Promicromonospora endophytica sp. nov., an endophytic actinobacterium isolated from the root of an Australian native Grey Box tree

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A novel aerobic actinobacterium, strain EUM 273^T, was isolated from the root of a Grey Box tree (*Eucalyptus microcarpa* Maiden). Cells were Gram-staining-positive with well-developed substrate mycelia which were non-motile and rod-like, with coccoid elements. Phylogenetic evaluation based on 16S rRNA gene sequence analysis placed the isolate as a member of the family *Promicromonosporaceae* that was most closely related to *Promicromonospora xylanilytica* YIM 61515^T (98.2 %) and *Promicromonospora vindobonensis* V45^T (98 %). Chemotaxonomic data including cell wall components, major menaquinone and major fatty acids confirmed the affiliation of strain EUM 273^T to the genus *Promicromonospora*. 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 273^T from the closest related species with validly published names. The name proposed for the novel species is *Promicromonospora endophytica* sp. nov. The type strain is EUM 273^T (=DSM 23716^T=NRRL B-24816^T).

The genus *Promicromonospora* was first described by Krasil'nikov *et al.* (1961) and belongs to the family *Promicromonosporaceae*, which includes six other genera: *Cellulosimicrobium*, *Isoptericola*, *Myceligenans*, *Xylanibacterium*, *Xylanimicrobium* and *Xylanimonas* (Zhi *et al.*, 2009). At the time of writing, the genus *Promicromonospora* contained eight species: *P. citrea* (Krasil'nikov *et al.*, 1961), *P. sukumoe* (Takahashi *et al.*, 1987), *P. aerolata* and *P. vindobonensis* (Busse *et al.*, 2003), *P. kroppenstedtii* (Alonso-Vega *et al.*, 2008), *P. flava* (Jiang *et al.*, 2009), *P. umidemergens* (Martin *et al.*, 2010) and *P. xylanilytica* (Qin *et al.*, 2012). *P. enterophila* (Jäger *et al.*, 1983) was transferred to the genus *Oerskovia* as *Oerskovia enterophila* (Stackebrandt *et al.*, 2002) and *P. pachnodae* (Cazemier *et al.*, 2003) was reclassified as *Xylanimicrobium pachnodae* (Stackebrandt & Schumann, 2004). Three species, *P. citrea*, *P. sukumoe* and *P. kroppenstedtii*, were isolated from soil, while *P. flava* was isolated from sea sediment. *P. aerolata* and *P. vindobonensis* were isolated from air; *P. umidemergens* was isolated from indoor wall material (Martin

et al., 2010); and *P. xylanilytica* was isolated from surface-sterilized leaves of a medicinal plant (Qin *et al.*, 2012).

During the course of our research isolating endophytic actinobacteria from crop plants and native trees (Coombs & Franco, 2003; Kaewkla & Franco, 2010), a *Promicromonospora*-like strain, EUM 273^T, was isolated from the root of a Grey Box tree (*Eucalyptus microcarpa* Maiden), a species endemic to Australia. In this study, the taxonomic position of this strain, including morphological, physiological, chemotaxonomic and phylogenetic characteristics, was determined, and showed that strain EUM 273^T represents a novel species of the genus *Promicromonospora*.

Root samples of a Grey Box tree were collected from the grounds of Flinders University, Adelaide, South Australia, and processed within 4 h. The roots were sterilized with 70 % (v/v) ethanol and 6 % (v/v) sodium hypochlorite for 5 min each, rinsed several times with sterile water, then with 10 % (w/v) NaHCO₃ for 10 min followed by further rinsing with sterile water. Crushed root tissue was placed onto VL70 medium containing 0.05 % (w/v) carboxymethyl cellulose and solidified with 0.8 % (w/v) gellan gum (Schoenborn *et al.*, 2004). The pH was adjusted to 7.2. The medium was supplemented with (ml⁻¹) 20 µg nalidixic acid and 100 U nystatin to control bacterial and fungal contamination, respectively. Plates were kept in plastic

Abbreviation: DAP, Diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EUM 273^T is GU434253.

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

sealed boxes, containing wet paper towels to maintain moisture, and incubated at 27 °C. Growth of strain EUM 273^T was observed after incubation for 3 weeks.

Extraction of genomic DNA from strain EUM 273^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 of strain EUM 273^T was analysed using BLAST (Altschul *et al.*, 1997) and subsequently aligned with the 16S rRNA gene sequences of representatives of related genera available from GenBank/EMBL by using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods using the MEGA version 4 (Tamura *et al.*, 2007) software package. Pairwise distances for the neighbour-joining algorithm were calculated according to the maximum composite likelihood model (Tamura *et al.*, 2004) and min-mini heuristic (factor=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.

