Pseudonocardia eucalypti sp. nov., an endophytic actinobacterium with a unique knobby spore surface, isolated from roots of a native Australian eucalyptus tree

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A novel strain, designated EUM 374^T, was isolated from the root of a native Australian eucalyptus tree, *Eucalyptus microcarpa*, and subjected to a range of morphological, phylogenetic and chemotaxonomic analyses. The strain was Gram-reaction-positive with well-developed aerial mycelia, which fragmented into rod-shaped spores that had unique knobby protrusions on the spore surface. Substrate mycelia were not present in the media used. Strain EUM 374^T grew as a film on the surface of static liquid culture medium but did not grow under shaking conditions. Phylogenetic evaluation based on 16S rRNA gene sequences identified the new isolate as belonging to the family *Pseudonocardiaceae* with sequence similarities of 96.1 and 96.3% to *Pseudonocardia acaciae* GMKU095^T and *Pseudonocardia spinosisporea* LM 141^T, respectively, and 93–96% sequence similarity to other members of the genus *Pseudonocardia*. The results of comprehensive phylogenetic analyses, including physiological and biochemical tests, differentiated strain EUM 374^T from related members of the genus *Pseudonocardia*. Based on the phenotypic, phylogenetic and chemotaxonomic evidence, strain EUM 374^T represents a novel species of the genus *Pseudonocardia*, for which the name *Pseudonocardia eucalypti* sp. nov. is proposed. The type strain is EUM 374^T (=DSM 45351^T =ACM 5285^T).

The genus *Pseudonocardia* was originally proposed by Henssen (1957). Members of the genus possess type IV cell walls that are of the non-mycolate type and contain meso-diaminopimelic acid, arabinose and galactose. The major menaquinone is MK-8(H₄) and phospholipids are either type PII or PIII. Members of the genus have DNA G+C contents ranging from 68 to 79 mol% and, in phylogenetic trees, form a consistent cluster within the evolutionary radiation of the family *Pseudonocardiaceae* (McVeigh *et al.*, 1994; Warwick *et al.*, 1994; Reichert *et al.*, 1998; Lee *et al.*, 2000). At the time of the writing, the genus contained more than 30 species with validly published names (<http://www.bacterio.cict.fr/p/pseudonocardia.html>). Most strains of these species were isolated from soil but some strains were isolated from a variety of sources such as coastal sediment (Liu *et al.*, 2006), tree bark compost (Reichert *et al.*, 1998), a gold mine (Lee *et al.*, 2001) and from 1,4-dioxane-contaminated industrial sludge (Mahendra & Alvarez-Cohen, 2005). Recent reports have described five novel

species of endophytic origin in this genus: *Pseudonocardia alni*, isolated from root nodules of an alder tree (Evtushenko *et al.*, 1989; Warwick *et al.*, 1994); *Pseudonocardia oroxyli*, from a surface-sterilized root of *Oroxylum indicum* (Gu *et al.*, 2006); *Pseudonocardia endophytica*, from the inner tissue of *Lobelia clavata* (Chen *et al.*, 2009); *Pseudonocardia acaciae*, from roots of *Acacia auriculiformis* (Duangmal *et al.*, 2009) and *Pseudonocardia adelaidensis*, also isolated from the stem of a eucalyptus tree (Kaewkla & Franco, 2010).

Strain EUM 374^T was isolated from a root of a eucalyptus tree, *Eucalyptus microcarpa*, during the course of research into molecular and culture-based methods of identifying endophytic actinobacteria (Coombs & Franco, 2003; Conn & Franco, 2004). In this paper we describe the morphological, physiological, chemotaxonomic and phylogenetic characteristics of this organism and propose that, based on the phenotypic and genotypic data, strain EUM 374^T represents a novel species of the genus *Pseudonocardia*.

Samples of leaves, stems and roots were collected from a eucalyptus tree growing on the grounds of the Flinders University campus, Adelaide, South Australia, and processed within 4 h of collection. After removing the bark from the roots, the plant samples were sterilized with 70% ethanol and 6% hypochlorite for 5 min each followed by

Abbreviations: FAME, fatty acid methyl esters; SEM, scanning electron microscopy.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain EUM 374^T is FJ805426.

