Actinopolymorpha pittospori sp. nov., an endophyte isolated from surface-sterilized leaves of an apricot tree (*Pittosporum phylliraeoides*)

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A member of the genus *Actinopolymorpha*, designated PIP 143^T, was isolated from the leaves of an Australian native apricot tree (*Pittosporum phylliraeoides*). The isolate was a Gram-reaction-positive, aerobic actinobacterium, with a well-developed substrate mycelium that fragmented into small rods. Phylogenetic evaluation based on 16S rRNA gene sequences placed the isolate in the family *Nocardioideae*. Strain PIP 143^T was most closely related to *Actinopolymorpha cephalotaxi* I06-2230^T (98.7 %) and *Actinopolymorpha rutila* YIM 45725^T (98.1 %). Chemotaxonomic data, including cell-wall components, menaquinones and fatty acids, confirmed the affiliation of strain PIP 143^T to the genus *Actinopolymorpha*. Phylogenetic analysis and physiological and biochemical studies, in combination with DNA–DNA hybridization studies, allowed the differentiation of strain PIP 143^T from its closest phylogenetic neighbours with validly published names. Therefore, a novel species is proposed, with the name *Actinopolymorpha pittospori* sp. nov. The type strain is PIP 143^T (=DSM 45354^T =ACM 5288^T =NRRL B-24810^T).

The genus *Actinopolymorpha* was first described by Wang *et al.* (2001) and belongs to the family *Nocardioideae*, which includes the genera *Nocardioides* (Prauser, 1976), *Aeromicrobium* (Miller *et al.*, 1991), *Kribbella* (Park *et al.*, 1999), *Marmoricola* (Urzì *et al.*, 2000) and *Jiangella* (Song *et al.*, 2005). At the time of writing, the genus *Actinopolymorpha* contained four species, all of which were isolated from soil samples: *A. singaporensis* (Wang *et al.*, 2001), *A. rutila* (Wang *et al.*, 2008), *A. alba* (Cao *et al.*, 2009) and *A. cephalotaxi* (Yuan *et al.*, 2010). All members of the genus contain LL-diaminopimelic acid, but the major menaquinones and cell-wall sugars vary between the species: *A. singaporensis* and *A. alba* contain MK-9(H₆), ribose, glucose and rhamnose (Wang *et al.*, 2001; Cao *et al.*, 2009); *A. rutila* contains MK-9(H₄), ribose, glucose and galactose (Wang *et al.*, 2008); and *A. cephalotaxi* contains MK-9(H₄) and glucose (Yuan *et al.*, 2010). The phospholipid pattern for these four species corresponds to type PI (Lechevalier *et al.*, 1977).

During the course of our research on isolating endophytic actinobacteria from crop plants and native trees (Coombs & Franco, 2003; Kaewkla & Franco, 2010), an

Actinopolymorpha-like strain, designated PIP 143^T, was isolated from a leaf sample from a native apricot tree (*Pittosporum phylliraeoides*), collected from the grounds of Flinders University, Adelaide, South Australia, and processed within 4 h of collection. Leaves were sterilized with 70 % ethanol and 6 % hypochlorite for 5 min each and rinsed repeatedly with sterile water, 10 % NaHCO₃ for 10 min and again with sterile water. Crushed leaf tissue was placed on VL70 medium containing a mixture of 17 amino acids and solidified with 0.8 % gellan gum (Hudson *et al.*, 1989; Schoenborn *et al.*, 2004). The medium was adjusted to pH 7.2 and supplemented with (ml⁻¹) 20 µg nalidixic acid and 100 U nystatin to control bacterial and fungal contamination, respectively. Plates were kept in plastic sealed boxes, which contained wet paper towels to maintain moisture, and incubated at 27 °C. Strain PIP 143^T was isolated from the leaf tissue after 11 weeks of growth on solid medium.

Extraction of genomic DNA from strain PIP 143^T and amplification and sequencing of the 16S rRNA gene were carried out as described previously (Coombs & Franco, 2003). A nearly complete 16S rRNA gene sequence (1407 bp) was obtained and analysed using BLAST (Altschul *et al.*, 1997) and was subsequently aligned with 16S rRNA gene sequences of representatives of related genera available from public databases using CLUSTAL X (Thompson *et al.*, 1997). Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) tree-making methods using MEGA

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Abbreviation: meso-A₂pm, meso-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PIP 143^T is FJ805429.

