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Pseudonocardia artemisiae sp. nov., isolated from surface-sterilized *Artemisia annua* L.

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A novel actinomycete strain, designated YIM 63587^T, was isolated from 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 63587^T was affiliated to the genus *Pseudonocardia*. 16S rRNA gene sequence similarities between strain YIM 63587^T and type strains of species of the genus *Pseudonocardia* were 96.6–93.8 %. The diagnostic cell-wall diamino acid in the peptidoglycan layer of strain YIM 63587^T was *meso*-diaminopimelic acid and the whole-cell sugars were arabinose, galactose, mannose and ribose. The predominant menaquinone was MK-8(H_4) (97.7 %). The phospholipids were diphosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol mannosides, phosphatidylinositol and an unknown phospholipid. The major cellular fatty acids (>5 %) were iso-$C_{16:0}$ (44.7 %), iso-$C_{14:0}$ (10.3 %), iso-$C_{16:1}$ H (9.8 %) and iso-$C_{15:0}$ (7.7 %). The G+C content of the genomic DNA was 68.2 mol%. On the basis of phylogenetic, physiological and chemotaxonomic data, strain YIM 63587^T represents a novel species of the genus *Pseudonocardia*, for which the name *Pseudonocardia artemisiae* sp. nov. is proposed. The type strain is YIM 63587^T (=DSM 45313^T=CCTCC AA 208081^T).

The genus *Pseudonocardia* was first 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 encompasses 33 species with validly published names (Sakiyama *et al.*, 2010; Qin *et al.*, 2010). Members of the genus *Pseudonocardia* have the following characteristics: vegetative and aerial mycelia with spore chains produced by acropetal budding or fragmentation, cell wall type IV, predominant menaquinone MK-8(H_4), DNA G+C content 68–79 mol%, no mycolic acids and phospholipid pattern type II or III.

In the course of our research on new actinobacterial sources, a new isolate, strain YIM 63587^T, was isolated from the roots of *Artemisia annua* L. collected in Yunnan province, south-west China. Root samples were washed in running water to remove soil particles and sterilized by an established procedure (Coombs & Franco, 2003; Li *et al.*, 2008). After being surface-sterilized, the samples were sliced, placed on TWYE agar (containing l^{-1} tap water: 0.25 g yeast extract, 0.5 g K_2HPO_4 and 18 g agar; pH 7.2) and incubated at 28 °C until the outgrowth of endophytic actinomycetes from plant segments was discernable. Pure cultures were obtained by repeated streaking on TWYE agar. Strain YIM 63587^T was maintained on tryptic soy agar (TSA) slants at 4 °C and in 20 % (w/v) glycerol suspensions at −80 °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^{-1} tap water: 15 g tryptone, 5 g soya peptone and 5 g NaCl; pH 7.2) at 28 °C for 1 week.

Extraction of genomic DNA and PCR amplification and sequencing of the 16S rRNA gene of strain YIM 63587^T were performed as described by Li *et al.* (2007) and the sequence was compared with corresponding sequences of other bacterial strains in the GenBank database. Multiple alignments with sequences of the most closely related actinobacteria and calculations of sequence similarity were carried out using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic trees were constructed with the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) tree-making algorithms

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Abbreviation: ISP, International *Streptomyces* Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 63587^T is GU227146.

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

using MEGA version 4.0 (Tamura *et al.*, 2007) and PHYLIP version 3.6. Topology was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

The nearly complete 16S rRNA gene sequence of strain YIM 63587^T (1426 bp) was determined. The neighbour-joining phylogenetic tree (Fig. 1) showed that strain YIM 63587^T formed a separate lineage within the genus *Pseudonocardia*, which was supported by a bootstrap value of 65 %. Strain YIM 63587^T clustered with *Pseudonocardia saturnea* IMSNU 20052^T and did not cluster with other closely related members of the genus *Pseudonocardia*. The same affiliation between strain YIM 63587^T and *P. saturnea* IMSNU 20052^T was also observed in trees generated with the maximum-parsimony and maximum-likelihood algorithms, with bootstrap values of 59 and 68 %, respectively (Fig. 1 and Supplementary Figs. S1 and S2, available in IJSEM Online). 16S rRNA gene sequence similarity between strain YIM 63587^T and members of the genus *Pseudonocardia* was less than 97 %; for example, *P. saturnea* IMSNU 20052^T, 96.6 %; *P. sulfidoxydans* DSM 44248^T, 96.5 %; *P. hydrocarbonoxydans* IMSNU 22140^T, 96.5 %; and *P. benzenivorans* B5^T, 96.2 %. A cut-off value of 97.0 % 16S rRNA gene sequence similarity was proposed by Stackebrandt & Goebel (1994) as a criterion for species discrimination. Taking this into consideration, we concluded that the 16S rRNA gene sequence similarities between strain YIM 63587^T and the type strains of species of the genus *Pseudonocardia* were low enough to exclude the assignment of the isolate to any of the recognized species of the genus *Pseudonocardia*.