The phylogenetic evaluation showed clearly that strain EUM 273^T was a member of the genus *Promicromonospora* with the highest 16S rRNA gene sequence similarity to *P. xylanilytica* YIM 61515^T (98.2%) followed by *P. vindobonensis* V45^T (98%). The affiliation between strain EUM 273^T and its closest neighbour, *P. xylanilytica* YIM 61515^T, was supported by both neighbour-joining and maximum-parsimony algorithms with bootstrap values of 58 and 54 %, respectively (Fig. 1 and Fig. S1, available in IJSEM Online). However, strain EUM 273^T was in a different cluster to *P. xylanilytica* YIM 61515^T, which suggested that strain EUM 273^T represents a novel genomic species.

Fig. 1. 16S rRNA gene-based neighbour-joining tree showing the phylogenetic relationships between *Promicromonospora endophytica* sp. nov. EUM 273^T and selected micro-organisms belonging to the family *Promicromonosporaceae*. Asterisks indicate branches of the tree that were also recovered by using the maximum-parsimony algorithm (Fig. S1). The numbers on the branches indicate the percentage bootstrap values of 1000 replicates. Bar, 0.005 changes per nucleotide.

The level of DNA–DNA relatedness between strain EUM 273^T and the two closest neighbours was determined according to the colorimetric microdilution plate method using biotinylated DNA (Ezaki *et al.*, 1989; Kusunoki *et al.*, 1991). The DNA hybridization experiment was performed by labelling the DNA of strain EUM 273^T and then studying reciprocal binding with the type strains. The relatedness was calculated from quadruplicate hybridization experiments and expressed as a mean of the corresponding reciprocal values. The DNA–DNA relatedness between strain EUM 273^T and *P. xylanilytica* YIM 61515^T was 62.6 %, and between strain EUM 273^T and *P. vindobonensis* V45^T was 43.7 %, both of which are below the 70 % cut-off point for recognition of genomic species (Wayne *et al.*, 1987).

The DNA G + C content of strain EUM 273^T was determined by HPLC (Mesbah *et al.*, 1989) to be 71.7 mol%, which is marginally higher than other members of the genus (70–70.8 mol%) (Busse *et al.*, 2003; Alonso-Vega *et al.*, 2008).

Polar lipids were extracted according to Minnikin *et al.* (1984). Major lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, and three unknown glycolipids (Fig. S2). Diaminopimelic acid (DAP) was analysed by TLC (Bousfield *et al.*, 1985) and whole cell sugars were analysed by TLC (Hasegawa *et al.*, 1983). No DAP was present and the whole cell sugars contained galactose, rhamnose and glucose, while the whole cell sugars of the closest type strain, *P. xylanilytica* YIM 61515^T contained galactose and glucose. Extraction and purification of isoprenoid quinones was performed using the method of Collins *et al.* (1977) with analysis of the samples by reverse phase LC-MS employing UV detection and electrospray mass spectrometry (ESI). The LC solvent system was 2-propanol:methanol (1:1) at a flow rate of 1.0 ml min⁻¹. Strain EUM 273^T contained MK-9(H₈) (41 %) as the predominant menaquinone, with MK-9(H₆) (37 %) and MK-9(H₄) (22 %) also present. The menaquinone profile of the closest type strain, *P. xylanilytica* YIM 61515^T, was very different; it contained MK-9(H₆) (46 %), MK-9(H₄) (29 %), MK-8(H₄) (19 %), MK-9(H₂) (3 %) and MK-9(H₈) (3 %) (Qin *et al.*, 2012). There have been no previous reports of MK-9(H₈) in any recognized species of the genus *Promicromonospora* apart from a small amount in *P. xylanilytica* YIM 61515^T (Qin *et al.*, 2012). For the analysis of whole-cell fatty acids, strain EUM 273^T and the type strains of the two closest members of the genus *Promicromonospora* were grown for 10 days at 25 °C in tryptic soy broth (Oxoid) in an Erlenmeyer flask at 150 r.p.m., and harvested by centrifugation. Fatty acids of 100 mg washed cells were saponified, methylated and extracted, and the fatty acid methyl esters (FAMES) were determined following the protocols described by Microbial Identification Inc. (MIDI) (Sasser, 2009). The Sherlock MIS SITE2 software version 6.1 (RTSBA6 database) was used for analysis. The whole-cell fatty acid pattern of strain EUM 273^T was of the iso-anteiso-branched type (Table 1). The major cellular fatty acid of strain EUM 273^T was anteiso-C_{15:0}.

(54.48 %), which is similar to the amount in the closest type strain *P. xylanilytica* YIM 61515^T (47.69 %). However, iso-C_{15:1} G, anteiso-C_{15:1} A, C_{17:0} and C_{18:0} fatty acids were detected in cells of *P. xylanilytica* YIM 61515^T but not in strain EUM 273^T. Based on chemotaxonomic data, strain EUM 273^T was different from other recognized species of the genus *Promicromonospora*.

Morphological characteristics of the isolate were observed on eight different media: ISP 2, ISP 3, ISP 4, ISP 5, ISP 7 (Atlas, 1993; Shirling & Gottlieb, 1966), Bennett's agar, half-strength potato dextrose agar (HPDA) and nutrient agar (NA) (Atlas, 1993), and are described in Table S1. Mycelium was non-motile, Y- or V-shaped, rod-like with coccoid elements. Oval spores (0.5 × 0.6 µm) were observed (Fig. S3). The strain did not produce diffusible pigments on any of the media used. Colony morphology of strain EUM 273^T and the closest type strain, *P. xylanilytica* YIM 61515^T was different on ISP 4 and HPDA. Substrate mycelium of *P. xylanilytica* YIM 61515^T was dark brown on these media while colonies of strain EUM 273^T were yellowish white.

