Pseudonocardia adelaidensis sp. nov., an endophytic actinobacterium isolated from the surface-sterilized stem of a grey box tree (*Eucalyptus microcarpa*)

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An aerobic, actinobacterial strain with rod-shaped spores, EUM 221^T, which was isolated from the surface-sterilized stem of a grey box tree (*Eucalyptus microcarpa*), is described. Phylogenetic evaluation based on 16S rRNA gene sequence similarity showed that this isolate belongs to the family *Pseudonocardiaceae*, with the closest neighbour being *Pseudonocardia zijingensis* 6330^T (98.7 %). The level of 16S rRNA gene sequence similarity between the isolate and species of the genus *Pseudonocardia* with validly published names ranged from 95 to 98 %. Chemotaxonomic data (meso-diaminopimelic acid; major menaquinone MK-8(H₄); major fatty acid iso-C_{16:0}) confirmed the affiliation of strain EUM 221^T to the genus *Pseudonocardia*. The results of the phylogenetic analysis, including physiological and biochemical studies in combination with DNA–DNA hybridization, allowed the genotypic and phenotypic differentiation of strain EUM 221^T from the closest described species. Therefore, this strain represented a novel species and the name proposed is *Pseudonocardia adelaidensis* sp. nov. The type strain is EUM 221^T (=DSM 45352^T =ACM 5286^T).

The genus *Pseudonocardia* was originally proposed by Henssen (1957). Phylogenetic analysis shows that this genus forms a consistent cluster within the evolutionary radiation of the family *Pseudonocardiaceae* (Lee *et al.*, 2000; McVeigh *et al.*, 1994; Reichert *et al.*, 1998; Warwick *et al.*, 1994). Members of the genus are non-motile and their aerial mycelium fragments to form chains of straight or zigzag spores. Chemotaxonomic characteristics show a lack of mycolic acids and a type IV cell wall containing meso-diaminopimelic acid (meso-A₂pm), arabinose and galactose. The major menaquinone is MK-8(H₄), the phospholipid pattern is type PII or PIII and iso-branched hexadecanoic acid (iso-C_{16:0}) is the predominant fatty acid. The DNA G + C content is in the range 68–79 mol%. At the time of writing, the genus comprises 31 species with validly published names (Euzéby, 1997).

During the course of our research on molecular and culture-based methods to identify endophytic actinobacteria (Coombs & Franco, 2003; Conn & Franco, 2004), we

have studied populations and identified novel strains of actinobacteria from native Australian plants. In this report, the description of the morphological, physiological, chemotaxonomic and phylogenetic characteristics of a *Pseudonocardia*-like strain, EUM 221^T, is presented. Phenotypic and genotypic data show that this strain represents a novel species.

Strain EUM 221^T was isolated from the internal tissue of a surface-sterilized stem sample of a grey box tree (*Eucalyptus microcarpa*) which was collected from the grounds of Flinders University. Samples were processed within 4 h of collection by using the following procedure, according to Kaewkla & Franco (2010). Briefly, the bark of the stems was removed and samples were sterilized with 70 % ethanol and 6 % sodium hypochlorite for 5 min each and sterile 10 % NaHCO₃ for 10 min. Crushed plant tissues were plated onto 10 isolation media. All media were adjusted to pH 7.2 and media were 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 kept in sealed plastic boxes, which contained moist paper towels to maintain moisture, and incubated at 27 °C. After incubation for 8 weeks, a *Pseudonocardia*-like strain, EUM 221^T, emerged from a surface-sterilized stem sample which was placed on VL 70 medium with a mixture of D-galacturonate, D-glucuronate, L-ascorbate and D-gluconate (GGAG) at concentrations of 0.5 mM of each growth

Abbreviation: A₂pm, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EUM 221^T is FJ805427.

An extended maximum-parsimony tree and a neighbour-joining tree based on 16S rRNA gene sequences and a scanning electron micrograph of spores of strain EUM 221^T are available as supplementary material with the online version of this paper.

substrate (Joseph *et al.*, 2003; Schoenborn *et al.*, 2004). This strain was purified and maintained on half-strength potato dextrose agar (HPDA).