Three supplementary figures are available with the online version of this paper.

10 % NaHCO₃ for 10 min. Crushed plant tissues were plated onto humic acid–vitamin B (HV) medium (Hayakawa & Nonomura, 1987), solidified with 0.8 % gellan gum instead of agar. The medium was adjusted to pH 7.2 and supplemented with 20 µg nalidixic acid ml⁻¹ and 100 U nystatin ml⁻¹ to repress the growth of bacteria and fungi, respectively. Plates were incubated at 27 °C for up to 12 weeks in sealed plastic boxes containing wet paper towels to maintain moisture. After incubation for 9 weeks, a *Pseudonocardia*-like strain, EUM 374^T, emerged on the HV–gellan gum plate. This strain was isolated, purified and maintained on half-strength potato dextrose agar (HPDA).

Genomic DNA from strain EUM 374^T was extracted and the 16S rRNA gene was amplified and sequenced as described previously (Coombs & Franco, 2003). The DNA G+C content was determined by HPLC (Mesbah *et al.*, 1989). The nearly complete 16S rRNA gene sequence was corrected manually and aligned with sequences of closely related species obtained from the GenBank database by using BLAST (Altschul *et al.*, 1997). The results were analysed by using the CLUSTAL_X program (Thompson *et al.*, 1997) with *Streptomyces griseus* NRRL-ISP 5236^T as the outgroup. Phylogenetic trees were reconstructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) methods in MEGA version 4 (Tamura *et al.*, 2007) software. 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. The topology of the tree was evaluated by performing a bootstrap analysis (Felsenstein, 1985) based on 1000 replications.

The nearly complete 16S rRNA gene sequence of strain EUM 374^T (1409 bp) was compared with 32 available sequences of species of the genus *Pseudonocardia*, showing sequence similarities of 93–96 %. Strain EUM 374^T showed the highest 16S rRNA gene sequence similarities to *P. spinosipora* LM 141^T (96.3 %) and *P. acaciae* GMKU095^T (96.1 %). Phylogenetic analysis determined that strain EUM 374^T belonged to the genus *Pseudonocardia*, grouping this strain with other members of the genus. The nearest phylogenetic neighbour was *P. acaciae* GMKU095^T, which formed a distinct clade with strain EUM 347^T, supported by a bootstrap value of 92 %. The next closest phylogenetic neighbour was *P. spinosipora* LM 141^T with bootstrap support of 84 % (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online). A phylogenetic tree reconstructed using all 32 available sequences of species of the genus *Pseudonocardia* is given in Supplementary Fig. S2. DNA–DNA relatedness studies were not carried out between strain EUM 374^T and its closest phylogenetic relatives as the level of 16S rRNA gene sequence similarity between them was less than 97 %, the cut-off point recommended for the delineation of bacterial species (Stackebrandt & Goebel, 1994).

The genomic DNA G+C content of strain EUM 374^T was 72.9 mol%, which is within the range of values typical of members of the genus *Pseudonocardia* (Huang *et al.*, 2002).

Strain EUM 374^T was subjected to a range of chemotaxonomic analyses. In order to quantify whole-cell fatty acid composition, cells were grown under static conditions for 14 days at 25 °C in TSB and harvested by centrifugation. Wet cells (100 mg) were saponified, methylated and extracted according to Sasser (2001) and the resultant fatty acid methyl esters (FAME) were determined by following the protocols in the MIDI system (MIDI, 1993). Isoprenoid quinones were extracted and purified using the method of Collins *et al.* (1977) and analyzed by reversed-phase LC–MS employing UV detection and electrospray MS. The solvent system was 2-propanol: methanol (1:1) at a flow rate of 1 ml min⁻¹. Whole cell hydrolysates were analysed for diaminopimelic acid isomers (Bousfield *et al.*, 1985) and for sugars (Hasegawa *et al.*, 1983). Mycolic acids were determined according to Minnikin *et al.* (1975, 1980) and acyl cell-wall analysis was performed according to Uchida *et al.* (1999).

