Pseudonocardia kunmingensis sp. nov., an actinobacterium isolated from surface-sterilized roots of *Artemisia annua* L.

Guo-Zhen Zhao,[1]† Jie Li,[1,2]† Hai-Yu Huang,[1] Wen-Yong Zhu,[1] Dong-Jin Park,[3] Chang-Jin Kim,[3] Li-Hua Xu[1] and Wen-Jun Li[1,2]

Correspondence
Wen-Jun Li
wjli@ynu.edu.cn *or*
liact@hotmail.com

[1]The Key Laboratory for Microbial Resources of the Ministry of Education, PR China and Laboratory for Conservation and Utilization of Bio-resources, Yunnan Institute of Microbiology, Yunnan University, Kunming, 650091, PR China

[2]Key Laboratory of Marine Bio-resources Sustainable Utilization/Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, PR China

[3]Korea Research Institutes of Biosciences and Biotechnology 52, Eoeun-dong, Yuseong gu, Daejeon, 305-333, Republic of Korea

A Gram-positive, aerobic, actinobacterial strain with rod-shaped spores, designated YIM 63158^T, was isolated from the surface-sterilized roots of *Artemisia annua* L. collected from Yunnan province, south-west China. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain YIM 63158^T belonged to the genus *Pseudonocardia*. The closest neighbours were '*Pseudonocardia sichuanensis*' KLBMP 1115 (99.9 % 16S rRNA gene sequence similarity), *Pseudonocardia adelaidensis* EUM 221^T (99.1 %) and *Pseudonocardia zijingensis* DSM 44774^T (98.8 %); sequence similarities to other members of the genus *Pseudonocardia* ranged from 98.6 to 94.4 %. The chemotaxonomic characteristics, such as the cell-wall diaminopimelic acid, whole-cell sugars, fatty acid components and major menaquinones, suggested that the isolate belonged to the genus *Pseudonocardia*. The G+C content of the genomic DNA was 73.3 mol%. On the basis of physiological, biochemical and chemotaxonomic data, including low DNA–DNA relatedness between the isolate and other members of the genus *Pseudonocardia*, it is proposed that strain YIM 63158^T represents a novel species in this genus, with the name *Pseudonocardia kunmingensis* sp. nov. The type strain is YIM 63158^T (=DSM 45301^T =CCTCC AA 208081^T).

The genus *Pseudonocardia* within the family *Pseudonocardiaceae* was described by Henssen (1957) and since then the description of the genus has been emended repeatedly (Warwick *et al.*, 1994; McVeigh *et al.*, 1994; Reichert *et al.*, 1998; Huang *et al.*, 2002; Park *et al.*, 2008). At the time of writing, the genus *Pseudonocardia* encompasses 38 species with validly published names (http://www.bacterio.cict.fr/; Ara *et al.*, 2011; Kaewkla & Franco, 2010, 2011; Qin *et al.*, 2010; Sakiyama *et al.*, 2010; Zhao *et al.*, 2011). Members of the genus *Pseudonocardia* have the following characteristics. Aerial mycelium may be present. The vegetative mycelium may fragment. The spores are normally smooth and form chains by acropetal budding or septation on the substrate or aerial mycelium. Alternatively, the spores are formed in longitudinal pairs on vegetative hyphae and singly or in longitudinal pairs on aerial hyphae. The chemotaxonomic characteristics are a type-IV cell wall, predominant menaquinone MK-8(H_4) or MK-9(H_0), DNA G+C content of 68–79 mol%, no mycolic acids and phospholipid type II, III or IV. Strains of this genus have been isolated from various environmental samples, such as activated sludge, soils and plant samples (Gu *et al.*, 2006; Chen *et al.*, 2009; Duangmal *et al.*, 2009; Kaewkla & Franco, 2010, 2011; Qin *et al.*, 2010, 2011; Zhao *et al.*, 2011).

In the course of our research on new actinobacterial sources, strain YIM 63158^T was isolated from the roots of *Artemisia annua* L. collected in Kunming, Yunnan province, south-west China. Samples were washed in running water to remove soil particles, surface-sterilized by an established procedure (Li *et al.*, 2008), sliced and placed on HV agar (Hayakawa & Nonomura, 1987). The plates were incubated at 28 °C for 4–6 weeks until the

†These authors contributed equally to this work.

