Pseudonocardia bannaensis *sp. nov., a novel actinomycete isolated from the surface-sterilized roots of* Artemisia annua *L.*

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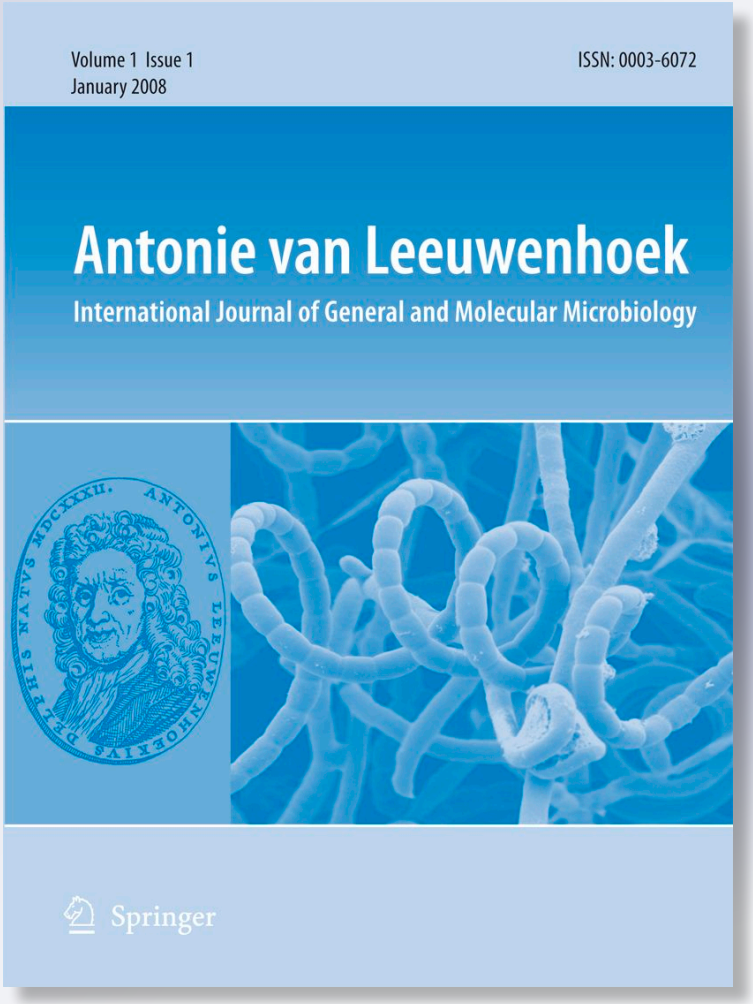

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ORIGINAL PAPER

Pseudonocardia bannaensis sp. nov., a novel actinomycete isolated from the surface-sterilized roots of *Artemisia annua* L.

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Abstract During the course of our research on new actinobacterial sources, a novel actinomycete strain YIM 63101^T was isolated from the surface-sterilized roots of *Artemisia annua* L. collected from Xishuangbanna, Yunnan province, south-west China and characterized by using a polyphasic approach. The strain formed well-differentiated aerial and substrate mycelia. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain YIM 63101^T belongs to the genus *Pseudonocardia*, with highest similarity to "*Pseudonocardia artemisiae* YIM 63587^T" (99.4%). Sequence similarities between strain YIM 63101^T and the other *Pseudonocardia* species ranged from 97.0 (*Pseudonocardia saturnea* IMSNU 20052^T) to 94.0% (*Pseudonocardia compacta* IMSNU 20111^T). The chemotaxonomic characteristics, such as cell wall diaminopimelic acid, whole-cell sugars, fatty acid components and the major menaquinones suggested that the organism belonged to the genus *Pseudonocardia*. The G + C content of the genomic DNA was 69.4 mol%. Based on comparative analysis of physiological, biochemical and chemotaxonomic data, including low DNA–DNA hybridization results, it is proposed that strain YIM 63101^T represents a novel species of the genus *Pseudonocardia*, named *Pseudonocardia bannaensis* sp. nov. The type strain is YIM 63101^T (= CCTCC AA 208077 ^{T} = DSM 45300^T).

