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ORIGINAL ARTICLE

Pseudonocardia antimicrobica sp. nov., a novel endophytic actinomycete associated with *Artemisia annua* L. (sweet wormwood)

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A Gram-reaction-positive, non-motile, endophytic actinomycete, designated strain YIM 63235^T, was isolated from the surface-sterilized stems of *Artemisia annua* L., and characterized to determine its taxonomic position. The strain YIM 63235^T formed well-differentiated aerial and substrate mycelia on media tested. The phylogenetic tree based on 16S rRNA gene sequences showed that the new isolate formed a distinct lineage within the genus *Pseudonocardia*, and the strain YIM 63235^T was closely related to *Pseudonocardia parietis* 04-St-002^T (99.1%). However, DNA–DNA relatedness demonstrated that strain YIM 63235^T was distinct from the closest phylogenetic neighbor. The chemotaxonomic properties of strain YIM 63235^T were consistent with those of the genus *Pseudonocardia*: the diagnostic diamino acid of the cell-wall peptidoglycan was *meso*-diaminopimelic acid and MK-8(H_4) was the predominant menaquinone. The major fatty acids were iso-$C_{16:0}$ and iso-$C_{16:1}$ H. The DNA G + C content of strain YIM 63235^T was 71.0 mol%. On the basis of the phenotypic and phylogenetic distinctiveness, the novel isolate was identified as representing a novel species of the genus *Pseudonocardia*, for which the name *Pseudonocardia antimicrobica* sp. nov. (type strain YIM 63235^T = CCTCC AA 208080^T = DSM 45303^T) is proposed.
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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. There are more than 300 000 plant species on the earth, and each individual plant is host to one or more endophytes.[1] However, only a few of these plants have ever been studied completely relative to their endophytic biology. Consequently, the opportunity to find novel and beneficial endophytic micro-organisms among the diversity of plants in different ecosystems is considerable.[2] As part of our long-term study on endophytic actinomcete diversity and bioactive metabolites isolated from tropical rainforest medicinal plants of Xishuangbanna, several novel species have been characterized: *Dietzia schimae* and *Dietzia cercidiphylli*,[3] *Plantactinospora mayteni*,[4] *Pseudonocardia artemisiae*[5] and *Streptomyces artemisiae*.[6] In this report, the description of the morphological, physiological, chemotaxonomic and phylogenetic characteristics of a *Pseudonocardia*-like strain YIM 63235^T is presented. Phenotypic and genotypic data show that the isolate YIM 63235^T represents a novel species of the genus *Pseudonocardia*, for which the name *Pseudonocardia antimicrobica* sp. nov. is proposed.

The genus *Pseudonocardia* within the family *Pseudonocardiaceae* was first described by Henssen,[7] and since then the description of the genus has been revised repeatedly.[8–12] Members of the genus *Pseudonocardia* displayed 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 and a DNA G + C content of 68–79 mol%. Members of the genus *Pseudonocardia* have been widely reported and recovered from several ecosystems, such as active sludge soil (including those polluted by industrial chemicals) and plant samples (including stems, leaves, root nodules, tree-bark compost and traditional Chinese medicinal plants). At the time of writing, the genus *Pseudonocardia* encompasses 46 species with validly published names.[13,14]

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MATERIALS AND METHODS

Strain and culture conditions

Stem samples of *Artemisia annua* L. (sweet wormwood) were collected from Xishuangbanna and Kunming City in Yunnan Province, between August 2006 and April 2007. The samples were maintained at 4 °C and transported to the laboratory for immediate analysis. Samples were washed in running water to remove soil particles and sterilized by the established procedure.[15] After being surface sterilized, the samples were sliced into pieces, followed by plating on tap water–yeast extract agar plates (containing 0.25 g of yeast extract, 0.5 g of K_2HPO_4 and 18 g of agar, per liter of tap water, pH 7.2) containing nalidixic acid (25 mg l^{-1}), nystatin (50 mg l^{-1}) and cycloheximide (50 mg l^{-1}) to repress growth of bacteria and fungi. The plates were incubated at 28 °C for 4–8 weeks until the outgrowth of endophytic actinomycetes were discerned. Colonies originating from plant segments were picked up and pure cultures were obtained by repeated streaking on tap water–yeast extract agar plates. The purified strain YIM 63235^T was picked and maintained on tryptic soy agar (containing 15 g of tryptone, 5 g of soya peptone, 5 g of NaCl and 15 g of agar, per liter of tap water, pH 7.2) slants at 4 °C and as 20% (w/v) glycerol suspensions at −80 °C.

Biomass for chemical and molecular studies was obtained by cultivation in shaken flasks (about 200 r.p.m.) using tryptic soy broth (containing 15 g of tryptone, 5 g of soya peptone and 5 g of NaCl, per liter of tap water, pH 7.2) medium at 28 °C for 1 week.