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A supplementary figure and two supplementary tables are available with the online version of this paper.

outgrowth of endophytic actinomycetes was discerned. Colonies originating from plant segments were selected and pure cultures were obtained by repeated streaking on TWYE agar [containing (l tap water)$^{-1}$: 0.25 g yeast extract, 0.5 g K_2HPO_4, 18 g agar; pH 7.2]. Strain YIM 63158^T was maintained on tryptic soy agar (TSA) slants at 4 °C and as 20 % (v/v) glycerol suspensions at −70 °C. Biomass for chemical and molecular studies was obtained by cultivation in shake flasks (about 200 r.p.m.) using tryptic soy broth [TSB; containing (l tap water)$^{-1}$: 15 g tryptone, 5 g soya peptone, 5 g NaCl; pH 7.2] at 28 °C for 1 week.

Gram staining was carried out using the standard Gram reaction and cell motility was confirmed by the development of turbidity throughout a tube containing semi-solid medium (Leifson, 1960). The morphological characteristics of strain YIM 63158^T, including spore-chain morphology, spore size and surface ornamentation, were assessed by light and scanning electron microscopy (XL30 and ESEM-TMP; Philips) of 14-day-old cultures prepared on YIM 38 medium (Zhao *et al.*, 2010). Aerial spore-mass colour, substrate mycelium pigmentation and coloration of the diffusible pigments of strain YIM 63158^T were recorded on International *Streptomyces* Project (ISP) media (Shirling & Gottlieb, 1966), Czapek's agar, potato–glucose agar and nutrient agar prepared as described by Dong & Cai (2001). Colours were determined using colour chips from the ISCC–NBS colour charts (standard sample no. 2106) (Kelly, 1964). Growth at 4, 10, 20, 28, 37, 40, 42, 45, 50 and 55 °C was tested on TSA for 21 days. Growth at pH 4–10 (in increments of 1 pH unit using the buffer system described by Xu *et al.* 2005) and growth with 0, 1, 3, 5, 7, 8, 9, 10, 15 and 20 % (w/v) NaCl were tested in TSB at 28 °C for 14–21 days. Catalase, oxidase and gelatinase activities, starch hydrolysis, nitrate reduction and urease were assessed as described by Smibert & Krieg (1994). Other physiological and biochemical tests were performed as described by Gordon *et al.* (1974).

Cells of strain YIM 63158^T were Gram-positive, aerobic and non-motile. Strain YIM 63158^T grew well on ISP 2, ISP 3, ISP 4, ISP 5, Czapek's agar, nutrient agar and potato–glucose agar. White aerial mycelium was produced on ISP 2, ISP 3, ISP 4, ISP 5, Czapek's agar and potato–glucose agar, but no aerial mycelium was formed on nutrient agar. The substrate mycelium varied from orange to orange–yellow on the media tested and a diffusible pigment (brown–yellow) was observed on potato–glucose agar (Supplementary Table S1, available in IJSEM Online). Morphological observation of a 14-day-old culture of strain YIM 63158^T revealed that both aerial and vegetative hyphae were abundant, well-developed and fragmented. The mycelia displayed long spore chains, containing up to 10 rod-shaped and smooth-surfaced spores (0.9–1.0 × 1.1–2.5 μm; Supplementary Fig. S1). The results showed that strain YIM 63158^T had morphological properties typical of the genus *Pseudonocardia*. The isolate grew at 10–40 °C, pH 6.0–9.0 and 0–7 % (w/v) NaCl. Optimal growth was observed at 28 °C, pH 7.0–8.0 and 1–3 % NaCl. The isolate was catalase-positive and oxidase-negative. Detailed physiological and biochemical properties are given in Table 1 and the species description. It is evident from Table 1 that there were some phenotypic differences between strain YIM 63158^T and its closest phylogenetic neighbours.

The isomer of diaminopimelic acid and sugar analyses of whole-cell hydrolysates were performed according to the procedures described by Hasegawa *et al.* (1983), Lechevalier & Lechevalier (1970) and Tang *et al.* (2009). Phospholipids were extracted, examined by two-dimensional TLC and identified using procedures described elsewhere (Minnikin *et al.*, 1979; Collins & Jones, 1980). Menaquinones were isolated according to Collins *et al.* (1977) and separated by HPLC (Tamaoka *et al.*, 1983). Mycolic acids were extracted and analysed by one-dimensional TLC as described by Minnikin *et al.* (1980). Cellular fatty acids were extracted and methylated using the Sherlock Microbial Identification System (MIDI) according to the manufacturer's instructions. The fatty

Table 1. Differential characteristics of strain YIM 63158^T and its closest phylogenetic neighbours

Strains: 1, *Pseudonocardia kunmingensis* sp. nov. YIM 63158^T; 2, '*P. sichuanensis*' KLBMP 1115; 3, *P. adelaidensis* DSM 45352^T; 4, *P. zijingensis* DSM 44774^T. All data were taken from this study unless otherwise indicated. All strains are Gram-positive, non-motile and catalase- and urease-positive, grow under aerobic conditions, with 5 % (w/v) NaCl, at pH 6.0–8.0 and at 10–40 °C, and utilize glucose, D-mannitol, D-mannose, raffinose, L-rhamnose and ribose. No strains utilize dulcitol. +, Positive; W, weakly positive; −, negative.