Keywords *Pseudonocardia bannaensis* sp. nov. · 16S rRNA · Endophyte

Guo-Zhen Zhao and Jie Li contributed equally to this work.

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Introduction

Endophytic bacteria can be defined as those bacteria that colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host (Holliday 1989; Schulz and Boyle 2006). There are more than 300 000 plant species on the earth, however only a few of these plants have ever been studied completely relative to their endophytic

biology (Strobel et al. 2004). Consequently, the opportunity to find novel and beneficial endophytic micro-organisms among the diversity of plants in different ecosystems is considerable (Ryan et al. 2008). Recent studies have indicated that endophytic bacteria are common among the resident microflora of the healthy inner tissues of various species of plants (Coombs and Franco 2003; Gu et al. 2006; Trujillo et al. 2006; Tian et al. 2007), in which these micro-organisms play an important role in plant growth.

The genus *Pseudonocardia* within the family *Pseudonocardiaceae* was first described by Henssen (1957), and since then the description of the genus has been revised repeatedly (Warwick et al. 1994; McVeigh et al. 1994; Reichert et al. 1998; Huang et al. 2002; Park et al. 2008). Members of the genus *Pseudonocardia* are composed of vegetative and aerial mycelium with spore chains produced by acropetal budding or fragmentation, type IV cell wall, major menaquinone is MK-8(H_4) or MK-9, DNA G + C content of 68–79 mol%. At the time of writing, the genus *Pseudonocardia* encompasses 41 species with validly published names (http://www.bacterio.cict.fr/; Ara et al. 2010; Kaewkla and Franco 2010a, b; Li et al. 2010; Qin et al. 2010a, b; Sakiyama et al. 2010; Zhao et al. 2010a, b).

In the course of our research on new actinobacterial sources, we obtained a novel isolate YIM 63101^T. Based on phenotypic and genotypic evidence, it is proposed that this isolate represents a novel species of the genus *Pseudonocardia*, for which the name *Pseudonocardia bannaensis* sp. nov., is proposed.

Materials and methods

Strain and culture conditions

Strain YIM 63101^T was isolated from the roots of *Artemisia annua* L., collected in Xishuangbanna, Yunnan province, south-west China. Samples were washed in running water to remove soil particles and sterilized by the established procedure (Li et al. 2008). After being surface-sterilized, the root samples were sliced into pieces, followed by plating on HV agar plates (Hayakawa and Nonomura 1987). The plates were incubated at 28°C for 4–6 weeks until the outgrowth of endophytic actinomycetes was discerned. Colonies originating from plant segments were selected and pure cultures were obtained by repeated streaking on TWYE plates (containing 0.25 g of yeast extract, 0.5 g of K_2HPO_4 and 18 g of agar per litre of tap water, pH 7.2). The purified strain YIM 63101^T was maintained on tryptic soy agar (TSA, containing 15 g of tryptone, 5 g of soya peptone, 5 g of NaCl and 15 g of agar, per litre of tap water pH 7.2) slants at 4°C and as 20% (v/v) glycerol suspensions at −80°C. Strain YIM 63101^T was deposited in the Collection Center of Typical Cultures, China (CCTCC) as strain CCTCC AA 208077^T and in the German Collection of Microorganisms and Cell Cultures (DSMZ) as strain DSM 45300^T.

Biomass for chemical and molecular studies was obtained by cultivation in shaken flasks (about 200 rpm) using tryptic soy broth (TSB, containing 15 g of tryptone, 5 g of soya peptone and 5 g of NaCl per litre of tap water pH 7.2) medium at 28°C for one week.