The results showed that the diaminopimelic acid in whole-cell hydrolysates of strain EUM 374^T was in the *meso*-configuration and the whole-cell sugars were arabinose and galactose. These characteristics suggested that strain EUM 374^T had a type IV cell wall and belonged to the family *Pseudonocardiaceae*. The cell wall was of the acetyl type and did not contain mycolic acids. Strain EUM 374^T contained MK-8(H₄) as the predominant menaquinone. The whole-cell fatty acid profile of EUM 374^T was of the *iso*/*anteiso* branched type and consisted of *iso*-C_{15:0} (26.68 %), *iso*-C_{16:0} (21.93 %), C_{16:0} (10.54 %), C_{17:1ω8c} (10.38 %), C_{16:1ω7c} (7.76 %), C_{17:0} (6.59 %), *iso*-C_{17:0} (4.04 %), C_{16:0} 10-methyl (1.45 %), *iso*-C_{14:0} (1.31 %) and *anteiso*-C_{17:0} (1.05 %).

Morphological characteristics of strain EUM 374^T were observed on eight different media: ISP 2, ISP 3, ISP 4, ISP 5, ISP 7, Bennett's agar, HPDA and nutrient agar (NA) (Atlas, 1993). To determine cell and spore morphology, cultures grown on ISP 2 for 14 days were mounted on a carbon adhesive tab without using a fixing agent, gold coated and viewed by scanning electron microscopy (SEM; ETEC Autoscan).

Cells of strain EUM 374^T had a morphology typical of members of the genus *Pseudonocardia* and possessed aerial mycelia which fragmented into zig-zag-shaped spore chains. The rod-shaped spores had a knobby surface (Supplementary Fig. S3), which is unique in members of this genus. Colony morphologies of strain EUM 374^T grown on different media are given in Table 1. On the media used, substrate mycelia were not present. The colour of aerial mycelium varied from white to brownish orange and spore colour varied from white through greyish yellow and orange to greenish grey. The strain did not produce any diffusible pigment. Growth occurred in liquid media under static conditions but not under shaking conditions.

Fig. 1. Neighbour-joining tree showing the relationship between strain EUM 374^T and related species of the genus *Pseudonocardia* using *S. griseus* as the outgroup. Asterisks indicate branches of the tree that were also recovered by using the maximum-parsimony algorithm (Supplementary Fig. S1). Bootstrap percentages based on 1000 resamplings are given at nodes. Bar, 0.01 substitutions per nucleotide position.

Strain EUM 374^T had similar growth characteristics to its second closest phylogenetic neighbour, *P. spinosissima* LM 141^T; both were unable to grow under submerged conditions and did not produce substrate mycelia. On the other hand, the morphological features of these strains were different. Strain EUM 374^T produced a zig-zag spore chain but *P. spinosissima* LM 141^T did not produce spore chains with this morphology. Strain EUM 374^T was also different from its closest neighbour, *P. acaciae* GMKU095^T, as this type strain could grow under both static and shaking conditions and formed substrate mycelia on ISP 2 medium. Moreover, spores of both *P. spinosissima* LM 141^T and *P. acaciae* GMKU095^T had spiny surfaces, whereas strain EUM 374^T displayed spores with a knobby surface (Supplementary Fig. S3). It should be noted that, to date, among the genus *Pseudonocardia*, only these three species have been reported as having spores with protrusions on their surfaces.

Physiological and biochemical characterization was carried out as described by Gordon *et al.* (1974) and Kurup & Schmitt (1973). The physiological characteristics that differentiate strain EUM 374^T from the type strains of the two most closely related species of the genus *Pseudonocardia* (Lee *et al.*, 2002; Duangmal *et al.*, 2009)

are displayed in Table 2. The three strains had different profiles of acid production from galactose, *myo*-inositol, mannitol and sorbitol (Table 2), differed in their ability to produce hydrogen sulfide, decompose tyrosine and hydrolyse gelatin and grew at different temperature ranges. Strain EUM 374^T grew at 15–37 °C, whereas *P. spinosissima* LM 141^T and *P. acaciae* GMKU095^T grew at 4–30 and 18–42 °C, respectively. Strain EUM 374^T and *P. acaciae* GMKU095^T grew in the presence of 3% (w/v) NaCl, whereas *P. spinosissima* LM 141^T did not grow under these conditions. Furthermore, strain EUM 374^T could grow at pH 5–10, whereas *P. acaciae* GMKU095^T could only grow at pH 5–8.

