Dactylosporangium tropicum sp. nov., isolated from soil

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Two novel actinomycete strains, designated KB2-4^T and KB9-2, were isolated from soil samples. Both isolates formed finger-shaped sporangia on short sporangiophores that emerged directly from substrate hyphae. The cell-wall peptidoglycan contained glutamic acid, glycine, alanine and meso-diaminopimelic acid; the whole-cell sugars were galactose, glucose, mannose, rhamnose, ribose and xylose. The diagnostic phospholipid was phosphatidylethanolamine. The predominant menaquinones were MK-9(H₈) and MK-9(H₆). Mycolic acids were not detected. The predominant cellular fatty acids (>20 %) were iso-C_{16:0} and anteiso-C_{17:0}. For both strains, the G + C content of the genomic DNA was about 72 mol%. The morphological and chemotaxonomic characteristics of the two strains were typical of members of the genus *Dactylosporangium*. Phylogenetic analysis using 16S rRNA gene sequences also indicated that these strains should be classified in the genus *Dactylosporangium* and showed that the closest relative was *Dactylosporangium salmoneum* NRRL B-16294^T (99.1 % 16S rRNA gene sequence similarity). DNA–DNA relatedness and some physiological and biochemical properties indicated that the isolates could be readily distinguished from their closest phylogenetic relatives. On the basis of these phenotypic and genotypic data, the isolates represent a novel species, for which the name *Dactylosporangium tropicum* sp. nov. is proposed. The type strain is strain KB2-4^T (=BCC 34760^T =JCM 15673^T).

The genus *Dactylosporangium* Thiemann *et al.* (1967) belongs to the family *Micromonosporaceae* in the order *Actinomycetales* (Zhi *et al.*, 2009). This genus has proved to be an important source of pharmaceutically useful metabolites, notably antibiotics (Goodfellow, 1988). Members of the genus *Dactylosporangium* normally form both finger-shaped sporangia and globose bodies directly on substrate hyphae. At the time of writing, the genus *Dactylosporangium* encompasses 11 species with validly published names: *D. aurantiacum* (Thiemann *et al.*, 1967), *D. thailandense* (Thiemann *et al.*, 1967; Thiemann, 1970), *D. matsuzakiense* (Shomura *et al.*, 1980), *D. vinaceum* (Shomura *et al.*, 1983), *D. roseum* (Shomura *et al.*, 1985),

D. fulvum (Shomura *et al.*, 1986), *D. darangshiense* (Seo & Lee, 2010), *D. maewongense* (Chiaraphongphon *et al.*, 2010) and the three species described by Kim *et al.* (2010), *D. luridum*, *D. luteum* and *D. salmoneum*. During an investigation of novel actinomycetes from soil near a hot spring in a tropical forest in Kanchanaburi Province, Thailand, we isolated strains KB2-4^T and KB9-2 that showed morphological and chemotaxonomic characteristics typical of the genus *Dactylosporangium*, but the isolates were genotypically and phenotypically distinct from all *Dactylosporangium* species with validly published names.

Samples were taken from the soil surface and kept at 4 °C. The samples were air-dried at room temperature for 8 days. Dried soil (1 g) was treated with 1.5 % (v/v) phenol in sterile distilled water (9 ml) and diluted serially in distilled water as recommended by Hayakawa *et al.* (1991). Both strains were isolated on humic acid–vitamin agar supplemented with (l⁻¹) 25 mg nalidixic acid, 50 mg

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KB2-4^T and KB9-2 are AB454508 and AB543492, respectively.

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

cycloheximide and 1 mg terbinafin. Purified cultures were preserved at -80°C and by freeze-drying.