Morphological, cultural, physiological and biochemical characteristics

Morphological, cultural, physiological and biochemical characterization of the strain YIM 63101^T were studied by following the guidelines of the International *Streptomyces* Project (Shirling and Gottlieb 1966). The morphological characteristics of strain YIM 63101^T, including spore-chain morphology, spore size and surface ornamentation, were assessed by light and scanning electron microscopy (Philips XL30; ESEM-TMP) of 14 day-old cultures on ISP 2 medium. Aerial spore-mass colour, substrate mycelium pigmentation and coloration of the diffusible pigments of strain YIM 63101^T were recorded on ISP media (International *Streptomyces* Project), Czapek's agar, potato-glucose agar and nutrient agar prepared as described by Dong and Cai (2001). Colours were determined by using the colour chips from the ISCC–NBS colour charts (standard samples, no. 2106) (Kelly 1964). Growth at different temperatures (4, 10, 20, 28, 37, 42, 45, 50 and 55°C) was tested on TSA plates by incubating the cultures for 21 days. The pH range for growth (pH 4, 5, 6, 7, 8, 9 and 10, using the buffer system described by Xu et al. 2005) and NaCl tolerance (0, 1, 2, 3, 4, 5, 6, 7, 10, 15 and 20% w/v)

were tested at 28°C for 14–21 days by culturing the strains in TSB medium. Catalase activity, oxidase and gelatinase activities, starch hydrolysis, nitrate reduction and urease were assessed as described by Smibert and Krieg (1994). Media and procedures used for the determination of other physiological characteristics, carbon source utilization and acid production from carbohydrates were those described by Gordon et al. (1974).

Chemotaxonomy

The isomer of diaminopimelic acid and sugar analysis of whole-cell hydrolysates were performed according to the procedures described by Hasegawa et al. (1983); Lechevalier and Lechevalier (1970) and Tang et al. (2009). Phospholipids were extracted, examined by two-dimensional TLC and identified using previously described procedures (Minnikin et al. 1979; Collins and Jones 1980). Menaquinones were isolated according to Collins et al. (1977) and separated by HPLC (Tamaoka et al. 1983). Mycolic acids were extracted and analyzed by one-dimensional TLC described by Minnikin et al. (1980). Cellular fatty acids were extracted, methylated and analysed by using the Sherlock Microbial Identification System (MIDI) according to the manufacturer's instructions. The fatty acid methyl esters were analysed by using the Microbial Identification software package (Sherlock Version 4.0; MIDI database: TSBA40).The G + C content of the genomic DNA was determined by using the HPLC method (Mesbah et al. 1989) with *E. coli* JM-109 as the reference strain.

Molecular analysis

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were performed as described by Li et al. (2007). The 16S rRNA gene sequence of strain YIM 63101^T was compared against a database of cultured species via BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the EzTaxon Database (http://www.eztaxon.org, Chun et al. 2007) of type strains in order to retrieve most similar sequences of recognized bacteria. Multiple alignments with sequences of the most closely related actinobacteria and calculations of levels of sequence similarity were carried out using CLUSTAL_X (Thompson et al. 1997). The phylogenetic trees were constructed by the neighbour-joining (Saitou and Nei 1987), maximum-parsimony (Fitch 1971) and maximum-likelihood (Felsenstein 1981) tree-making algorithms by using the software packages MEGA version 4.0 (Tamura et al. 2007), PHYLIP version 3.6 (Felsenstein 2002) and PHYML (Guindon and Gascuel 2003). The topologies of the phylogenetic trees were evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. The strain *Kutneria kofuensis* NRRL B-24061^T (AF114801) was used as the outgroup. DNA–DNA relatedness was studied according to the fluorometric micro-well method (Ezaki et al. 1989; Christensen et al. 2000; He et al. 2005), and the hybridizations were performed with six replications.

The 16S rRNA gene sequence of strain YIM 63101^T has been deposited in GenBank under the accession number FJ817375.