The G+C content of the genomic DNA of strain YIM 63587^T was determined by the HPLC method (Mesbah *et al.*, 1989) with *Escherichia coli* JM-109 as the reference strain. The G+C content was 68.2 mol%, which was in accordance with the range for the genus *Pseudonocardia* (68–79 mol%).

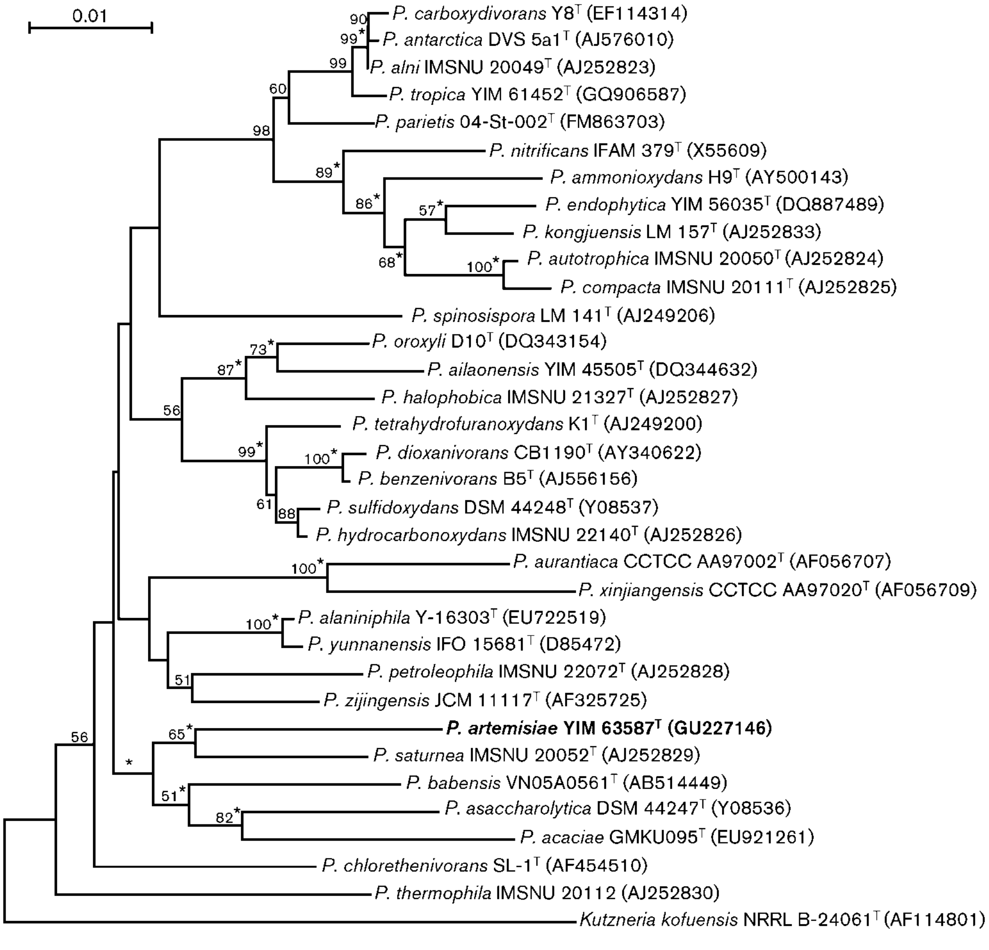

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the relationships between strain YIM 63587^T and other members of the genus *Pseudonocardia*. Bootstrap values (>50 %) based on 1000 replicates are shown at branch nodes. Asterisks indicate that the corresponding branches were also recovered in trees generated with the maximum-parsimony and maximum-likelihood methods. *Kutzneria kofuensis* NRRL B-24061^T was used as the outgroup. Bar, 0.01 substitutions per nucleotide position.

