Flindersiella endophytica gen. nov., sp. nov., an endophytic actinobacterium isolated from the root of Grey Box, an endemic eucalyptus tree

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A novel endophytic actinobacterium, designated strain EUM 378^T, was isolated from the surface-sterilized root tissue of *Eucalyptus microcarpa*, a eucalyptus tree known as Grey Box. Phylogenetic evaluation based on 16S rRNA gene sequence analysis, including alignment with taxon-specific 16S rRNA gene signature nucleotides, placed this isolate as a member of the family *Nocardioideae*. Strain EUM 378^T showed >5.5 % 16S rRNA gene sequence divergence from other members of this family and was related most closely to *Actinopolymorpha alba* YIM 48868^T (94.2 %) and *Actinopolymorpha singaporensis* IM 7744^T (94.4 %). This Gram-positive, aerobic actinobacterium has well-developed substrate mycelia that fragment into small rods. Chemotaxonomic data revealed that the cell wall contains LL-diaminopimelic acid, ribose, glucose and rhamnose. MK-10(H₈) is the predominant menaquinone. Chemotaxonomic and phylogenetic evidence confirmed that strain EUM 378^T represents a novel species of a new genus, for which the name *Flindersiella endophytica* gen. nov., sp. nov. is proposed. The type strain is EUM 378^T (=DSM 45355^T=ACM 5289^T).

The family *Nocardioideae* was proposed by Nesterenko *et al.* (1985), with two genera, *Nocardioides* (Prauser, 1976) and *Pimelobacter* (Suzuki & Komagata, 1983), belonging to this family. Later, the species of the genus *Pimelobacter* were reclassified to the genera *Terrabacter* and *Nocardioides* (Collins *et al.*, 1989). At the time of writing, the family *Nocardioideae* comprises six genera: *Nocardioides*, *Aeromicrobium* (Miller *et al.*, 1991), *Kribbella* (Park *et al.*, 1999), *Marmoricola* (Urzi *et al.*, 2000), *Actinopolymorpha* (Wang *et al.*, 2001) and *Jiangella* (Song *et al.*, 2005).

During the course of our research to identify endophytic actinobacteria from Australian plants using molecular- and culture-based methods (Conn & Franco, 2004; Coombs & Franco, 2003), an endophytic nocardioform-like strain (EUM 378^T) was isolated from the surface-sterilized root tissue of an endemic Australian tree, *Eucalyptus microcarpa*, known as Grey Box. Detailed polyphasic taxonomic analysis revealed that this strain forms a novel taxon

within the family *Nocardioideae* for which the name *Flindersiella* gen. nov. is proposed, with the type species *Flindersiella endophytica* sp. nov.

Root samples of a Grey Box (*E. microcarpa*) tree were collected from the grounds of the Flinders University campus, Adelaide, South Australia, and processed within 4 h. The bark was removed from the roots and the samples were sterilized with 70 % ethanol and 6 % hypochlorite for 5 min each, washed thoroughly with sterile water and then treated with sterile 10 % NaHCO₃ for 10 min before being washed with sterile water. The root tissue was crushed in a sterile mortar and pestle and plated onto VL70 medium containing a mixture of 17 aa and solidified with 0.8 % gellan gum (Hudson *et al.*, 1989; Schoenborn *et al.*, 2004; Song *et al.*, 2005). The pH was adjusted to 7.2. The medium was supplemented with 20 µg nalidixic acid ml⁻¹ and 100 U nystatin ml⁻¹ to control bacterial and fungal contamination, respectively. Plates were kept in plastic sealed boxes that contained moist paper towels to maintain moisture, and incubated at 27 °C for 16 weeks. One actinobacterium-like strain, EUM 378^T, emerged from the surface-sterilized internal root tissue of the Grey Box tree (*E. microcarpa*) after incubation for 12 weeks.