Results and discussion

The cells of the novel strain YIM 63101^T were Gram-positive, aerobic and non-motile. Morphological observation of the 14 day-old culture of the strain YIM 63101^T revealed that both aerial and vegetative hyphae were abundant and well developed. The mycelia produced long spore chains, which containing more than 20 spores per chain. The spores were rod-shaped, smooth and around 0.5–1.0 × 0.5–1.5 μm in size (Fig. S1 in supplementary material). Strain YIM 63101^T grew well on ISP 2, ISP 3, ISP 4, ISP 5 and potato-glucose agar media. It exhibited moderate growth on Czapek's agar and nutrient agar media. Abundant white or yellow-pink aerial mycelium was produced on ISP 2, ISP 3, ISP 4, ISP 5 and Czapek's agar media, but sparse white or gray aerial mycelia were formed with cultivation on nutrient agar and potato-glucose agar media. The substrate mycelium differs from orange yellow/yellow to brown on the media tested and diffusible pigments were absent on these media (Table S1a in supplementary material). Growth was observed at 10–42°C, 0–4% NaCl (w/v) and pH 4.0–9.0. The optimal growth pH, temperature and NaCl concentration were pH 7.0–8.0, 20–28°C and 1%, respectively. Strain YIM 63101^T was nutritionally versatile, utilizing a wide range of substrates such as L-arabinose, D-cellobiose, D-fructose, glucose, glycerol,

myo-inositol, maltose, D-mannitol, D-mannose, L-rhamnose, D-sorbitol, D-xylose, L-alanine, L-arginine, L-asparagine, L-hydroxyproline, hypoxanthine, L-phenylalanine, L-serine, L-tyrosine and L-valine, whereas dulcitol, D-galactose, lactose, D-raffinose, D-ribose, sodium acetate, sucrose, glycine, L-lysine and xanthine were not utilized. Acid was produced from L-arabinose, D-fructose, glucose, maltose and D-mannitol. Catalase activity, hydrolysis of Tweens 20 and 40, milk coagulation and milk peptonization were positive, while hydrogen sulfide production and activities of urease, nitrate reduction, oxidase, gelatin liquefaction, cellulose and starch hydrolysis were negative. The other physiological and biochemical properties are given in the Table 1 and the species description. Several test results were obtained that enable the differentiation of strain YIM 63101^T from the most closely related *Pseudonocardia* species (Table 1).

The cell wall diamino acid in the peptidoglycan layer of strain YIM 63101^T was *meso*-diaminopimelic acid (*meso*-DAP) and the whole-cell sugars were arabinose, glucose, galactose and mannose. The phospholipid profile comprised diphosphatidyl glycerol, phosphatidylethanolamine, phosphatidyl methyl ethanolamine, phosphatidylcholine, phosphatidylinositol and phosphatidylinositol mannosides. The predominant menaquinone was MK-8(H_4) (96.9%), minor component was also detected as MK-8(H_6) (3.1%). Mycolic acids were absent. The major fatty acids were iso-$C_{16:0}$ (46.3%), $C_{16:1}$ iso H (11.7%), iso-$C_{15:0}$ (7.4%), $C_{16:1}\omega7c/_{15:0}$ iso 2-OH (6.9%), iso-$C_{14:0}$ (6.0%), $C_{16:0}$ 10-methyl (5.3%), anteiso-$C_{17:0}$ (2.4%), $C_{16:0}$ (2.3%), $C_{17:1}$ $\omega8c$ (2.2%), iso-$C_{17:0}$ (1.8%), $C_{17:0}$ 10-methyl (1.4%) and $C_{15:1}$ $\omega6c$ (1.2%). The complete fatty acid compositions of the novel strain YIM 63101^T and "*P. artemisiae* YIM 63587^T" are detailed in Table S2 in supplementary material. The fatty acid profiles of strain YIM 63101^T were similar to those of "*P. artemisiae* YIM 63587^T" but differed based on the presence or proportions of $C_{15:0,}$ $C_{18:0,}$ $C_{15:1}\omega6c$ and iso-$C_{14:0}$. The G + C content of the DNA of strain YIM 63101^T was 69.4 mol%, which is in accordance with the values for the genus *Pseudonocardia* (68–79 mol%). The chemotaxonomic characteristics of strain YIM 63101^T, such as amino acid and sugar analysis of whole-cell hydrolysates, menaquinones, major fatty acids and phospholipids were consistent with its assignment to the genus *Pseudonocardia*.