A supplementary figure is available with the online version of this paper.

version 4 (Tamura *et al.*, 2007). Pairwise distances for the neighbour-joining algorithm were calculated according to the Kimura two-parameter model (Kimura, 1980) and close-neighbour-interchange (search level=2, random addition=100) was applied in the maximum-parsimony analysis. Tree topology was evaluated by using bootstrap analysis (Felsenstein, 1985) with 1000 replications. The phylogenetic evaluation clearly showed that strain PIP 143^T was a member of the genus *Actinopolymorpha*. The highest sequence similarity was observed with *A. cephalotaxi* I06-2230^T (98.7 % 16S rRNA gene sequence similarity), followed by *A. rutila* YIM 45725^T (98.1 %), *A. singaporensis* IM 7744^T (97.7 %) and *A. alba* YIM 48868^T (97.3 %). The affiliation between strain PIP 143^T and its closest neighbour was supported by both the neighbour-joining and maximum-parsimony algorithms, with bootstrap values of 97 and 89 %, respectively (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online).

DNA–DNA relatedness between strain PIP 143^T and its two closest phylogenetic neighbours was determined according to the colorimetric microdilution-plate method using biotinylated DNA (Ezaki *et al.*, 1989; Kusunoki *et al.*, 1991) and reciprocal experiments. DNA–DNA relatedness was calculated from four repeated experiments and expressed as a mean with the corresponding reciprocal values. DNA–DNA relatedness values between strain PIP 143^T and *A. cephalotaxi* I06-2230^T and *A. rutila* YIM 45725^T were 18.63 and 12.17 %, respectively, which were well below the 70 % cut-off point recommended for the recognition of bacterial species (Wayne *et al.*, 1987).

The DNA G + C content of strain PIP 143^T was determined by HPLC (Mesbah *et al.*, 1989). The DNA G + C content of strain PIP 143^T was 69.6 mol%, which was similar to other members of the genus *Actinopolymorpha* with validly published names (66.6–69.5 mol%) (Wang *et al.*, 2001, 2008; Cao *et al.*, 2009; Yuan *et al.*, 2010).

For the analysis of whole-cell fatty acids, strain PIP 143^T and the four type strains of species of the genus *Actinopolymorpha* were grown for 10 days at 25 °C in tryptic soy broth (Oxoid) at 150 r.p.m. and then harvested by centrifugation. Washed

cells (100 mg) were saponified, the fatty acids were methylated and extracted and the fatty acid methyl esters were determined by the protocols described by Sasser (1990). Sherlock Microbial Identification System SITE1 version 6.0 was used for analysis. Extraction and purification of isoprenoid quinones was performed using the method of Collins *et al.* (1977), with analysis of the samples by reversed-phase LC–MS employing UV detection and electrospray MS. The LC solvent system was 2-propanol/methanol (1 : 1) at a flow rate of 1.0 ml min⁻¹. Whole-cell hydrolysates were analysed for diaminopimelic acid isomers by TLC (Bousfield *et al.*, 1985) and for sugars using the method of Hasegawa *et al.* (1983). The presence of mycolic acids was determined according to Minnikin *et al.* (1975, 1980). Acyl cell-wall analysis was performed according to Uchida & Seino (1997). The diaminopimelic acid in whole-cell hydrolysates of strain PIP 143^T was in the LL-configuration and the whole-cell sugars were ribose, glucose and galactose. Strain PIP 143^T contained MK-9(H₄) as the predominant menaquinone, with MK-9(H₆), MK-10(H₄) and MK-10(H₈) also present. *A. cephalotaxi* I06-2230^T had a similar menaquinone composition, although it only contained glucose in its cell wall (Yuan *et al.*, 2010). The whole-cell fatty acids of strain PIP 143^T were chiefly of the iso- or anteiso-branched types (Table 1). The major cellular fatty acid of strain PIP 143^T was iso-C_{16:0} (29.16 %), which was the same as for *A. cephalotaxi* I06-2230^T (40.46 %); however, *A. cephalotaxi* I06-2230^T also contained iso-C_{13:0}, anteiso-C_{17:1}ω9c, C_{17:0}, C_{18:0}, C_{16:1}ω7c and anteiso-C_{17:1} B, which were not detected in strain PIP 143^T.

