Pseudonocardia acaciae sp. nov., isolated from roots of *Acacia auriculiformis* A. Cunn. ex Benth.

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A novel Gram-positive-staining actinomycete designated strain GMKU095^T was isolated from surface-sterilized roots of *Acacia auriculiformis* A. Cunn. ex Benth. (earpod wattle). The organism produced branching mycelium. The spores were non-motile and had a spiny surface. Growth of strain GMKU095^T occurred at 18–42 °C, pH 5.0–8.0 and at NaCl concentrations up to 5 %. Whole-cell hydrolysates contained arabinose and galactose as major characteristic sugars. The diagnostic diamino acid of the peptidoglycan was *meso*-diaminopimelic acid. The glycan moiety of the murein contained acetyl residues. The menaquinone was MK-8(H₄); mycolic acids were not detected. The G+C content of the DNA was 71.6 mol%. iso-C_{16:0} was detected as the major cellular fatty acid. Comparative studies of 16S rRNA gene sequences indicated that the strain was phylogenetically related to members of the genus *Pseudonocardia*. The most closely related type strain is *Pseudonocardia spinosisporea* IMSNU 50581^T, which is 96.2 % similar in 16S rRNA gene sequence. On the basis of the genotypic and phenotypic properties presented, a novel species of the genus *Pseudonocardia* is proposed, *Pseudonocardia acaciae* sp. nov. The type strain is strain GMKU095^T (=NRRL B-24609^T =BCC 28481^T =TISTR 1862^T =NBRC 104274^T).

The genus *Pseudonocardia* was proposed by Henssen (1957) and its description has been emended by Warwick *et al.* (1994), McVeigh *et al.* (1994), Reichert *et al.* (1998) and Huang *et al.* (2002). At the time of writing, the genus contains 27 species with validly published names. Strains have been isolated from a wide range of habitats such as tree-bark compost (Reichert *et al.*, 1998), a gold mine (Lee *et al.*, 2001), industrial sludge contaminated with 1,4-dioxane (Mahendra & Alvarez-Cohen, 2005) and coastal sediment (Liu *et al.*, 2006). The most recently reported species was *Pseudonocardia oroxyli*, which was isolated from surface-sterilized roots of the higher plant *Oroxylum indicum* (Gu *et al.*, 2006). In this paper, a novel *Pseudonocardia* strain isolated from roots of *Acacia auriculiformis* A. Cunn. ex Benth. (earpod wattle) is described.

Strain GMKU095^T was isolated from surface-sterilized roots of *Acacia auriculiformis* A. Cunn. ex Benth. using starch-casein agar (Küster & Williams, 1964) supplemented

with antibacterial and antifungal agents (nalidixic acid and ketokonazole at 25 and 50 µg ml⁻¹, respectively). The plates were incubated at 28 °C for 21 days. Small colonies with white spore mass were the predominant actinomycete colonies growing on the isolation plates. One of these colonies was isolated and purified on glucose-yeast extract agar (GYE agar) [containing 1.0 % glucose, 1.0 % yeast extract and 1.5 % agar (w/v)]. The pure culture was maintained as a 20 % glycerol suspension at –20 °C or as lyophilized cells for long-term preservation.

The morphological characteristics of strain GMKU095^T were observed by light microscopy and scanning electron microscopy (JEOL JSM 5600 LV) of 10-day-old cultures grown on ISP 3 medium (International *Streptomyces* Project; Shirling & Gottlieb, 1966). Acid production from carbohydrates was determined using media and methods described by Gordon *et al.* (1974). Enzyme activity profiles were tested using API ZYM (bioMérieux), covering 19 enzyme activities. Urease activity was determined by a colour change in urea broth (Gordon *et al.*, 1974). The temperature range for growth was determined on ISP 2 (Shirling & Gottlieb, 1966) using a temperature gradient

Abbreviation: A₂pm, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GMKU095^T is EU921261.

incubator (Tokyo Kagaku Sangyo) set with low and high temperatures of 5 and 50 °C. Casein and gelatin hydrolysis and reduction of nitrate were evaluated using the methods of Gordon & Mihm (1957). Production of melanoid pigment was determined by the method of the ISP (Shirling & Gottlieb, 1966). NaCl tolerance was studied on ISP 2 containing NaCl at a final concentration of 1–9 % (w/v). pH tolerance was studied at pH 4–10.