The almost complete 16S rRNA gene sequence of strain YIM 63101^T (1422 bp) was determined in this study. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain YIM 63101^T should be assigned to the genus *Pseudonocardia*. A phylogenetic tree was constructed based on 16S rRNA gene sequences to show the comparative relationship between strain YIM 63101^T and other related *Pseudonocardia* species (Fig. 1). The comparative analysis of 16S rRNA gene sequence and phylogenetic relationships showed that strain YIM 63101^T lies in a subclade in the tree with "*P. artemisiae* YIM 63587^T", with which it shares a 16S rRNA gene sequence similarity of 99.4%. Levels of the 16S rRNA gene sequence similarity between strain YIM 63101^T and the other *Pseudonocardia* species were less than 97%, ranged from 97.0% [*P. saturnea* IMSNU 20052^T (AJ252829)] to 94.0% [*P. compacta* IMSNU 20111^T (AJ252825)]. In the phylogenetic trees (Fig. 1, Figs. S2, S3 and S4 in Supplementary material), the affiliation between strain YIM 63101^T and its closest neighbour, "*P. artemisiae* YIM 63587^T", was supported with high bootstrap values of 100, 99, 100 and 100% in the neighbour-joining, maximum-parsimony and maximum-likelihood (using PHYLIP version 3.6 and PHYML) trees, respectively. DNA–DNA hybridization between strain YIM 63101^T and "*P. artemisiae* YIM 63587^T" was carried out by applying the fluorometric micro-well method under optimal hybridization conditions. The determined DNA–DNA relatedness value of 58.0 ± 2.0%, which is well below the 70% cut-off point for recognition of genomic species (Stackebrandt and Goebel 1994), thus suggesting that the strain YIM 63101^T should be considered as a different genomic species of the genus *Pseudonocardia.*

The combination of phylogenetic, chemotaxonomic and phenotypic data indicated that strain YIM 63101^T was a member of the genus *Pseudonocardia*. However, it could be differentiated readily from "*P. artemisiae* YIM 63587^T" based on several phenotypic characteristics (Table 1): the ability to grow at 42°C; a different pH range for growth (i.e. 4.0–9.0 and 5.0–9.0 for strain YIM 63101^T and "*P. artemisiae* YIM 63587^T", respectively) and utilization of L-arabinose, glucose, glycerol, D-sorbitol and D-xylose; acid is produced from L-arabinose and glucose. Strain YIM 63101^T produced white aerial mycelium on ISP 2 and nutrient agar, but "*P. artemisiae* YIM 63587^T" failed

Table 1 Different characteristics of strain YIM 63101^T and its closely related *Pseudonocardia* species, "*P. artemisiae* YIM 63587^T"

Characteristic	1	2
Utilization of:		
L-arabinose	+	−
Dulcitol	−	+
D-galactose	−	+
Glucose	+	−
Glycerol	+	−
Lactose	−	+
D-sorbitol	+	−
D-xylose	+	−
Xanthine	−	+
Acid produced from:		
L-arabinose	+	−
Glucose	+	−
Lactose	−	+
Hydrolysis of Tween 40	+	−
Growth at/on:		
42 °C	+	−
pH 4.0	+	−
G + C content (mol%)	69.4	68.2
Diagnostic sugars	Arabinose, galactose glucose and mannose	Arabinose, galactose, mannose and ribose
Fatty acids	iso-$C_{16:0}$ (46.27), $C_{16:1}$ iso H (11.74), iso-$C_{15:0}$ (7.37), iso-$C_{14:0}$ (6.09)	iso-$C_{16:0}$ (44.67), iso-$C_{14:0}$ (10.31), $C_{16:1}$ iso H(9.82), iso-$C_{15:0}$ (7.68)