Extraction of genomic DNA and PCR amplification and sequencing of the 16S rRNA gene from strain EUM 221^T were achieved as described previously by Coombs & Franco (2003). The resultant nearly full-length 16S rRNA gene sequence (1412 bp) was aligned manually to the GenBank databases by using BLAST (Altschul *et al.*, 1997) and the result showed that strain EUM 221^T was closely related to members of the genus *Pseudonocardia*. The sequence of this strain was multiply aligned with 16S rRNA gene sequences available from GenBank/EMBL/DBJ of representatives of the closest related genus by using the CLUSTAL_X program (Thompson *et al.*, 1997). 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 Kimura's two-parameter model (Kimura, 1980) and Min-mini heuristic with search factor of 1 was applied in maximum-parsimony analysis. The topology of the tree was evaluated by performing a bootstrap analysis (Felsenstein, 1985) based on 1000 replications.

Strain EUM 221^T showed the highest 16S rRNA gene sequence similarity to *Pseudonocardia zijingensis* 6330^T (98.7 %, a value that corresponds to 16 differences over 1412 locations). The strain shared 95–98 % 16S rRNA gene sequence similarity with all other *Pseudonocardia* species with validly published names. The phylogenetic position of strain EUM 221^T was evaluated, showing clearly that strain EUM 221^T was a member of the genus *Pseudonocardia* within the family *Pseudonocardiaceae*. The affiliation

Fig. 1. Neighbour-joining tree showing the relationship between strain EUM 221^T and closely related members of the genus *Pseudonocardia* based on 16S rRNA gene sequences. *Streptomyces griseus* ISP 5236^T was used 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 listed at nodes. Bar, 0.01 changes per nucleotide position. A tree including a wider selection of reference strains is available as Supplementary Fig. S2.

between strain EUM 221^T and its closest neighbour, *P. zijingensis* 6330^T, was supported by both the neighbour-joining and maximum-parsimony algorithms, with bootstrap values of 97 and 96 %, respectively (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online). A neighbour-joining tree incorporating strain EUM 221^T and representatives of all species of the genus with validly published names is presented as Supplementary Fig. S2.

The G+C content of the genomic DNA of strain EUM 221^T was determined by HPLC as described by Mesbah *et al.* (1989). The result showed that the DNA G+C content was 78.8 mol%.

DNA–DNA relatedness experiments were carried out between strain EUM 221^T and its closest phylogenetic neighbour, *P. zijingensis* DSM 44774^T, by measuring the divergence between the thermal denaturation midpoint of homoduplex DNA and heteroduplex DNA (ΔT_m), as described by Gonzalez & Saiz-Jimenez (2005). The result showed that strain EUM 221^T represented a distinct taxon, as the DNA–DNA relatedness analyses revealed differences in melting temperature of 6 °C with its closest phylogenetic neighbour, which corresponds to 60 % relatedness, which is well below the 70 % guideline recommended for the delineation of genomic species (Wayne *et al.*, 1987; Rosselló-Mora & Amann, 2001).

For chemotaxonomic analyses, strain EUM 221^T and the type strains *P. zijingensis* DSM 44774^T and *Pseudonocardia yunnanensis* DSM 44253^T were studied together for comparative purposes. For the analysis of whole-cell fatty acids, cells of all strains were grown for 10 days at 25 °C in TSB at 150 r.p.m. and harvested and washed by repeated centrifugation in sterile water. Wet cells (100 mg) were saponified and methylated and the fatty acid methyl esters (FAMES) were analysed using the MIDI (Microbial Identification) system (Sasser, 2001). Extraction and purification of isoprenoid quinones were performed using the method of Collins *et al.* (1977) and samples were analysed by reversed-phase LC-MS employing UV detection and electrospray mass spectrometry (ESI). The solvent system was 2-propanol/methanol (1:1) at a flow rate of 1.0 ml min⁻¹. Whole-cell hydrolysates were analysed for A₂pm isomers (Bousfield *et al.*, 1985) and for sugars using the method of 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 A₂pm in whole-cell hydrolysates of strain EUM 221^T was in the *meso* configuration and that the whole-cell sugars were arabinose and galactose. These data confirmed that strain EUM 221^T belongs to the family *Pseudonocardiaceae*, members of which have type IV cell walls. Whole cells were of the non-mycolic acid type and acetyl type. Strain EUM 221^T contained MK-8(H₄) as the predominant menaquinone; no other menaquinone components could be detected. The whole-cell fatty acids of EUM 221^T were of the iso- and anteiso-branched type. The major fatty acid was iso-C_{16:0}.