Biomass for chemotaxonomic studies was obtained after growing strain GMKU095^T in GYE broth in shake flasks for 10 days at 28 °C. The resultant cells were washed three times in distilled water and freeze-dried. Standard procedures were used to determine the isomer of diaminopimelic acid (A₂pm) (Hasegawa *et al.*, 1983). The acyl type of the cell wall was analysed according to the method of Uchida & Aida (1984). Whole-cell sugars were analysed according to the method of Becker *et al.* (1965). Menaquinones were extracted and purified by the method of Collins *et al.* (1977), and isoprene units were analysed by HPLC using a JASCO 802-SC chromatograph equipped with a Shiseido CAPCELL PAK C18 column (Tamaoka *et al.*, 1983). Mycolic acids were detected by TLC according to the method of Tomiyasu (1982). The G + C content of the DNA, isolated following the method of Marmur (1961), was determined by HPLC according to the method of Tamaoka & Komagata (1984). Analysis of fatty acids in cells grown on ISP 2 at 37 °C for 10 days was performed according to the procedures of the Sherlock Microbial Identification System (Microbial ID).

The 16S rRNA gene of strain GMKU095^T was amplified as described by Duangmal *et al.* (2005). The PCR products were purified and sequenced using an ABI PRISM BigDye Terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems) and universal primers (Lane, 1991). The almost-complete 16S rRNA gene sequence (1483 bp) was aligned with the corresponding sequences of representatives of the genus *Pseudonocardia* and related genera, retrieved from the GenBank database, using the CLUSTAL_X (Thompson *et al.*, 1997) and PHYDIT (<http://plaza.snu.ac.kr/~jchun/phydit/>) programs.

Phylogenetic trees were inferred by using the least-squares (Fitch & Margoliash, 1967), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) tree-making methods from the PHYLIP suite of programs (Felsenstein, 1993). The resultant tree topologies were evaluated by using bootstrap analyses (Felsenstein, 1985) based on 1000 resampled datasets using the SEQBOOT and CONSENSE options from the PHYLIP package. The resultant phylogenetic trees were viewed using the TreeView program (Page, 1996).

Strain GMKU095^T was a Gram-positive-staining, aerobic, filamentous actinobacterium with white to yellowish-white aerial mycelium and yellowish-brown substrate mycelium on ISP 2 agar after 10 days at 37 °C. Chains of oval spores with spiny surfaces were produced on ISP 2 agar (Fig. 1). Growth occurred in both standing and shake-flask cultures.

Fig. 1. Scanning electron micrograph of strain GMKU095^T grown for 10 days at 28 °C on ISP 3 agar. Bar, 1 μm.