Strains 1, Strain YIM 63101^T, *2* "*P. artemisiae* YIM 63587^T". Both strains were able to utilize D-cellobiose, D-fructose, *myo*-inositol, maltose, D-mannitol, D-mannose and L-rhamnose as sole carbon sources, but not D-raffinose, sodium acetate or sucrose as sole carbon sources. Acid was produced from D-fructose, maltose and D-mannitol. The strains could grow at 3% (w/v) NaCl, pH between 5.0 and 9.0 and temperatures between 10 and 37°C

Note: + Positive or present, − negative or absent

to produce aerial mycelium on these media; strain YIM 63101^T produced gray aerial mycelium and yellow substrate mycelium on potato-glucose agar, which is different from "*P. artemisiae* YIM 63587^T" (Table S1a, b in supplementary material). Based on the phenotypic and genotypic differences between strain YIM 63101^T and its closest relative, strain YIM 63101^T represents a novel species of the genus *Pseudonocardia*, for which the name *Pseudonocardia bannaensis* sp. nov. is proposed.

Description of *Pseudonocardia bannaensis* sp. nov.

Pseudonocardia bannaensis (ban.na.en′sis. N.L. adj. bannaensis of Banna, referring to the geographical origin of the strain).

Aerobic, non-motile, Gram-positive actinomycete that forms extensively branched substrate mycelia and aerial mycelia which carried smooth-surfaced rod-shaped spores (0.5–1.0 × 0.5–1.5 μm in size). Forms white or yellow-pink or gray aerial mycelia and orange-yellow/yellow or brown substrate mycelia on media tested. Temperature range for growth is 10–42°C, with optimal growth occurring at 20–28°C. The pH range for growth is 4.0–9.0 (optimum, pH 7.0–8.0). The NaCl concentration range for growth is 0–4% (optimum, 1%; w/v). Tween 20 and 40 are degraded, but not Tween 80. 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. Utilizes L-arabinose, D-cellobiose, D-fructose, glucose, glycerol, *myo*-inositol, maltose, D-mannitol, D-mannose, L-rhamnose, D-sorbitol and D-xylose as sole carbon sources. Dulcitol, D-galactose, lactose, D-raffinose, D-ribose, sodium acetate and sucrose are not utilized. Acid is produced from L-arabinose, D-fructose, glucose, maltose and D-mannitol. L-alanine, L-arginine, L-asparagine, L-hydroxyproline, hypoxanthine, L-phenylalanine, L-serine, L-tyrosine and