Extraction of genomic DNA from strain EUM 378^T and amplification and sequencing of the 16S rRNA gene were carried out as described previously (Coombs & Franco, 2003). The nearly complete resultant 16S rRNA gene sequence (1401 bp) of strain EUM 378^T was analysed using

Abbreviations: A₂pm, diaminopimelic acid; ESI, electrospray mass spectrometry; HPDA, half-strength potato dextrose agar; NA, nutrient agar; SEM, scanning electron microscopy.

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The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain EUM 378^T is FJ805430.

Supplementary tables and figures are available with the online version of this paper.

BLAST (Altschul *et al.*, 1997) and subsequently aligned with the 16S rRNA gene sequences of representatives of related genera available from GenBank by using CLUSTAL_X (Thompson *et al.*, 1997), with *Streptomyces griseus* NRRL B-2165^T as the outgroup. Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) tree-making methods by using the software package MEGA 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 maximum-parsimony analysis. The topology of the tree was evaluated by performing a bootstrap analysis (Felsenstein, 1985) based on 1000 replications.

The phylogenetic position of strain EUM 378^T was determined by analysis of the almost-complete 16S rRNA gene sequence (GenBank accession no. FJ805430) with sequences of representative members of the family *Nocardioideae*. This evaluation shows clearly that strain EUM 378^T resides within this family, but shows a line of descent distinct from other validly described members (Fig. 1; Supplementary Fig. S2, available in IJSEM Online). This strain forms a new cluster with its closest neighbours *Actinopolymorpha alba* YIM 48868^T, *Actinopolymorpha singaporensis* IM 7744^T, *Actinopolymorpha cephalotaxi* 06-2230^T and *Actinopolymorpha rutila* YIM 45725^T, with low nucleotide sequence similarity (94.2, 94.4, 94.5 and

94.4%, respectively). The closest neighbouring genus was *Jiangella*, sharing only 91% 16S rRNA gene sequence similarity. Analysis of 16S rRNA gene signature nucleotides of strain EUM 378^T confirmed that this isolate belongs to the family *Nocardioideae* (Zhi *et al.*, 2009).

DNA-relatedness studies were not carried out between strain EUM 378^T and its closest phylogenetic relatives, as the relatively low level of 16S rRNA gene sequence similarity, of <95%, clearly differentiated strain EUM 378^T from its closest neighbours and is lower than the cut-off point recommended for delineation of genomic species (Stackebrandt & Goebel, 1994).

The G+C content of the DNA of strain EUM 378^T was determined by HPLC (Mesbah *et al.*, 1989) to be 68.8 mol%.

For the analysis of whole-cell fatty acids, strain EUM 378^T and all four *Actinopolymorpha* type strains were grown for 10 days at 25 °C in tryptic soya broth at 150 r.p.m. and harvested by centrifugation at 5000 g. Washed cells (100 mg) were saponified, methylated and extracted and the fatty acid methyl esters were determined by following the protocols described by Microbial Identification Inc. (MIDI) using the Sherlock Microbial Identification System (Sasser, 2001). Extraction and purification of isoprenoid quinones were performed by using the method of Collins *et al.* (1977) and analysis of the samples was by reverse-phase LC-MS employing UV detection and electrospray mass spectrometry (ESI). The LC solvent system was

Fig. 1. 16S rRNA gene-based neighbour-joining tree showing the phylogenetic relationships between *Flindersiella endophytica* EUM 378^T and selected micro-organisms belonging to the family *Nocardioideae*. Asterisks indicate branches of the tree that were also recovered by using the maximum-parsimony algorithm (Supplementary Fig. S2, available in IJSEM Online). The length of the sequences is 1390 bp. Numbers on the branches indicate percentage bootstrap values of 1000 replicates. Bar, 0.01 changes per nucleotide.