Based on phenotypic and genotypic evidence, strain EUM 374^T represents a novel species of the genus *Pseudonocardia*, for which the name *Pseudonocardia eucalypti* sp. nov. is proposed.

Description of *Pseudonocardia eucalypti* sp. nov.

Pseudonocardia eucalypti (eu.ca.lyp'ti. N.L. gen. n. *eucalypti* of *Eucalyptus*, isolated from *Eucalyptus microcarpa*).

Cells are aerobic, Gram-reaction-positive, non-acid/alcohol-fast and catalase- and urease-positive. Grows at 15–37 °C

Table 1. Culture characteristics of strain EUM 374^T

ISP, International *Streptomyces* project. No soluble pigments or substrate mycelia were observed on any of the media tested.

Medium	Growth	Aerial mycelium*
Yeast extract malt extract agar (ISP 2)	Good	Pale, grey spore
Oatmeal agar (ISP 3)	Moderate	White, fine spore
Inorganic salt starch agar (ISP 4)	Poor	Pale, yellow spore
Glycerol asparagine agar (ISP 5)	Moderate	Greyish, orange spore
Tyrosine agar (ISP 7)	Moderate	Greying, yellow spore
Bennett's agar	Good	Greenish, grey spore
HPDA	Good	Yellowish, white spore
Nutrient agar	Moderate	Yellowish, white spore

*Colour determination based on Methuen Handbook of Colour (Kornerup & Wanscher, 1978).

Table 2. Characteristics that differentiate strain EUM 374^T from closely related type strains of species of the genus *Pseudonocardia*

Strains: 1, EUM 374^T (data from this study); 2, *P. acaciae* GMKU095^T (data from Duangmal *et al.*, 2009); 3, *P. spinosipora* LM 141^T (data from Lee *et al.*, 2002). K, Knobby; S, spiny; +, positive; W, weakly positive; –, negative; NR, not reported. All strains could produce acid from L-arabinose, D-fructose D-glucose, D-mannose, L-rhamnose, D-xylose and adonitol but not from maltose, melezitose or sucrose. All strains were positive for catalase. Urease production was positive for all strains but they could not hydrolyse casein.

Characteristic	1	2	3
Spore surface	K	S	S
Acid production from:			
D-Galactose	+	–	–
myo-Inositol	+	W	+
D-Mannitol	+	W	–
D-Sorbitol	W	–	–
Enzyme activity:			
H ₂ S production	–	–	+
Decomposition of tyrosine	+	+	–
Hydrolysis of:			
Gelatin	–	+	–
Starch	+	+	–
Growth at/in:			
Temperature range (°C)	15–37	18–42	4–30
pH range	5–10	5–8	NR
37 °C	W	+	–
3 % NaCl	+	+	–

and pH 5–10 (weakly at 37 °C and pH 5). Does not produce hydrogen sulfide. Does not produce diffusible pigment. Aerial mycelia are white on ISP 3 but produce yellowish–greyish spores on other media and fragment to zig-zag-shaped spore chains with rod-shaped spores that have knobby spore surfaces. Does not produce substrate mycelia. Grows on the surface of liquid media under static conditions but not under shaking conditions. Acid is produced from L-arabinose, D-fructose, D-galactose, D-glucose, D-mannose, L-rhamnose, trehalose, D-xylose, adonitol, i-erythritol, myo-inositol and D-mannitol, weakly from D-sorbitol but not from cellobiose, maltose, melezitose, methyl α-D-glucoside or sucrose. Tyrosine and urea are degraded. Starch is hydrolysed but casein and gelatin are not. The cell wall contains meso-diaminopimelic acid. Whole-cell hydrolysate contains galactose and arabinose. The major menaquinone is MK-8(H₄). Predominant whole-cell fatty acids are iso-C_{15:0} and iso-C_{16:0}.

The type strain, EUM 374^T (=DSM 45351^T =ACM 5285^T), is an endophytic actinobacterium isolated from the root of a eucalyptus tree, *Eucalyptus microcarpa*, which was collected from the grounds of the Flinders University campus, Adelaide, South Australia. The DNA G+C content of the type strain is 72.9 mol%.

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