Acid production from carbohydrates was examined by the methods of Gordon *et al.* (1974). Growth at different temperatures (4, 15, 27, 37 and 45 °C), NaCl concentrations (1, 3, 5, 10, 15 and 20 %, w/v) and pH 4 to 10 (in 1 pH unit intervals) was assessed after incubation at 27 °C for 7–14 days on ISP 2 medium (Kurup & Schmitt, 1973). Catalase production was determined as described by Kurup & Schmitt (1973). Decomposition of starch and casein was examined according to the method of Gordon *et al.* (1974).

Table 1. Whole-cell fatty acid composition (%) of strain EUM 273^T and related type strains of members of the genus *Promicromonospora*

Strains: 1, EUM 273^T; 2, *P. xylanilytica* YIM 61515^T; 3, *P. vindobonensis* V45^T. Only fatty acids detected at >0.1 % of the total are presented. —, Not detected. All data are from this study.

Fatty acid	1	2	3
iso-C _{14:0}	1.46	0.86	0.80
C _{14:0}	0.45	0.55	0.22
iso-C _{15:1} G	—	0.17	0.93
anteiso-C _{15:1} A	—	0.14	0.63
iso-C _{15:0}	33.56	35.59	28.23
anteiso-C _{15:0}	54.48	47.69	51.72
iso-C _{16:0}	4.96	3.74	6.63
C _{16:0}	0.41	1.06	0.70
anteiso-17:1ω9c	0.17	—	0.20
iso-C _{17:0}	0.65	1.84	1.32
anteiso-C _{17:0}	3.69	7.20	8.10
C _{17:0}	—	0.17	—
C _{18:1} ω9c	—	—	0.20
C _{18:0}	—	0.28	0.19

The physiological properties of strain EUM 273^T and its closest neighbour, *P. xylanilytica* YIM 61515^T, were significantly different in terms of acid production from *myo*-inositol and adonitol (Table 2). Moreover, strain EUM 273^T could grow well at pH 6 and with 5 % NaCl while the closest type strain, *P. xylanilytica* YIM 61515^T, could not.

Based on the results of this study, isolate EUM 273^T is proposed as a novel species of the genus *Promicromonospora*, with the name *Promicromonospora endophytica* sp. nov.

Description of *Promicromonospora endophytica* sp. nov.

Promicromonospora endophytica (en.do.phy'ti.ca. Gr. pref. *endo* within; Gr. n. *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 tissues).

Gram-staining-positive, aerobic, non-acid-alcohol-fast, and catalase-positive. The strain grows between 15–27 °C. Good growth occurs at pH 6.0–10.0 and in the presence of 1, 3 and 5 % (w/v) NaCl. Colony morphology is wrinkly with a shiny surface. Substrate mycelium develops well on most media used and aerial mycelium is formed on some media. Colony colour is yellowish white. Diffusible pigments are not produced. Mycelium is non-motile, Y- or V-shaped, rod-like with coccoid elements. Monospores (0.5 × 0.6 µm) with an oval shape are observed. Contains MK-9(H₈) (41 %) as the predominant menaquinone, with MK-9(H₆) (37 %) and MK-9(H₄) (22 %) also present. The whole-cell fatty acid profile is shown in Table 1 and physiological properties are listed in Table 2.

Table 2. Differential characteristics between strain EUM 273^T and related species of the genus *Promicromonospora*

Strains: 1, EUM 273^T; 2, *P. xylanilytica* YIM 61515^T; 3, *P. vindobonensis* V45^T. +, Positive or present; w, weakly positive; —, negative or absent. All strains were positive for the following: catalase production; hydrolysis of starch and skimmed milk; acid production from cellobiose, fructose, galactose, glucose, maltose, mannose, mannitol, ribose, sucrose, xylose and salicin. All strains were negative for acid production from 1,2-propanediol and D-sorbitol. All strains could grow at 1 and 3 % (w/v) NaCl, at pH 7–10, and at 15 °C and 27 °C, but not at 10, 15 or 20 % (w/v) NaCl, at pH 4 or pH 5, or at 4 °C or 45 °C.

Characteristic	1	2	3
Acid production from:			
D-Arabinose	+	+	—
<i>myo</i> -Inositol	+	—	+
Adonitol	+	—	—
Growth at:			
5 % (w/v) NaCl	+	—	+
pH 6	+	—	—

The type strain, EUM 273^T (=DSM 23716^T=NRRL B-24816^T), is an endophytic actinobacterium isolated from the root of *Eucalyptus microcarpa* Maiden growing on the campus of Flinders University, Adelaide, South Australia. The DNA G+C content of the type strain is 71.7 mol%.

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