Table 1. Whole-cell fatty acid compositions of strain EUM 221^T and related *Pseudonocardia* type strains

Strains: 1, *Pseudonocardia adelaidensis* sp. nov. EUM 221^T; 2, *P. zijingensis* DSM 44774^T; 3, *P. yunnanensis* DSM 44253^T. Data are percentages of total fatty acids and were obtained in this study; only fatty acids detected at $\geq 0.5\%$ of the total are presented. ND, Not detected.

Fatty acid	1	2	3
iso-C _{14:0}	3.44	1.54	ND
C _{14:0}	ND	0.51	0.92
iso-C _{15:1} <i>cis</i> 5	ND	ND	0.95
iso-C _{15:0}	6.68	19.76	ND
anteiso-C _{15:0}	1.05	1.74	ND
C _{15:1} <i>cis</i> 6	0.90	1.50	ND
iso-C _{16:1} H	14.71	5.01	ND
iso-C _{16:0}	53.19	25.38	ND
C _{16:1} <i>cis</i> 7	0.87	3.12	11.68
C _{16:1} <i>cis</i> 5	ND	ND	1.55
C _{16:0}	1.10	2.82	29.57
10-Methyl C _{16:0}	4.05	ND	1.25
iso-C _{17:1} <i>cis</i> 9	ND	9.45	ND

(53.19%); the fatty acid compositions of strain EUM 221^T and related type strains are shown in Table 1. According to the whole-cell fatty acid profile, strain EUM 221^T has a similar profile to *P. zijingensis* DSM 44774^T. However, iso-C_{17:1} *cis*9 was not detected in the whole-cell extract of strain EUM 221^T, though it was observed in significant amounts (9.45%) in the cell extract of *P. zijingensis* DSM 44774^T (Table 1).

Morphological characteristics of strain EUM 221^T and reference strains *P. zijingensis* DSM 44774^T and *P. yunnanensis* DSM 44253^T were observed as described by Shirling & Gottlieb (1966) on ISP 2, 3, 4, 5 and 7, Bennett's agar, HPDA and nutrient agar (Atlas, 1993). The strain was grown on ISP 4 for 21 days and mounted, unfixed, on a carbon adhesive tab, gold coated and viewed by scanning electron microscopy (ETEC Autoscan 1974). Strain EUM

221^T showed morphology consistent with its membership of the genus *Pseudonocardia*, with well-developed substrate mycelium but with aerial mycelium formed only on some media, such as ISP 3 and ISP 4. Substrate mycelium was yellowish white on all media used. Cultural characteristics on different media are shown in Table 2. The strain did not produce any diffusible pigment on any of the media tested. Electron microscopy revealed that the mycelium fragmented to form spores, which were straight long rods with a smooth surface (approx. $0.5 \times 1 \mu\text{m}$; Supplementary Fig. S3).

The physiological characteristics of strain EUM 221^T and its closest relatives, *P. zijingensis* DSM 44774^T and *P. yunnanensis* DSM 44253^T, were studied together by using the following methods. Carbohydrate utilization was examined as described by Shirling & Gottlieb (1966). Growth at 15, 27, 37 and 45 °C, at 2, 5, 10 and 15% (w/v) NaCl and at pH 4.0–10.0 was assessed after incubation for 7–21 days on ISP 2 agar at 27 °C, unless otherwise stated (Kurup & Schmitt, 1973). Decomposition of adenine, hypoxanthine, tyrosine, xanthine, aesculin and hippurate was evaluated according to Gordon *et al.* (1974). Hydrolysis of casein, starch and gelatin, catalase production and organic acid assimilation were assessed as described by Kurup & Schmitt (1973). The physiological properties that differentiated strain EUM 221^T from *P. zijingensis* DSM 44774^T and *P. yunnanensis* DSM 44253^T are shown in Table 3. There were many differences between strain EUM 221^T and its closest relative, *P. zijingensis* DSM 44774^T. *P. zijingensis* DSM 44774^T utilized arabinose, cellulose and maltose as sole carbon sources while strain EUM 221^T could not. Also, *P. zijingensis* DSM 44774^T used cellobiose and lactose weakly, while strain EUM 221^T gave a negative test. *P. zijingensis* DSM 44774^T decomposed xanthine and starch and assimilated citrate and tartrate, whereas strain EUM 221^T did not. Moreover, EUM 221^T could grow at NaCl concentrations of up to 15% (w/v) and growth occurred well at pH 6–10 and at 15–37 °C. On the other hand, *P. zijingensis* DSM 44774^T could not tolerate >5% NaCl, good growth occurred at pH 6–9 and it grew weakly at 45 °C (Table 3).