Characteristic	1	2	3	4
Utilization of:				
L-Arabinose	+	+	−	+
Cellobiose	+	−	−	+
Glycerol	−	−	+	+
myo-Inositol	+	−	+	+
Lactose	+	+	−	+
Maltose	+	+	−	+
Sucrose	−	+	+	+
Acid from:				
D-Galactose	−	−	+	+
D-Mannose	+	+	−	−
D-Ribose	+	−	−	+
Hydrolysis of:				
Starch	−	+	−	+
Tween 40	+	+	−	+
Growth at/with:				
45 °C	−	+	W	+
10 % NaCl	−	−	W	−
pH 5.0	−	+	W	−
pH 10	−	−	+	W
DNA G+C content (mol%)*	73.3	69.8	78.8	70.9

*Data for columns 2–4 were taken from Qin *et al.* (2011), Kaewkla & Franco (2010) and Huang *et al.* (2002), respectively.

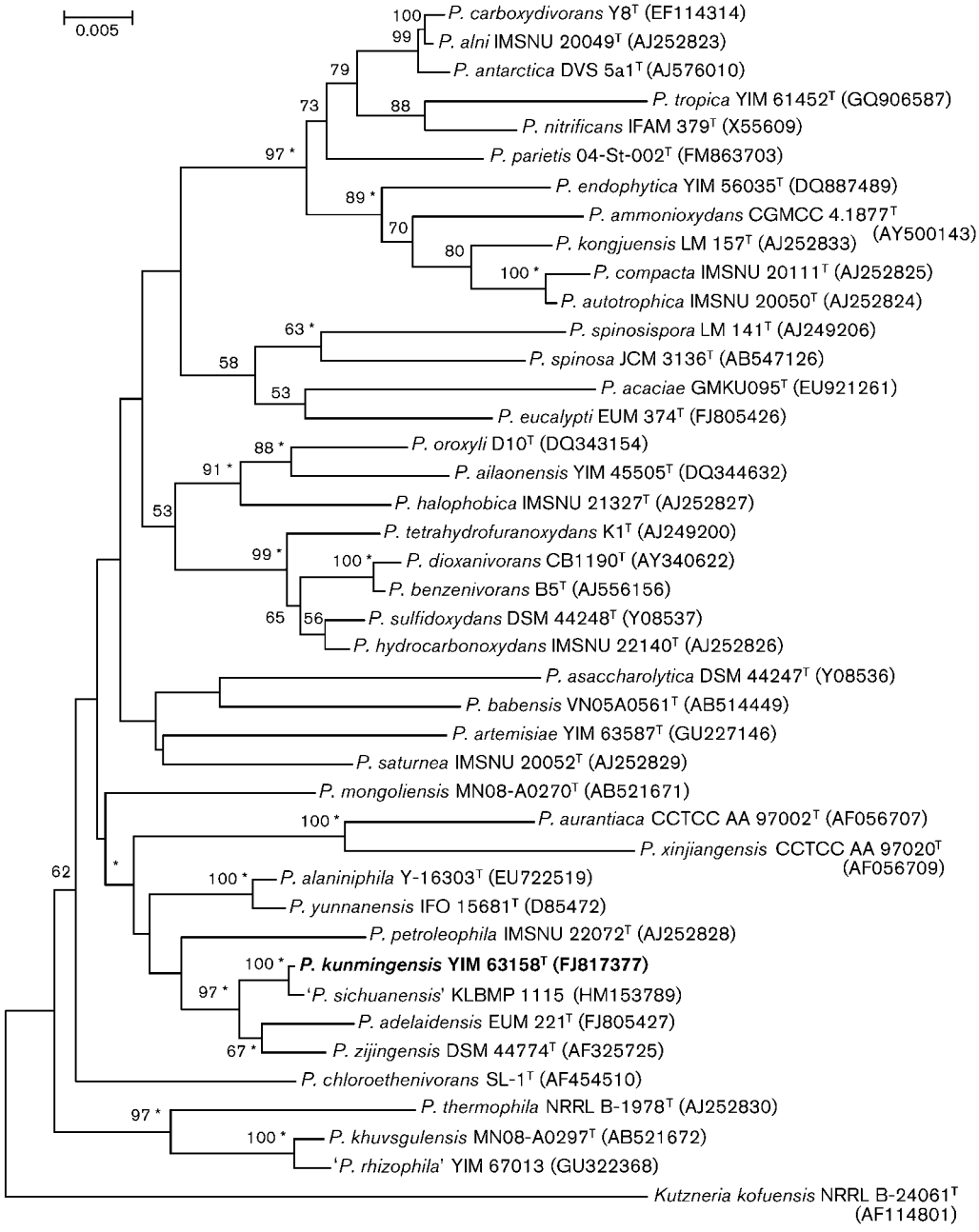

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain YIM 63158^T with members of the genus *Pseudonocardia*. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. Asterisks indicate that the corresponding nodes were also recovered in trees generated using the maximum-parsimony and maximum-likelihood methods. Bar, 0.005 substitutions per nucleotide position.

acid methyl esters were analysed by GC (7890A GC System; Agilent Technologies) using the Microbial Identification software package (Sherlock version 6.1, MIDI database TSBA6).

The results indicated that the cell-wall diamino acid in the peptidoglycan layer of strain YIM 63158^T was *meso*-diaminopimelic acid and the whole-cell sugars were glucose, arabinose, galactose, mannose and ribose. The phospholipids consisted of diphosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylglycerol and an unknown glycolipid. MK-8(H_4) (95.5 %) was the predominant menaquinone; MK-8(H_6) (2.8 %) and MK-8(H_2) (1.7 %) were detected as minor components. The predominant menaquinone of strain YIM 63158^T was similar to those of closely related strains; in

particular, MK-8(H_4) is a predominant menaquinone in most recognized species of the genus *Pseudonocardia*. Mycolic acids were absent. The major fatty acids were iso-$C_{16:0}$ (37.14 %), $C_{16:0}$ 10-methyl (13.76 %), anteiso-$C_{17:0}$ (10.51 %), $C_{16:1}\omega6c/\omega7c$ (6.40 %), $C_{16:0}$ (5.73 %) and iso-$C_{15:0}$ (4.63 %), which were similar to those described for the genus *Pseudonocardia*. However, there were differences with the reference strains (Supplementary Table S2): for example, strain YIM 