2-propanol:methanol (1:1) at a flow rate of 1.0 ml min⁻¹. Polar lipids were extracted and purified as described by Minnikin *et al.* (1984). Analysis of the samples was by LC-MS employing an SGE Wakosil C18 HPLC column (150 × 2 mm), UV detection at 210 nm and ESI. Isocratic elution was performed with a solvent consisting of acetonitrile:methanol (3:7) containing 5 µM ethanalamine at a flow rate of 0.2 ml min⁻¹ (Brouwers *et al.*, 1999; Fang & Barcelona, 1998; Mazzella *et al.*, 2004). Whole-cell hydrolysates were analysed for diaminopimelic acid (A₂pm) isomers by TLC (Bousfield *et al.*, 1985) and for sugars using the method of Hasegawa *et al.* (1983). Mycolic acid was determined according to Minnikin *et al.* (1975, 1980) and acyl cell-wall analysis was performed according to Uchida & Seino (1997). The A₂pm in whole-cell hydrolysates of strain EUM 378^T was in the LL configuration, and the whole-cell sugars were ribose, glucose and rhamnose. Strain EUM 378^T contained MK-10(H₆) as the predominant menaquinone, with MK-10(H₈), MK-10(H₄) and MK-10(H₂) present in small amounts. Phospholipids present were diphosphatidylglycerol and phosphatidylglycerol. The cell wall of strain EUM 378^T was of the non-mycolate and acetyl type, and the whole-cell fatty acid pattern was of the iso-anteiso-branched type. The predominant cellular fatty acid of this strain was iso-C_{16:0} (34.65 %); the fatty acid composition is shown in Supplementary Table S1, available in IJSEM Online.

Morphological characteristics of strain EUM 378^T and related type strains were observed by comparison on eight different media: five recommended by the International *Streptomyces* Project (ISP) (Shirling & Gottlieb, 1966), i.e. ISP 2, 3, 4, 5 and 7, plus Bennett's agar, half-strength potato dextrose agar (HPDA) and nutrient agar (NA) (Atlas, 1993). Cell and spore morphologies of cultures grown on HPDA for 21 days were viewed by scanning electron microscopy (SEM; ETEC Autoscan 1974). Strain EUM 378^T showed morphology characteristic of a nocardioform actinobacterium with well-developed substrate mycelium, but aerial mycelium formation was only achieved on some media. Spores are tiny rods (approx. 0.25–0.40 × 0.30 µm) on short chains that develop from aerial mycelium on colonies cultured on HPDA (see Supplementary Fig. S1, available in IJSEM Online). Colony characteristics on different media are presented in Supplementary Table S2. Colonies varied in colour on different media from yellow to olive green and, after 30 days growth on HPDA, colonies changed to a bluish purple. Also, this strain produced a greyish orange pigment on tyrosine agar.

Physiological and biochemical characterization of strain EUM 378^T and *A. alba* YIM 48868^T, *A. cephalotaxi* I06-2230^T, *A. rutila* YIM 45725^T and *A. singaporensis* IM 7744^T was performed by the following procedures. Acid production from carbohydrates was examined by the methods of Gordon *et al.* (1974). Growth at different temperatures (15, 27, 37 and 45 °C), NaCl concentrations (2, 5, 10, 15 and 20 %, w/v) and pH (4.0–10.0) was assessed after incubation

at 27 °C for 7–14 days on ISP 2 medium (Kurup & Schmitt, 1973). Hydrolysis of casein, starch and gelatin, and catalase production were determined as described by Kurup & Schmitt (1973). Physiological differentiation of strain EUM 378^T from the most closely related type strains is shown in Table 1.