Cell morphology was observed by light and scanning electron microscopy (JSM-5410 LV; JEOL) after growth on modified soil extract agar [0.5 g $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 0.25 g $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.05 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g K_2SO_4 , 0.02 g KH_2PO_4 , 0.1 g NaHCO_3 , 0.02 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g yeast extract, 0.1 g Casamino acids, 0.2 g glucose, 100 ml soil extract (Hamaki *et al.*, 2005), 18 g agar, 900 ml distilled water; pH 7.0]. Cells for scanning electron microscopy were prepared as described elsewhere (Itoh *et al.*, 1989). Phenotypic characteristics were examined by using several standard methods. Cultural characteristics were tested using 14-day-old cultures grown at 30°C on various agar media. The Jacal Colour Card L2200 (Japan Colour Research Institute) was used for determining colour designations. Hydrolysis of various compounds and acid production from carbon sources were examined using the basal medium recommended by Gordon *et al.* (1974). Conditions for growth (temperature, pH and NaCl concentration) were determined on yeast extract–malt extract agar (ISP 2; Shirling & Gottlieb, 1966) for 14 days at 30°C (varied for growth temperature determination). Carbon-source utilization was tested on ISP 9 (Shirling & Gottlieb, 1966) supplemented with a final concentration of 1% carbon source and 0.05% Casamino acids. Gelatin liquefaction, milk peptonization, nitrate reduction and starch hydrolysis were determined through cultivation on various media as described by Arai (1975) and Williams & Cross (1971). The results are given in detail in the species description and Table 1. Strains KB2-4^T and KB9-2 possessed some distinct chemotaxonomic and phenotypic characteristics that separated them from their closest phylogenetic neighbour, *D. salmoneum* NRRL B-16294^T. In particular, the absence of 3-hydroxy-*meso*-diaminopimelic acid in the cell-wall hydrolysates, the peptonization of milk, the inability to liquefy gelatin and the inability to utilize cellobiose, D-fructose, melibiose, D-xylose, lactose and L-rhamnose were effective to discriminate between strains KB2-4^T and KB9-2 and closely related organisms.

For chemotaxonomic analysis, freeze-dried cells were obtained from cultures grown in ISP 2 broth on a rotary shaker at 30°C for 4 days. The cell-wall peptidoglycan was prepared and hydrolysed by the method of Kawamoto *et al.* (1981) and the amino acid composition was analysed by TLC (Lechevalier & Lechevalier, 1980). The isomer of diaminopimelic acid in the cell wall was determined by the method of Stanek & Roberts (1974). 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 by the method of Minnikin *et al.* (1984). Fatty acid methyl ester analysis was performed by GLC according to the instructions of the Sherlock Microbial Identification System version 4.5 (Sasser, 1990; Kämpfer & Kroppenstedt, 1996) with the

Table 1. Differential characteristics of strains KB2-4^T and KB9-2 and their closest phylogenetic relatives

Strains: 1, *Dactylosporangium tropicum* sp. nov. KB2-4^T; 2, *D. tropicum* sp. nov. KB9-2; 3, *D. salmoneum* NRRL B-16294^T; 4, *D. vinaceum* NBRC 14181^T; 5, *D. matsuzakiense* NBRC 14259^T. All data were taken from this study. DR, Deep red; O, orange; POY, pale orange–yellow; PY, pale yellow; VR, vivid red; Y, yellow; +, positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5
Colony colour (ISP 2)	POY	Y	Y	VR	O
Soluble pigment colour (ISP 2)	PY	PY	–	DR	PY
3-OH- <i>meso</i> -diaminopimelic acid	–	–	+	+	+
Gelatin liquefaction	–	–	+	+	–
Nitrate reduction	+	+	+	–	–
Milk peptonization	+	+	–	+	–
Starch hydrolysis	+	+	+	+	–
Growth at 37°C	+	+	+	–	–
Minimum pH tolerance	5	5	4	6	6
Utilization of:					
Cellobiose	–	–	+	w	w
D-Fructose	–	–	+	+	w
D-Galactose	+	+	w	+	–
D-Mannitol	+	w	+	+	+
Melibiose	–	–	+	w	w
Raffinose	w	w	+	w	+
D-Xylose	–	–	+	+	–
Glycerol	+	+	+	w	+
Lactose	–	–	+	w	–
L-Arabinose	+	+	w	w	w
L-Rhamnose	–	–	w	+	w

TSBA40 MIDI database. The presence of mycolic acids was investigated by the method of Minnikin *et al.* (1975). Isoprenoid quinones were extracted by the method of Collins *et al.* (1977) and analysed by HPLC using a Cosmosil 5C₁₈ column (4.6 × 150 mm; Nacalai Tesque). The elution solvent was methanol/2-propanol (2:1, v/v).