The results of chemical analysis indicated that the organism has chemotaxonomic markers typical of the genus *Pseudonocardia* (Huang *et al.*, 2002). Strain GMKU095^T contained *meso*-A₂pm in the peptidoglycan and MK-8(H₄) as the major menaquinone. No mycolic acids were detected. The predominant cellular fatty acids were iso-C_{16:0} (23.13 %), anteiso-C_{17:0} (16.23 %), 10-methyl C_{16:0} (10.22 %), C_{17:1ω6c} (7.40 %), iso-C_{17:0} (7.10 %), 10-methyl C_{17:0} (6.21 %), iso-C_{15:0} (5.66 %) and C_{16:0} (5.57 %). Fatty acids present as minor components are C_{17:1ω8c} (3.73 %), C_{17:0} (2.00 %), anteiso-C_{15:0} (1.89 %), C_{15:0} (0.93 %), iso-C_{16:1} H (0.85 %), iso-C_{18:0} (0.64 %), C_{16:1} 2-OH (0.62 %), C_{18:1ω9c} (0.52 %), iso-C_{14:0} (0.40 %) and C_{12:0} 2-OH (0.30 %). The G + C content of the DNA was 71.6 mol%. Phylogenetic analysis of an almost-complete 16S rRNA gene sequence of strain GMKU095^T indicated that the isolate formed a distinct cluster with members of the genus *Pseudonocardia* (Fig. 2). The 16S rRNA gene sequence similarity between strain GMKU095^T and its nearest neighbours, *Pseudonocardia spinosipora* LM 141^T and *Pseudonocardia asaccharolytica* DSM 44247^T, was 96.2 and 96.0 %, respectively. The relatively low level of 16S rRNA gene sequence similarity clearly differentiated strain GMKU095^T from its closest phylogenetic relatives. A 16S rRNA gene sequence similarity of less than 97 % generally indicates an overall genomic relatedness of less than 70 %, the cut-off point recommended for delineation of genomic species (Stackebrandt & Goebel, 1994). Much higher 16S rRNA gene sequence similarities have been recorded between representatives of several *Pseudonocardia* species with validly published names that have DNA–DNA relatedness values well below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the circumscription of strains that belong to the same genomic species. The type strains of *Pseudonocardia tetrahydrofuranooxydans* and *Pseudonocardia hydrocarbonooxydans*, for example, share a 16S rRNA gene sequence similarity of 99.3 %, but have a DNA–DNA relatedness of 31.7 % (Kämpfer *et al.*, 2006). Similarly, the type strain of *Pseudonocardia kongjuensis* shows 98.8 % 16S rRNA gene sequence similarity to the

Fig. 2. Neighbour-joining tree derived from 16S rRNA gene sequences showing the relationship of strain GMKU095^T with members of the genus *Pseudonocardia*. The tree was based on a comparison of 1323 nucleotide positions. Asterisks indicate branches of the tree that were also found using the least-squares (Fitch & Margoliash, 1967) and maximum-parsimony (Kluge & Farris, 1969) tree-making algorithms. Numbers at nodes indicate percentage bootstrap support from an analysis of 1000 resampled datasets. Bar, 0.02 substitutions per site.

type strain of its closest neighbour, *Pseudonocardia autotrophica*, but they share a DNA–DNA relatedness of only 32 % (Lee *et al.*, 2001).

Strain GMKU095^T was positive for catalase and urease activities. Observation of cultures over 14 days revealed growth in a temperature range of 18–42 °C, with optimum growth at 36–38 °C. No growth was observed at or above 44 °C. The strain was able to grow at pH 5.0 and 8.0, with optimal growth at pH 6.0–7.0. NaCl was tolerated up to 5%. Melanin pigment was not produced. Strain GMKU095^T was readily differentiated from its closest relatives (*P. asaccharolytica* DSM 44247^T and *P. spinosipora* IMSNU 50581^T) on the basis of physiological properties (Table 1), especially the ability to produce acid from mannitol, the decomposition of tyrosine, hydrolysis of gelatin and starch, growth at 37 °C and growth with 3 % NaCl. Moreover, growth of strain GMKU095^T occurred between 18 and 42 °C, whereas the growth of *P. spinosipora* IMSNU 50581^T occurred at 4–30 °C.

In conclusion, strain GMKU095^T is clearly differentiated from *P. asaccharolytica* DSM 44247^T and *P. spinosipora* IMSNU 50581^T based on polyphasic taxonomic analysis. We conclude that strain GMKU095^T represents a novel species of *Pseudonocardia*, for which the name *Pseudonocardia acaciae* sp. nov. is proposed.