Phenotypic and genotypic data revealed that strain EUM 378^T is different from the described *Actinopolymorpha* species and other members of the family *Nocardioideaceae*. There are significant differences in culture characteristics between strain EUM 378^T and the most closely related type strain, *A. alba* YIM 48868^T. For example, the substrate mycelium colour of strain EUM 378^T was yellowish white to dark green on a variety of media, whereas for *A. alba* YIM 48868^T it varied from grey–white to milk white. Also, the type strain of *A. alba* did not grow on nutrient agar or ISP 4, whereas strain EUM 378^T could grow on these media. Sparse aerial hyphae of the type strain of *A. alba* were observed only on ISP 2, but no aerial hyphae were observed on any other media. On the other hand, aerial mycelia of strain EUM 378^T were observed on most media used, except for ISP 5 and NA. The type strain of *A. alba* produced a brown pigment on tyrosine agar, but strain EUM 378^T did not. The type strain of *A. alba* did not produce spores, whereas strain EUM 378^T produced spores on four media tested (Supplementary Table S2). Furthermore, physiological properties of

Table 1. Physiological characteristics of *Flindersiella endophytica* EUM 378^T and *Actinopolymorpha* reference strains

Strains: 1, *Flindersiella endophytica* EUM 378^T; 2, *A. alba* YIM 48868^T; 3, *A. singaporensis* IM 7744^T; 4, *A. cephalotaxi* I06-2230^T; 5, *A. rutila* YIM 45725^T. All data were generated in this study. +, Positive or present; w, weakly positive; –, negative or absent. All strains could decompose gelatin, and catalase activity was positive. All strains could produce acid from D-arabinose, cellobiose, fructose, galactose, glucose, maltose, mannose, ribose, sucrose, trehalose, xylose and salicin, but not from 1,2 propanediol. They could grow at 1 % (w/v) NaCl. All strains could grow well at pH 6–10 and at 27 °C.

Characteristic	1	2	3	4	5
Acid production from:					
Dulcitol	–	–	w	–	w
myo-Inositol	+	w	+	+	w
Mannitol	+	–	+	+	+
Adonitol	+	+	+	–	+
Sorbitol	+	–	+	+	+
Decomposition of starch	+	–	–	–	–
Growth at:					
3 % NaCl	+	–	+	+	+
5 % NaCl	w	–	–	+	–
pH 5	w	–	w	+	+
15 °C	+	–	–	+	+
37 °C	+	–	+	w	+
45 °C	–	–	w	–	–

both strains were different. Strain EUM 378^T produced acid from *myo*-inositol, mannitol and sorbitol, whereas the type strain of *A. alba* showed a weak result for *myo*-inositol and negative results for mannitol and sorbitol. Growth of strain EUM 378^T occurred between pH 5 and 10 and between 15 and 37 °C, whereas *A. alba* could grow between pH 6 and 10 and at 27 °C (Table 1).

Chemotaxonomic characteristics differentiated strain EUM 378^T from related genera of the family *Nocardioideae* (Table 2). The predominant menaquinone of strain EUM 378^T differs from those of *Actinopolymorpha* spp., as it contains menaquinone MK-10(H₆), whereas the four described *Actinopolymorpha* species have MK-9(H₆) or MK-9(H₄) (Cao *et al.*, 2009; Wang *et al.*, 2001, 2008; Yuan *et al.*, 2010).

Also, strain EUM 378^T can be distinguished from representatives of the genus *Actinopolymorpha* by its whole-cell fatty acid profile, as it contains iso-C_{10:0} (5.86 %), whereas this fatty acid was detected in low amounts (0.98 %) in *A. rutila*. In contrast, the four *Actinopolymorpha* type strains contain iso-C_{15:0} and iso-C_{17:0} at much higher levels than strain EUM 378^T (Supplementary Table S1).

On the basis of phenotypic and genotypic data, strain EUM 378^T can be distinguished from all validly described genera of the family *Nocardioideae*. The evidence presented in this study is sufficient to propose a new genus and novel species for strain EUM 378^T: *Flindersiella endophytica* gen. nov., sp. nov.

Description of *Flindersiella* gen. nov.

Flindersiella (Flin.der.si.el'la. N.L. fem. dim. n. *Flindersiella* named after Flinders University, signifying the site of the host tree from which the type strain originated).