63158^T contained high amounts of anteiso-$C_{17:0}$ and $C_{16:0}$ 10-methyl, but '*P. sichuanensis*' KLBMP 1115, *P. adelaidensis* DSM 45352^T and *P. zijingensis* DSM 44774^T contained lower amounts of these fatty acids. The chemotaxonomic characteristics of strain YIM 63158^T, such as the diaminopimelic acid isomer and sugars in whole-cell hydrolysates, menaquinones, major fatty acids and phospholipids, were consistent with its assignment to the genus *Pseudonocardia*.

Extraction of genomic DNA and PCR amplification and sequencing of the 16S rRNA gene were performed as described by Li *et al.* (2007). Multiple alignments with sequences of the most closely related actinobacteria and sequence similarity calculations were carried out using CLUSTAL X (Thompson *et al.*, 1997). The phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) tree-making algorithms using the software packages MEGA version 4.0 (Tamura *et al.*, 2007), PHYLIP version 3.6 (Felsenstein, 2002) and PHYML (Guindon & Gascuel, 2003). The topologies of the phylogenetic trees were evaluated using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

The nearly complete 16S rRNA gene sequence of strain YIM 63158^T (1388 bp) was determined and compared with corresponding sequences in public databases. The neighbour-joining phylogenetic analysis based on sequence data from strain YIM 63158^T and all recognized members of the genus *Pseudonocardia*, as well as *Kutzneria kofuensis* NRRL B-24061^T, revealed that strain YIM 63158^T should be assigned to the genus *Pseudonocardia* (Fig. 1). Strain YIM 63158^T formed a separate lineage within the cluster containing '*P. sichuanensis*' KLBMP 1115, *P. adelaidensis* EUM 221^T and *P. zijingensis* DSM 44774^T (97 % bootstrap value), with which it shared 99.9, 99.1 and 98.8 % 16S rRNA gene sequence similarity, respectively. Strain YIM 63158^T and other members of the genus *Pseudonocardia* shared <98.6 % 16S rRNA gene sequence similarity. The affiliations of strain YIM 63158^T, '*P. sichuanensis*' KLBMP 1115, *P. adelaidensis* EUM 221^T and *P. zijingensis* DSM 44774^T were also supported by the maximum-parsimony and maximum-likelihood algorithms, with high bootstrap values.