Chromosomal 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) and an equimolar mixture of nucleotides (Yamasa Shoyu) 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 using the colorimetric method (Verlander, 1992). PCR-mediated amplification of the 16S rRNA gene and sequencing of the PCR products were carried out as described elsewhere (Suriyachadkun *et al.*, 2009). The 16S rRNA gene sequence was aligned with selected sequences obtained from public databases using CLUSTAL W version 1.81 (Thompson *et al.*, 1994). The alignment was manually verified and adjusted prior to the construction of phylogenetic trees using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) methods in MEGA version

2.1 (Kumar *et al.*, 2001). The confidence values of nodes were determined by using bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. 16S rRNA gene sequence similarity among all members of recognized species of the genus *Dactylosporangium* was determined by using the EzTaxon server (Chun *et al.*, 2007) and, among closely related strains, was calculated manually after pairwise alignment using CLUSTAL X (Thompson *et al.*, 1997). Gaps and ambiguous nucleotides were eliminated from the calculations.

Strains KB2-4^T and KB9-2 exhibited morphological and chemical properties of the genus *Dactylosporangium* (Thiemann *et al.*, 1967; Shomura *et al.*, 1983). Colonies of both isolates were compact and raised and produced well-developed, branched substrate hyphae, but lacked aerial hyphae. Growth was good on ISP 2, oatmeal agar (ISP 3) and inorganic salts–starch agar (ISP 4). Colonies were pale orange–yellow. A pale-yellow soluble pigment was produced in ISP 2 medium. The isolates formed groups of finger-shaped sporangia on short sporangiophores that emerged directly from substrate hyphae (Fig. 1). The sporangium surface was smooth. Each sporangium contained a row of ovoid motile spores (0.9–1.1 µm). Globose bodies were observed on modified soil extract agar and humic acid–vitamin agar.

Strains KB2-4^T and KB9-2 had identical chemotaxonomic characteristics and these were similar to those of members of the genus *Dactylosporangium*. Cell-wall hydrolysates of both isolates contained glutamic acid, glycine, alanine and *meso*-diaminopimelic acid, indicating that this isolate had the cell-wall chemotype II of Lechevalier & Lechevalier (1970). The acyl type of the cell-wall muramic acid was glycolyl. Galactose, glucose, mannose, rhamnose, ribose and xylose were found as whole-cell sugars. The characteristic phospholipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylethanolamine, but not phosphatidylcholine. This pattern corresponds to phospholipid type PII of Lechevalier *et al.* (1977). The major

cellular fatty acids (>2 %) were iso-C_{16:0}, anteiso-C_{17:0}, iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{17:0}, C_{16:0}, C_{17:0} and C_{18:0} and the isolates also contained 10-methyl-C_{17:0} (Supplementary Table S1, available in IJSEM Online). This pattern corresponds to fatty acid type 3b of Kroppenstedt (1985). Mycolic acids were absent. The predominant menaquinones of strains KB2-4^T and KB9-2 were MK-9(H₈) (46.9 and 48.8 %, respectively) and MK-9(H₆) (39.9 and 41.1 %, respectively). The G + C content of the genomic DNA was 72.5 mol% for strain KB2-4^T and 72.2 mol% for strain KB9-2.

Almost-complete 16S rRNA gene sequences were obtained for strains KB2-4^T and KB9-2 (1452 and 1456 nt, respectively). The sequences exhibited a close relationship between the isolates and members of the family *Micromonosporaceae*. Both isolates were placed within the genus *Dactylosporangium* and formed a distinct lineage in a subcluster that also contained *D. salmoneum* NRRL B-16294^T, which was supported by a moderate bootstrap value (51 % in the neighbour-joining tree; Fig. 2). The relationship between the isolates and *D. salmoneum* NRRL B-16294^T was also supported by the result of the maximum-parsimony analysis. 16S rRNA gene sequence similarity between strains KB2-4^T and KB9-2 and the type strains of all species of the genus *Dactylosporangium* with validly published names ranged from 98.0 % (*D. thailandense* IFO 12593^T) to 99.1 % (*D. salmoneum* NRRL B-16294^T). The isolates shared 100 % 16S rRNA gene sequence similarity with each other.