Gram staining was carried out using the standard Gram reaction. The morphological characteristics of strain YIM 63587^{T}, including spore-chain morphology, spore size and surface ornamentation, were assessed by light and scanning electron microscopy (XL-30 ESEM-TMP; Philips) with 14-day-old cultures on YIM 38 medium (Zhao *et al.*, 2010). Aerial spore colour, substrate mycelium pigmentation and colouration of diffusible pigments were recorded after growth 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 Inter-Society Color Council – National Bureau of Standards colour charts (standard sample no. 2106; Kelly, 1964). Physiological tests, such as growth at 4, 10, 20, 28, 37, 45, 50 and 55 °C, at pH 4–10 (in increments of one pH unit) using the buffer system described by Xu *et al.* (2005) and with 0, 1, 3, 5, 7, 10, 15 and 20 % (w/v) NaCl, were performed in TSB. 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 63587^{T} were Gram-positive-staining, aerobic, non-motile and non-endospore-forming. The isolate showed good growth on ISP media 2–5, nutrient agar and potato-glucose agar and moderate growth on Czapek's agar. On most media, the isolate formed white aerial mycelia and orange–yellow or brown substrate mycelia (Table 1). The isolate formed an extensively branched substrate mycelium and aerial hyphae that carried smooth-surfaced rod-shaped spores (Fig. 2). The isolate grew at 10–37 °C, at pH 5–9 and with 0–3 % (w/v) NaCl. Optimal growth was observed at 20–28 °C and pH 6–8. The isolate was catalase-positive and oxidase-negative. Detailed physiological and biochemical properties are given in Table 2 and the species description. There were some phenotypic differences between strain YIM 63587^{T} and *P. saturnea* IMSNU 20052^{T}.

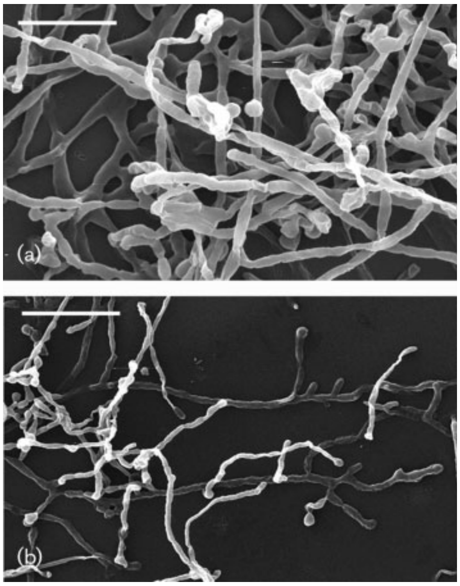

Fig. 2. Scanning electron micrographs of strain YIM 63587^{T} grown on YIM 38 medium for 2 weeks at 28 °C. Bars, 5 μm (a) and 10 μm (b).

Amino acid and sugar analysis of whole-cell hydrolysates was performed according to the procedures described by Hasegawa *et al.* (1983), Lechevalier & Lechevalier (1970)

Table 1. Cultural characteristics of strain YIM 63587^{T}

Colours are according to the Inter-Society Color Council – National Bureau of Standards colour charts (standard sample no. 2106; Kelly, 1964). −, No growth. No soluble pigment was produced on any of the media tested.

Agar medium	Colour of mycelium	
	Aerial	Substrate
Czapek's	White	Orange–yellow
Potato-glucose	White	Brown
Nutrient	−	Orange–yellow
Yeast extract-malt extract (ISP 2)	−	Orange–yellow
Oatmeal (ISP 3)	Yellow–white	Brown
Inorganic salt-starch (ISP 4)	White	Orange–yellow
Glycerol asparagine (ISP 5)	White	Orange–yellow

Table 2. Differential characteristics of strain YIM 63587^{T} and closely related type strains of species of the genus *Pseudonocardia*

Strains: 1, YIM 63587^{T}; 2, *P. saturnea* IMSNU 20052^{T}; 3, *P. babensis* NBRC 105793^{T}; 4, *P. asaccharolytica* DSM 44247^{T}; 5, *P. acaciae* GMKU 095^{T}. Data for columns 1 and 2 were taken from this study and for columns 3–5 from Park *et al.* (2008), Duangmal *et al.* (2009) and Sakiyama *et al.* (2010). +, Positive; w, weakly positive; −, negative; ND, no data available.

Characteristic	1	2	3	4	5
Growth at/with:					
45 °C	−	+	+	−	−
5 % (w/v) NaCl	−	+	−	−	w
Acid produced from:					
L-Arabinose	−	+	+	−	+
Lactose	+	−	−	−	−
Sucrose	−	+	+	−	−
D-Xylose	−	+	+	−	+
Hydrolysis of:					
Gelatin	−	+	ND	−	+
Tween 80	−	+	ND	ND	ND
H_2S production	−	+	ND	−	−
Urease	−	+	−	−	+

and Tang *et al.* (2009). Phospholipids were extracted, examined by two-dimensional TLC and identified using described procedures (Minnikin *et al.*, 1979; Collins & Jones, 1980). Menaquinones were isolated according to Collins *et al.* (1977) and separated by HPLC (Tamaoka *et al.*, 1983). Cellular fatty acid analysis was performed using the Sherlock Microbial Identification System (MIDI), according to the manufacturer's instructions.