Table 2. Cultural characteristics of strain EUM 221^T

ISP, International *Streptomyces* Project. Colour determinations based on Kornerup & Wanscher (1978). Soluble pigments were not detected on any medium.

Medium	Growth	Aerial mycelium	Substrate mycelium
Yeast extract-malt extract agar (ISP 2)	Good	Not detected	Good, light yellow
Oatmeal agar (ISP 3)	Poor	Moderate, white spore	Poor, yellowish white
Inorganic salts-starch agar (ISP 4)	Poor	Poor, white spore	Poor, light yellow
Glycerol-asparagine agar (ISP 5)	Poor	Not detected	Poor, yellowish white
Tyrosine agar (ISP 7)	Moderate	Not detected	Medium, yellowish white
Bennett's agar	Moderate	Not detected	Medium, light yellow
HPDA	Good	Not detected	Good, pale yellow
Nutrient agar	Poor	Not detected	Poor, light yellow

Table 3. Characteristics that differentiate strain EUM 221^T from related *Pseudonocardia* type strains

Strains: 1, *P. adelaidensis* sp. nov. EUM 221^T; 2, *P. zijingensis* DSM 44774^T; 3, *P. yunnanensis* DSM 44253^T. +, Positive or present; w, weakly positive; –, negative or absent. Data were obtained in this study. All strains could hydrolyse aesculin and hippurate and were catalase-positive. They could assimilate acetate, malate and propionate but could not decompose adenine, casein or gelatin. All strains were able to utilize glucose, glycerol, mannitol and ribose but not dextran or dulcitol as sole carbon sources. All strains could grow at 5 % (w/v) NaCl, pH 5–9 and 15–37 °C.

Characteristic	1	2	3
Decomposition of:			
Xanthine	–	+	–
Hypoxanthine	+	+	–
L-Tyrosine	+	+	–
Starch	–	+	+
Acid assimilation of:			
Benzoate	w	w	–
Citrate	–	+	+
Lactate	+	+	–
Tartrate	–	+	–
Growth at:			
10 % NaCl	w	–	–
15 % NaCl	w	–	–
pH 10	+	w	+
45 °C	w	+	+
Sole carbon sources			
Arabinose	–	+	–
Cellobiose	–	w	–
Cellulose	–	+	–
Galactose	+	+	w
myo-Inositol	+	+	–
Lactose	–	w	–
Maltose	–	+	w
Melibiose	+	+	–
Mannose	+	+	w
Raffinose	+	+	–
Rhamnose	+	+	–
Sorbitol	+	+	–
Sucrose	+	+	–
Trehalose	w	+	+
Xylose	+	+	–

Based on this polyphasic study, strain EUM 221^T could be readily differentiated from other members of *Pseudonocardia* species and represents a novel species, for which the name *Pseudonocardia adelaidensis* sp. nov. is proposed.

Description of *Pseudonocardia adelaidensis* sp. nov.

Pseudonocardia adelaidensis (a.de.lai.den'sis. N.L. fem. adj. *adelaidensis* relating to Adelaide, South Australia, the source of the tree from which the type strain was isolated).

Aerobic, non-acid–alcohol-fast and catalase-positive. Grows well at 15–37 °C, but weakly at 45 °C. Grows at pH 6.0–10.0 and in the presence of 15 % (w/v) NaCl. Colonies are small and tough, yellowish white to pale white, and substrate mycelium develops well, but aerial mycelium is rarely formed. Aerial mycelium fragments into long, rod-shaped spores with smooth surfaces (approx. 0.5 × 1 µm). Diffusible pigments are not produced. Additional physiological properties are listed in Table 3 and the whole-cell fatty acid profile is shown in Table 2. The DNA G+C content of the type strain is 78.8 mol%.

The type strain, EUM 221^T (=DSM 45352^T =ACM 5286^T), is an endophyte, isolated from the surface-sterilized stem of a grey box tree (*Eucalyptus microcarpa*) growing on the Flinders University campus, Adelaide, South Australia.

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