Whole-cell hydrolysate of members of the genus contains A₂pm in the LL configuration, and whole-cell sugars are ribose, glucose and rhamnose. Diphosphatidylglycerol and phosphatidylglycerol are present. The major fatty acids are iso-C_{16:0}, anteiso-C_{17:0}, and anteiso-C_{15:0}. MK-10(H₆) is

the major menaquinone. The DNA G + C content of the type species of this genus is 68.8 mol%. Phylogenetically, the genus is positioned in the family *Nocardioideae*, as revealed by the presence of family-specific signature nucleotides (Zhi *et al.*, 2009). The type species is *Flindersiella endophytica*.

Description of *Flindersiella endophytica* sp. nov.

Flindersiella endophytica (en.do.phy'ti.ca. Gr. *endo* within; Gr. *phyton* plant; L. fem. suff. *-ica* adjectival suffix used with the sense of belonging to; N.L. fem. adj. *endophytica* within plant, endophytic, pertaining to the original isolation from plant tissue).

Aerobic, Gram-positive, non-acid–alcohol-fast, catalase-positive actinobacterium. Colony morphology ranges from smooth to rugose. Colonies are white on ISP 4 and yellowish white on ISP 5, Bennett's agar and NA. They are greyish yellow on ISP 3 and ISP 7 and pale yellow on ISP 2. Colonies are olive green on HPDA and change to a bluish purple colour after 30 days growth. The type strain produces a greyish orange pigment on tyrosine agar. Substrate mycelium develops well on most media, but aerial mycelium is rarely formed. Substrate mycelium is branched with irregular thickness and fragments into short chains or aggregates. Spores are tiny rods on short chains that develop from aerial mycelium. White spores are produced on ISP 3, ISP 4, ISP 7 and HPDA. Grows between 15 and 37 °C, between pH 5.0 and 10.0 and in the presence of up to 5 % (w/v) NaCl. Optimum growth is achieved at a temperature of 27–37 °C, between pH 6 and 10 and in the presence of <3 % (w/v) NaCl. Acid production from carbon sources is shown in Table 1.

The type strain, EUM 378^T (=DSM 45355^T=ACM 5289^T), was isolated from surface-sterilized root tissue of a *Eucalyptus microcarpa* tree that grows on the campus of Flinders University, Adelaide, South Australia. The DNA G + C content of the type strain is 68.8 mol%.

Table 2. Differential characteristics of *Flindersiella endophytica* EUM 378^T and related taxa in the family *Nocardioideae*

Taxon	Cell morphology	Major menaquinone	Polar lipids*	DNA G + C content (mol%)	Reference
Strain EUM 378 ^T	Hyphae, rods	MK-10(H ₆)	DPG, PG, PI	68.8	This study
<i>Actinopolymorpha</i>	Pleomorphism to hypha	MK-9(H ₆) or MK-9(H ₄)	PIM, DPG	66.6–69.5	Cao <i>et al.</i> (2009); Wang <i>et al.</i> (2001, 2008); Yuan <i>et al.</i> (2010)
<i>Jiangella</i>	Hyphae, rods	MK-9(H ₄)	PI, PIM, PG	70	Song <i>et al.</i> (2005)
<i>Kribbella</i>	Hyphae, rods, cocci	MK-9(H ₄)	DPG, PC, PG, PI	68–71	Park <i>et al.</i> (1999)
<i>Nocardioides</i>	Hyphae, rods, cocci	MK-8(H ₄)	PG, DPG, PL, PG-OH	66.5–71.7	Collins <i>et al.</i> (1989)
<i>Aeromicrobium</i>	Rods	MK-9(H ₄)	PE, PG	71–73	Miller <i>et al.</i> (1991)
<i>Marmoricola</i>	Cocci	MK-8(H ₄)	PI, DPG, PG	72	Urzi <i>et al.</i> (2000)

*DPG, Diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PG-OH, phosphatidylglycerol containing 2-hydroxy fatty acids; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; PL, unknown phospholipid(s).

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