The results indicated that the cell-wall diamino acid in the peptidoglycan layer of strain YIM 63587^T was *meso*-diaminopimelic acid and the whole-cell sugars were arabinose, galactose, mannose and ribose. The phospholipids consisted of diphosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol mannosides, phosphatidylinositol and an unknown phospholipid. The menaquinones were represented by MK-8(H_4) (97.7 %) and MK-8(H_6) (2.3 %). The fatty acids were iso-$C_{16:0}$ (44.7 %), iso-$C_{14:0}$ (10.3 %), iso-$C_{16:1}$ H (9.8 %), iso-$C_{15:0}$ (7.7 %), $C_{16:0}$ 10-methyl (4.7 %) and $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH (4.7 %) and some others in lesser amounts. The profile of the new isolate was very similar to those described for recognized species of the genus *Pseudonocardia*; however, the profile was different from that of *P. saturnea* IMSNU 20052^T (Table 3). The chemotaxonomic characteristics of strain YIM 63587^T, such as the diamino acid and sugars of whole-cell hydrolysates, menaquinones, major fatty acids and phospholipids, were consistent with its assignment to the genus *Pseudonocardia*.

The phenotypic and chemotaxonomic data, together with the 16S rRNA gene sequence data, provide sufficient evidence to support the proposal that strain YIM 63587^T represents a novel species of the genus *Pseudonocardia*, for which the name *Pseudonocardia artemisiae* sp. nov. is proposed.

Table 3. Fatty acid profiles of strain YIM 63587^T and *P. saturnea* IMSNU 20052^T

Fatty acid (%)	*P. artemisiae* sp. nov. YIM 63587^T	*P. saturnea* IMSNU 20052^T
$C_{14:0}$	0.52	0.83
$C_{15:0}$	1.98	1.01
$C_{16:0}$	2.01	5.24
$C_{15:1}\omega 6c$	2.40	–
$C_{17:1}\omega 8c$	2.07	–
iso-$C_{14:0}$	10.31	1.92
iso-$C_{15:0}$	7.68	27.47
iso-$C_{16:0}$	44.67	8.73
iso-$C_{17:0}$	1.82	10.81
iso-$C_{16:1}$ H	9.82	1.17
anteiso-$C_{15:0}$	0.56	29.33
anteiso-$C_{17:0}$	2.40	6.99
$C_{16:0}$ 10-methyl	4.72	–
$C_{17:0}$ 10-methyl	1.54	0.40
Summed feature 3*	4.67	–

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH.

Description of *Pseudonocardia artemisiae* sp. nov.

Pseudonocardia artemisiae (ar.te.mi′si.ae. L. n. *artemisia* mugwort, also a plant genus; L. gen. n. *artemisiae* of *Artemisia*, isolated from *Artemisia annua* L.).

Aerobic, non-motile, non-endospore-forming, Gram-positive-staining actinomycete that forms extensively branched substrate mycelium and aerial mycelium, which carry smooth-surfaced rod-shaped spores. Forms white aerial mycelia and orange–yellow or brown substrate mycelia on the media tested. No pigment is produced. Grows at 10–37 °C (optimum 20–28 °C), at pH 5–9 (optimum pH 6–8) and with 0–3 % NaCl (optimum 1 % NaCl). Positive for catalase, milk coagulation and milk peptonization, but negative for nitrate reduction, oxidase, urease, gelatin liquefaction, cellulose and starch hydrolysis and H_2S production. As sole carbon sources, utilizes cellobiose, D-fructose, D-galactose, *myo*-inositol, lactose, maltose, D-mannitol, D-mannose and L-rhamnose, but not glucose, glycerol, raffinose, sodium acetate, D-sorbitol or sucrose. As sole nitrogen sources, utilizes L-alanine, L-arginine, L-asparagine, L-hydroxyproline, hypoxanthine, L-phenylalanine, L-serine, L-tyrosine, L-valine and xanthine, but not glycine or L-lysine. Acid is produced from D-fructose, lactose, mannitol and maltose. The cell wall of strain YIM 63587^T contains *meso*-diaminopimelic acid and the whole-cell sugars are arabinose, galactose, mannose and ribose (cell wall type IV). The predominant menaquinone is MK-8(H_4). The phospholipids are diphosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol mannosides, phosphatidylinositol and an unknown phospholipid (phospholipid type PIII). The major fatty acids are iso-$C_{16:0}$, iso-$C_{14:0}$, iso-$C_{16:1}$ H, iso-$C_{15:0}$, $C_{16:0}$ 10-methyl and $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH.

The type strain, YIM 63587^T (=DSM 45313^T=CCTCC AA 208081^T), was isolated from surface-sterilized roots of *Artemisia annua* L., collected from Yunnan province, south-west China. The DNA G+C content of the type strain is 68.2 mol%.

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