DNA–DNA hybridization between strain YIM 63158^T and the reference strains was carried out by the fluorometric micro-well method at the optimal hybridization temperature (47 °C). DNA–DNA relatedness between strain YIM 63158^T and '*P. sichuanensis*' KLBMP 1115, *P. adelaidensis* DSM 45352^T and *P. zijingensis* DSM 44774^T was 51.0±1.5, 47.0±2.0 and 39±1.8 %, respectively, which was well below the 70 % cut-off point for recognition of genomic species (Stackebrandt & Goebel, 1994), thus suggesting that strain YIM 63158^T should be considered as a different genomic species of the genus *Pseudonocardia*. It has been shown that some members of the genus *Pseudonocardia* exhibit high 16S rRNA gene sequence similarities (97.8–99.7 %) but low DNA–DNA relatedness values (<70 %) (Prabahar *et al.*, 2004; Kämpfer *et al.*, 2006; Park *et al.*, 2008; Qin *et al.*, 2010). For example, *Pseudonocardia antarctica* and *Pseudonocardia alni* share 99.7 % 16S rRNA gene sequence similarity, but have 50 % DNA–DNA relatedness (Prabahar *et al.*, 2004). Similarly, *Pseudonocardia parietis* shares 99.2, 99.1 and 99.1 % 16S rRNA gene sequence similarity with *P. antarctica*, *P. alni* and *Pseudonocardia carboxydivorans*, respectively, but has 30.5–44.5 % DNA–DNA relatedness with these species (Schäfer *et al.*, 2009). For this reason, DNA–DNA hybridization between strain YIM 63158^T and members of the genus *Pseudonocardia* with which it shared <98.6 % 16S rRNA gene sequence similarity were not carried out.

The G+C content of the genomic DNA was determined using the HPLC method (Mesbah *et al.*, 1989) with *Escherichia coli* JM-109 as the reference strain. The DNA G+C content of strain YIM 63158^T was 73.3 mol%, which is in accordance with the range for the genus *Pseudonocardia* (68–79 mol%).

The phenotypic and chemotaxonomic data, together with the 16S rRNA gene sequence data, support the proposal that strain YIM 63158^T represents a novel species of the genus *Pseudonocardia*, for which the name *Pseudonocardia kunmingensis* sp. nov. is proposed.

Description of *Pseudonocardia kunmingensis* sp. nov.

Pseudonocardia kunmingensis (kun.min.gen′sis. N.L. fem. adj. *kunmingensis* of or pertaining to Kunming, a city of Yunnan in south-west China).

Aerobic, non-motile, Gram-positive actinomycete that forms extensively branched substrate and aerial mycelia. The aerial mycelium carries smooth-surfaced, rod-shaped spores. Forms white aerial mycelium and orange to orange–yellow substrate mycelium. A diffusible pigment (brown–yellow) is observed on potato–glucose agar. Grows at 10–40 °C (optimum 28 °C), at pH 6.0–9.0 (optimum pH 7.0–8.0) and with 0–7 % (w/v) NaCl [optimum 1–3 % (w/v) NaCl]. Positive for catalase and urease, but negative for milk coagulation and peptonization, nitrate reduction, oxidase, gelatin liquefaction, cellulose and starch hydrolysis and H_2S production. Tween 40 is hydrolysed, but Tweens 20 and 80 are not hydrolysed. As sole carbon sources, utilizes L-arabinose, cellobiose, D-fructose, D-galactose, glucose, *myo*-inositol, lactose, maltose, D-mannitol, D-mannose, raffinose, L-rhamnose, D-ribose, D-sorbitol and D-xylose, but not dulcitol, glycerol, sodium acetate or

sucrose. As sole nitrogen sources, utilizes L-alanine, L-arginine, L-asparagine, glycine, L-hydroxyproline, hypoxanthine, L-lysine, L-phenylalanine, L-serine, L-tyrosine, L-valine and xanthine. Produces acid from D-mannose and D-ribose. The cell wall contains *meso*-diaminopimelic acid. The whole-cell sugar pattern consists of glucose, arabinose, galactose, mannose and ribose. MK-8(H_4) is the predominant menaquinone. The phospholipids consist of diphosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylglycerol and an unknown glycolipid. Mycolic acids are absent. The major fatty acids are iso-$C_{16:0}$, $C_{16:0}$ 10-methyl, anteiso-$C_{17:0}$, $C_{16:1}\omega6c/\omega7c$, $C_{16:0}$ and iso-$C_{15:0}$. The G+C content of the type strain is 73.3 mol%.

The type strain, YIM 63158^T (=DSM 45301^T =CCTCC AA 208081^T), was isolated from surface-sterilized roots of *Artemisia annua* L. collected from Kunming, Yunnan province, south-west China.

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