Morphological characteristics of the isolate were observed on eight different media: ISP 2, 3, 4, 5 and 7, Bennett's agar, half-strength potato dextrose agar and nutrient agar (Atlas, 1993), the results of which are given in Table 2. Strain PIP 143^T showed a nocardioform morphology, with a well-developed substrate mycelium. Sparse aerial mycelia were formed on some media. The isolate did not produce diffusible pigments on any of the media used. The substrate mycelium was yellowish white on most media used, which is in contrast to *A. cephalotaxi*, the mycelium colour of which varies from yellow to brilliant orange.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the relationships between strain PIP 143^T and selected members of the family Nocardioidaceae. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. Asterisks indicate that the corresponding nodes were also recovered in the tree generated using the maximum-parsimony algorithm. Bar, 0.01 substitutions per nucleotide.

Table 1. Whole-cell fatty acid compositions of strain PIP 143^T and members of the genus *Actinopolymorpha*

Strains: 1, *Actinopolymorpha pittospori* sp. nov. PIP 143^T; 2, *A. cephalotaxi* I06-2230^T; 3, *A. rutila* YIM 45725^T; 4, *A. singaporensis* IM 7744^T; 5, *A. alba* YIM 48868^T. Data were taken from this study. Fatty acids representing <0.5 % of the total are not shown.

Fatty acid (%)	1	2	3	4	5
C _{9:0} 3-OH	0.71	1.16	—	1.15	0.62
iso-C _{10:0}	—	—	0.98	—	—
iso-C _{13:0}	—	0.91	—	—	0.50
iso-C _{14:0}	0.52	1.05	2.87	1.69	3.60
iso-C _{15:1} G	—	—	—	—	0.76
iso-C _{15:0}	16.99	17.46	15.25	33.65	32.33
anteiso-C _{15:0}	7.52	1.18	3.48	4.80	13.87
C _{16:1} ω7c alcohol	—	—	—	—	1.79
C _{16:0} N alcohol	—	—	1.58	—	—
iso-C _{16:0}	29.16	40.46	35.82	25.30	10.90
C _{16:1} ω9c	—	—	—	0.59	—
C _{16:0}	0.76	0.83	0.61	1.34	—
iso-C _{17:1} ω10c	—	—	2.93	—	0.98
anteiso-C _{17:1} ω9c	—	0.53	0.50	—	1.02
iso-C _{17:0}	16.35	10.64	9.97	12.11	10.86
anteiso-C _{17:0}	20.74	9.41	9.83	11.10	10.24
C _{17:0}	—	3.28	2.58	—	—
C _{17:0} 10-methyl	0.77	3.26	2.47	1.16	—
iso-C _{18:0}	1.61	0.66	0.69	—	—
C _{18:1} ω9c	0.74	0.55	—	—	—
C _{18:0}	—	0.61	0.75	—	—
iso-C _{15:1} H	—	—	—	—	—
C _{19:0}	—	—	0.95	—	—
C _{18:0} 2-OH	—	—	0.50	—	—
C _{16:1} ω7c	—	1.57	1.85	—	3.50
anteiso-C _{17:1} B	—	2.55	—	1.23	—
iso-C _{17:1}	0.80	—	—	—	—
iso-C _{17:1} I	—	—	1.17	—	—
iso-C _{18:2} ω6,9c	—	—	0.85	—	—
iso-C _{19:1} ω7c	—	—	0.54	—	—
iso-C _{20:2} ω6,9c	—	—	—	—	5.08
C _{12:0} aldehyde	—	—	—	—	—
C _{16:0} 10-methyl	0.59	2.51	—	2.23	—

Carbohydrate utilization was examined as described by Shirling & Gottlieb (1966). Acid production from carbohydrates was examined by using the methods of Gordon *et al.* (1974). Growth at 4, 15, 27, 37 and 45 °C, in 1, 3, 5, 10, 15 and 20 % (w/v) NaCl and at pH 4–10 (at intervals of one pH unit) was assessed after incubation at 27 °C for 7–14 days on ISP 2 medium (Kurup & Schmitt, 1973). Hydrolysis of gelatin and catalase production were determined as described by Kurup & Schmitt (1973).