Low DNA–DNA relatedness (from 20.6 ± 1.2 to 38.8 ± 0.9 %) was observed between the isolates and their close phylogenetic relatives (Supplementary Table S2, available in IJSEM Online). DNA–DNA relatedness between strains KB2-4^T and KB9-2 ranged from 91.2 ± 0.7 to 93.8 ± 1.3 % and between strain KB2-4^T and *D. salmoneum* JCM 3272^T was 36.7 ± 0.4 %.

It is evident from the phenotypic, chemotaxonomic and phylogenetic data that strains KB2-4^T and KB9-2 can be distinguished from the members of previously described species of the genus *Dactylosporangium*. It is therefore proposed that these strains be classified in a novel species, with the name *Dactylosporangium tropicum* sp. nov.

Description of *Dactylosporangium tropicum* sp. nov.

Dactylosporangium tropicum [tro'pi.cum. L. neut. adj. *tropicum* tropical, of or pertaining to the tropic(s), relating to isolation from a tropical forest].

Gram-positive, mesophilic actinomycete that forms smooth-surfaced finger-shaped sporangia on short sporangiophores directly from the substrate hyphae. Colonies are pale orange–yellow on ISP 2. Each sporangium contains a row of ovoid motile spores. Globose bodies are observed on modified soil extract agar and humic acid–vitamin agar. Aerial mycelium is absent. Spore surface appears smooth. A pale-yellow soluble pigment is produced on ISP 2. Nitrate is reduced to nitrite. Utilizes D-galactose, D-mannitol, glycerol and L-arabinose,

Fig. 1. Scanning electron micrograph of sporangia and globose bodies of strain KB2-4^T grown on humic acid–vitamin agar for 4 weeks at 28 °C. Bar, 5 µm.

Fig. 2. Neighbour-joining tree (Saitou & Nei, 1987) based on almost-complete (1401 nt) 16S rRNA gene sequences, showing the relationships between strains KB2-4^T and KB9-2 and members of the genera *Dactylosporangium*, *Micromonospora* and *Actinoplanes*. Bootstrap values (>50%) based on 1000 replicates are shown at branch nodes. Asterisks indicate that the corresponding nodes were also recovered in the tree generated using the maximum-parsimony method. *Streptomyces ambofaciens* ATCC 23877^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

weakly utilizes raffinose, but does not utilize cellobiose, D-fructose, melibiose, D-xylose, lactose or L-rhamnose. Peptonization of milk and hydrolysis of starch are positive. Gelatin liquefaction is negative. Growth is optimum at 25–30 °C; no growth occurs above 40 °C. The maximum NaCl concentration for growth is 3% (w/v). The cell wall contains glutamic acid, glycine, alanine and *meso*-diaminopimelic acid. The acyl type of the cell wall is glycolyl. The predominant menaquinones were MK-9(H₈) and MK-9(H₆). The characteristic whole-cell sugars are galactose, glucose, mannose, rhamnose, ribose and xylose. The phospholipid profile contains diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylethanolamine. The fatty acids are iso-C_{16:0}, anteiso-C_{17:0}, iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{17:0}, C_{16:0}, C_{17:0}, C_{18:0}, 10-methyl-C_{17:0}, C_{15:0}, iso-C_{14:0}, 10-methyl-C_{16:0}, 10-methyl-C_{18:0}, C_{18:1}ω9*c* and C_{17:1}ω8*c*. The G + C content of the type strain is 72.5 mol%.

The type strain is KB2-4^T (=BCC 34760^T =JCM 15673^T), isolated from soil near a hot spring in a tropical forest in Kanchanaburi Province, Thailand.

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