailand, where the type strain was isolated).

Gram-positive, mesophilic, non-motile actinomycete that forms well-developed and branched substrate hyphae. Colonies are pale yellow on ISP2 medium. Cylindrical sporangia containing four oval- to rod-shaped spores are formed at the ends of short sporangiospores on the aerial mycelium. Spores are non-motile. No soluble pigment is detected in ISP media 2, 3, 4 or 5. Optimal temperature for growth is between 25 and 30 °C. No growth occurs above 37 °C. The maximum NaCl concentration for growth is 1.5 %. Nitrate is not reduced. Utilizes cellobiose, D-lactose, D-mannitol, melibiose and glycerol, but not L-arabinose, D-fructose, D-galactose, raffinose, L-rhamnose, D-ribose, salicin or D-xylose. Acid is produced from D-galactose, D-glucose, D-mannitol and D-mannose, but not from D-lactose, maltose, melibiose, L-rhamnose, raffinose or D-xylose. Peptonization of milk, hydrolysis of starch and gelatin liquefaction are negative. Cell wall contains glutamic acid, alanine and *meso*-A₂pm. The predominant menaquinones are MK-9(H₄) and MK-9(H₂). The whole-

Fig. 1. Phylogenetic relationships based on neighbour-joining analysis (Saitou & Nei, 1987) of *Planotetraspora kaengkrachanensis* A-T 0875^T and *Planotetraspora phitsanulokensis* A-T 1383^T derived from 16S rRNA gene sequences of some members of the family Streptosporangiaceae. *Streptosporangium roseum* DSM 43021^T was used as an outgroup. Numbers at nodes indicate bootstrap percentages derived from 1000 replications (only values greater than 50 % are indicated). Bar, 0.005 substitutions per nucleotide position.

cell sugars are rhamnose, madurose, glucose, galactose and 3-*O*-methylmannose. The diagnostic phospholipid is phosphatidylethanolamine. The major fatty acids are iso-*C*_{16:0}, 10-methyl-*C*_{17:0}, iso-*C*_{15:0} and *C*_{15:0}.

The type strain is A-T 0875^T (=BCC 24832^T=NBRC 104272^T), isolated from soil. The DNA G+C content of the type strain is 71.0 mol%.

Description of *Planotetraspora phitsanulokensis* sp. nov.

Planotetraspora phitsanulokensis (phit.sa.nu.lok.en'sis. N.L. fem. adj. *phitsanulokensis* of Phitsanulok Province, located in Thailand, where the type strain was isolated).

Gram-positive, mesophilic, non-motile actinomycete that forms well-developed and branched substrate hyphae. Colonies are colourless on ISP2 medium. Cylindrical sporangia containing four oval- to rod-shaped spores are formed at the ends of short sporangiospores on the aerial mycelium. Spores are non-motile. No soluble pigment is detected in ISP media 2, 3, 4 or 5. Optimal temperature for growth is between 25 and 30 °C. No growth occurs above 37 °C. The maximum NaCl concentration for growth is less than 1 %. Nitrate is not reduced. Utilizes cellobiose, D-galactose, D-glucose, D-lactose, D-mannitol and glycerol, but not L-arabinose, D-fructose, melibiose, raffinose, L-rhamnose, D-ribose, salicin or D-xylose. Acid is produced from D-glucose, maltose, D-mannitol and D-mannose, but not from D-galactose, D-lactose, melibiose, L-rhamnose, raffinose or D-xylose. Peptonization of milk, hydrolysis of starch and gelatin liquefaction are negative. Cell wall

contains glutamic acid, alanine and *meso*-A₂pm. The predominant menaquinone is MK-9(H₄). The whole-cell sugars are rhamnose, madurose, glucose, galactose and 3-*O*-methylmannose. The diagnostic phospholipid is phosphatidylethanolamine. The major fatty acids are iso-*C*_{16:0}, 10-methyl-*C*_{17:0}, iso-*C*_{15:0} and anteiso-*C*_{17:0}.

The type strain is A-T 1383^T (=BCC 26045^T=NBRC 104273^T), isolated from soil. The DNA G+C content of the type strain is 70.9 mol%.

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