The physiological properties of strain PIP 143^T and *A. cephalotaxi* I06-2230^T were significantly different (Table 3) in terms of acid production from fructose, galactose, maltose, inositol, mannitol, sucrose, sorbitol, mannose, salicin and xylose, utilization of arabinose, raffinose, *myo*-inositol and mannitol and growth in 5 % (w/v) NaCl.

Based on the results of phylogenetic, phenotypic and chemotaxonomic analyses, strain PIP 143^T represents a novel species of the genus *Actinopolymorpha*, for which the name *Actinopolymorpha pittospori* sp. nov. is proposed.

Description of *Actinopolymorpha pittospori* sp. nov.

Actinopolymorpha pittospori (pit.to.spo'ri. N.L. gen. n. *pittospori* named after the genus of the host plant *Pittosporum phylliraeoides*, from which the type strain was isolated).

Cells are Gram-reaction-positive, aerobic and non-acid-alcohol-fast. Catalase-positive. Grows at 15–27 °C, at pH 6–10 and in 1 % (w/v) NaCl. Colonies are smooth to rugose and yellowish white to pale yellow. Substrate mycelium develops well on most media used but aerial mycelium is rarely formed. Diffusible pigments are not produced. Substrate mycelium is branched with irregular thickness which fragments into short chains or aggregates. In the later stages of growth, hyphae fragment to rod-like elements or V-shaped forms. Physiological properties are listed in Table 3 and whole-cell fatty acids are shown in Table 1.

The type strain, PIP 143^T (=DSM 45354^T =ACM 5288^T =NRRL B-24810^T), is an endophytic actinobacterium, which was isolated from the leaves of *Pittosporum*

Table 2. Cultural characteristics of strain PIP 143^T

Colour determination based on Methuen Handbook of Colour (Kornerup & Wanscher, 1978). Diffusible pigments were not detected on any media tested. —, Not detected.

Medium	Growth	Aerial mycelium	Substrate mycelium
Yeast extract malt extract agar (ISP 2)	Good	Moderate	Pale yellow
Oatmeal agar (ISP 3)	Poor	—	Yellowish white
Inorganic salt starch agar (ISP 4)	Poor	—	White
Glycerol asparagine agar (ISP 5)	Poor	—	Yellowish white
Tyrosine agar (ISP 7)	Poor	—	Yellowish white
Bennett's agar	Good	Moderate	Pale yellow
Half-strength potato dextrose agar	Good	Good	Yellowish white
Nutrient agar	Poor	—	Yellowish white

Table 3. Characteristics differentiating between strain PIP 143^T and members of the genus *Actinopolymorpha*

Strains: 1, *Actinopolymorpha pittospori* sp. nov. PIP 143^T; 2, *A. cephalotaxi* I06-2230^T; 3, *A. rutila* YIM 45725^T; 4, *A. singaporensis* IM 7744^T; 5, *A. alba* YIM 48868^T. All data were from this study. All strains are positive for gelatin decomposition, catalase, utilization of glucose as sole carbon source, production of acid from arabinose, glucose and trehalose and growth with 1% (w/v) NaCl, at pH 6–10 and at 27 °C. All strains are negative for production of acid from 1,2-propanediol and growth with 10, 15 and 20% (w/v) NaCl, at pH 4 and at 4 and 45 °C. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5
Carbon utilization of:					
Arabinose	w	+	+	–	+
Fructose	+	+	+	+	–
Galactose	+	+	+	+	–
Inositol	–	+	+	+	–
Mannitol	–	+	+	+	–
Raffinose	w	+	+	–	+
Acid production from:					
Fructose	–	+	+	+	+
Dulcitol	–	–	w	w	–
Galactose	–	+	+	+	+
Maltose	–	+	+	+	+
Mannose	w	+	+	+	+
Inositol	–	+	w	+	w
Mannitol	–	+	+	+	w
Salicin	w	+	+	+	+
Sucrose	–	+	+	+	+
Xylose	w	+	+	+	+
Adonitol	–	–	+	+	+
Sorbitol	–	+	+	+	–
Growth at/with:					
15 °C	+	+	w	–	–
37 °C	–	–	+	+	–
pH 5	w	+	+	w	–
3% NaCl	w	+	+	+	–
5% NaCl	–	+	–	–	–

phylliraeoides growing on the campus of Flinders University, Adelaide, South Australia. The DNA G+C content of strain PIP 143^T is 69.6 mol%.

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