Table 1. Differential characteristics of strain GMKU095^T and closely related strains of the genus *Pseudonocardia*

Strains: 1, strain GMKU095^T; 2, *P. asaccharolytica* DSM 44247^T; 3, *P. spinosipora* IMSNU 50581^T. +, Positive; w, weakly positive; –, negative; ND, no data. Data for reference strains were taken from Reichert *et al.* (1998) and Lee *et al.* (2002). All three strains have iso-C_{16:0} as the predominant fatty acid.

Characteristic	1	2	3
Spore surface	Spiny	Smooth	Spiny
Acid production from:			
Adonitol	+	–	+
L-Arabinose	+	–	+
D-Fructose	+	–	+
D-Glucose	+	–	+
myo-Inositol	w	–	+
Mannitol	w	–	–
L-Rhamnose	+	–	+
D-Xylose	+	–	+
H ₂ S production	–	–	+
Urease	+	–	+
Decomposition of tyrosine	+	–	–
Hydrolysis of:			
Gelatin	+	–	–
Starch	+	–	–
Growth temperature (°C)	18–42	ND	4–30
Growth at 37 °C	+	–	–
Growth with 3 % NaCl	+	–	–

Description of *Pseudonocardia acaciae* sp. nov.

Pseudonocardia acaciae (a.ca.ci'ae. L. n. *acacia* the acacia tree and also the name of a botanical genus; L. gen. n. *acaciae* of *Acacia*, referring to the isolation of the type strain from roots of *Acacia auriculiformis* A. Cunn. ex Benth.).

Aerobic, Gram-positive-staining, non-acid–alcohol-fast. Forms yellowish-white aerial mycelium and yellowish-brown substrate mycelium on ISP 2 agar. The spiny spores occur in chains. Growth occurs in both standing and shake-flask cultures. Catalase, nitrate reduction and urease are positive. H₂S is not produced. Acid is produced from adonitol, L-arabinose, D-fructose, D-glucose, D-mannose, L-rhamnose, ribose and D-xylose. No acid production from D-galactose, lactose, maltose, melezitose, raffinose, D-sorbitol or sucrose. Weak acid production from glycerol, myo-inositol and mannitol. Hydrolyses gelatin and starch, but not casein. Growth occurs at pH 5.0–8.0, with an optimum at pH 6.0–7.0. The temperature range for growth is 18–42 °C, with an optimum at 36–38 °C. On ISP 2 agar, good growth occurs at 0–1 % NaCl, moderate growth at 2 % NaCl and poor growth at 3–5 % NaCl. Esterase (C4), lipase (C8), leucine aminopeptidase and valine aminopeptidase are detected with the API ZYM enzyme assay; acid and alkaline phosphatases, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, trypsin, phosphoamidase, chymotrypsin, cystine aminopeptidase, α-fucosidase, β-

glucuronidase, lipase (C14), α -mannosidase and *N*-acetyl- β -glucosaminidase are not detected. The diagnostic diamino acid of the peptidoglycan is *meso*-A₂pm. Whole-cell sugars are arabinose and galactose. The glycan moiety of the murein is acetylated. The predominant menaquinone is MK-8(H₄). Mycolic acids are not detected. The fatty acid profile is dominated by iso-C_{16:0}, anteiso-C_{17:0}, 10-methyl C_{16:0}, C_{17:1}ω6c, iso-C_{17:0}, 10-methyl C_{17:0}, iso-C_{15:0} and C_{16:0}. Other cellular fatty acids detected as minor components (<5%) are C_{17:1}ω8c, C_{17:0}, anteiso-C_{15:0}, C_{15:0}, iso-C_{16:1} H, iso-C_{18:0}, C_{16:1} 2-OH, C_{18:1}ω9c, iso-C_{14:0} and C_{12:0} 2-OH. The G+C content of the DNA of the type strain is 71.6 mol%.

The type strain, strain GMKU095^T (=NRRL B-24609^T =BCC 28481^T =TISTR 1862^T =NBRC 104274^T), was isolated from roots of *Acacia auriculiformis* A. Cunn. ex Benth., collected from Kasetsart University, Bangkok, Thailand.

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