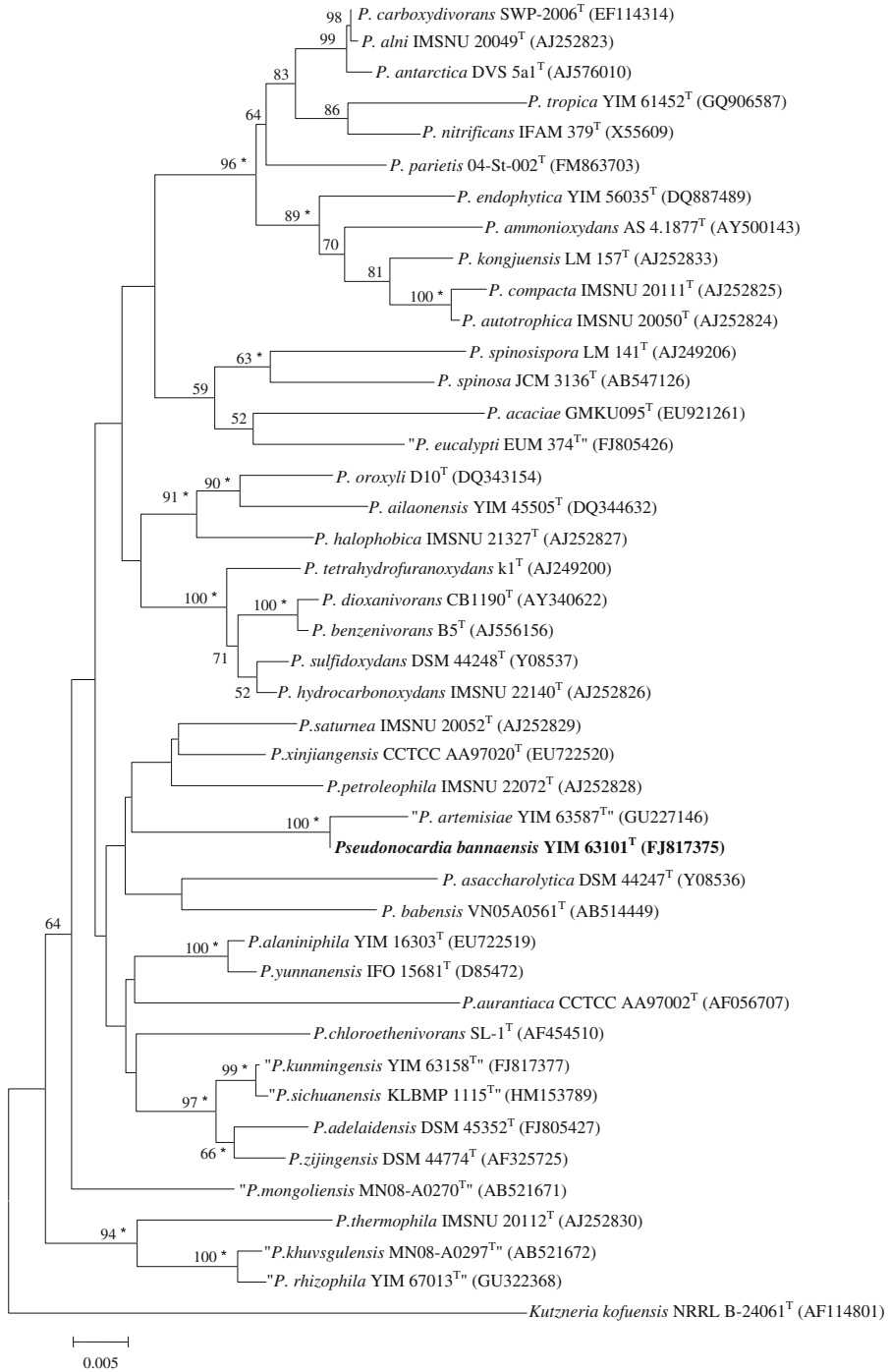

Fig. 1 Phylogenetic relationships of strain YIM 63101^T and other closely related *Pseudonocardia* species based on 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method. *Asterisks* indicate branches that were also recovered using the maximum-parsimony and maximum-likelihood methods. Bootstrap values (expressed as percentages of 1000 replications) of above 50% are shown at branch points. *Bar* 0.005 substitutions per nucleotide position

L-valine can be used as sole nitrogen sources, but not glycine, L-lysine and xanthine. The cell wall of strain YIM 63101^T contains *meso*-DAP. The whole-cell sugar pattern consists of arabinose, glucose, galactose and mannose. MK-8(H_4) is the predominant menaquinone. Mycolic acids are absent. The phospholipids consist of phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylinositol mannosides (PIM), phosphatidylmethylethanolamine (PME) and diphosphatidylglycerol (DPG) (type P III phospholipid). The major fatty acids are iso-$C_{16:0}$ (46.3%), $C_{16:1}$ iso H (11.7%), iso-$C_{15:0}$ (7.4%), $C_{16:1}\omega7c/_{15:0}$ iso 2-OH (6.9%), iso-$C_{14:0}$ (6.0%), $C_{16:0}$ 10-methyl (5.3%), anteiso-$C_{17:0}$ (2.4%), $C_{16:0}$ (2.3%), $C_{17:1}$ $\omega8c$ (2.2%), iso-$C_{17:0}$ (1.8%), $C_{17:0}$ 10-methyl (1.4%) and $C_{15:1}$ $\omega6c$ (1.2%). The G + C content of genomic DNA is 69.4 mol%.

The type strain, YIM 63101^T (= CCTCC AA 208077^T = DSM 45300^T) was isolated from the roots of *Artemisia annua* L., collected in Xishuangbanna, Yunnan province, south-west China.

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