Phenotypic characterization

Morphological, cultural, physiological and biochemical characterization of the strain YIM 63235^T was studied by following the guidelines of the International *Streptomyces* Project.[16] The morphological characteristics were observed by light microscopy (BH2; Olympus, Japan) and scanning electron microscopy (Quanta 200; FEI, USA) using cultures grown on ISP 2 medium at 28 °C for 7–14 days. Cultural characteristics were recorded on ISP media (International *Streptomyces* Project), Czapek's agar, potato–glucose agar and nutrient agar prepared as described by Dong and Cai.[17] Cell motility, Gram staining, growth parameter (temperature range, pH range and NaCl tolerance), starch hydrolysis, nitrate reduction and oxidase activity were determined.[18] The antimicrobial activities of strain YIM 63235^T were investigated by using media containing *Aspergillus niger*, *Bacillus subtilis* and *Escherichia coli*.[19]

Chemotaxonomy

The isomer of diaminopimelic acid and sugar analysis of whole-cell hydrolysates were performed according to the procedures described by Hasegawa *et al.*,[20] Lechevalier and Lechevalier[21] and Tang *et al.*[22] Phospholipids were extracted, examined by two-dimensional thin layer chromatography and identified using previously described procedures.[23,24] Mycolic acids were extracted and analyzed by one-dimensional thin layer chromatography described by Minnikin *et al.*[25] Menaquinones were isolated according to Collins *et al.*[26] and separated by HPLC.[27] Cellular fatty acids were extracted, methylated and analyzed by using the Sherlock Microbial Identification System (MIDI) according to the manufacturer's instructions. The fatty acid methyl esters were analyzed by using the Microbial Identification software package (Sherlock Version 4.0; MIDI database: TSBA40, MIDI Company, USA). The G + C content of the genomic DNA was determined by using the HPLC method[28] 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.*[29] The phylogenetic neighbors were identified and pairwise16S rRNA gene sequence similarities were calculated using the EzTaxon-e server Database[30] (http://eztaxon-e.ezbiocloud.net/). The almost-complete 16S rRNA gene sequence determined in this study was aligned with reference sequences of the genus *Pseudonocardia* by using the CLUSTAL_X program.[31] The phylogenetic trees were constructed by the neighbor-joining,[32] maximum-parsimony,[33] minimum-evolution[34] and maximum-likelihood[35] tree-making algorithms by using the software packages MEGA version 4.0[36] and PHYML.[37] The topologies of the phylogenetic trees were evaluated by using the bootstrap resampling method of Felsenstein[38] 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,[39–41] and the hybridizations were performed with six replications.

The 16S rRNA gene sequence of strain YIM 63235^T has been deposited in GenBank under the accession number FJ817380.

RESULTS AND DISCUSSION

Strain YIM 63235^T formed visible colonies within 5 days on ISP 2 incubated at 28 °C. Good growth occurred at 20–28 °C, which formed extensively branched substrate mycelia (orange–yellow/yellow–brown) and aerial mycelia (white) (Supplementary Table S1). Morphological observation of 7 day-old cultures of strain YIM 63235^T revealed that both aerial and vegetative hyphae were abundant, well developed and fragmented into rod-shaped elements (Supplementary Figure S1). Yellowish-brown soluble pigment is produced on the potato–glucose agar. Cells were aerobic, non-motile, Gram-stain-positive and catalase-positive. Temperature range for growth is 10–42 °C, with optimal growth occurring at 20–28 °C. The pH range for growth is 5.0–9.0 (optimum, pH 7.0–8.0). The NaCl concentration range for growth is 0–10% (optimum, 0–5% NaCl, w/v). Other physiological and biochemical characteristics are summarized in the species description. Strain YIM 63235^T was differentiated from *Pseudonocardia parietis* 04-St-002^T by the oxidase reaction, degradation of Tween 20, Tween 80 and starch, utilization of D-cellobiose, lactose, D-raffinose, ribose, xylose, hypoxanthine and L-phenylalanine and the range of temperature/pH/NaCl tolerance of growth (Table 1). Strain YIM 63235^T produced a substance that inhibited the growth of *B. subtilis* and *E. coli*.

Table 1 Differential physiological characteristics of strains *P. antimicrobica* YIM 63235^T and *P. parietis* 04-St-002^T

Characteristic	*1*	*2*
Utilization as sole carbon source		
D-Cellobiose	+	−
Lactose	−	w
D-Raffinose	+	−
Ribose	+	w
Xylose	+	−
Utilization as sole nitrogen source		
Hypoxanthine	+	−
L-Phenylalanine	+	−
Hydrolysis of:		
Tween 20	+	−
Tween 80	+	−
Starch	−	+
Oxidase activity	−	+
Growth at/on		
42 °C	w	−
pH 5.0	w	−
pH 9.0	w	−
7% NaCl (w/v)	+	−
10% NaCl (w/v)	w	−

Characteristics are scored as follows: +, positive; w, weakly positive; −, negative.
Strains: 1, *P. antimicrobica* YIM 63235^T; 2, *P. parietis* 04-St-002^T. Both strains were Gram-positive, non-motile, catalase positive and grew under aerobic conditions. Both strains were able to utilize L-arabinose, D-fructose, D-galactose, glucose, maltose, D-mannitol, D-mannose, ribose, D-sorbitol and sucrose as the sole carbon sources.

Strain YIM 63235^T was examined for chemical markers considered to be characteristic of *Pseudonocardia* strains. Strain YIM 63235^T contained *meso*-diaminopimelic acid (*meso*-DAP) as the diagnostic diamino acid and the whole-cell hydrolysates were rich in glucose, arabinose, galactose and mannose (type IV cell wall). The quinone system of YIM 63235^T was composed of menaquinones MK-8(H_4) (94.5%) and MK-8(H_2) (5.5%). Mycolic acids were absent. The predominant polar lipids contained of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylinositol mannoside and four unknown polar lipids (type PIII phospholipid, Supplementary Figure S2). The fatty acid profile of strain YIM 63235^T contained saturated, unsaturated, 10-methyl and hydroxyl components, with the major fatty acids being iso-$C_{16:0}$ (60.9%), iso-$C_{16:1}$ H (22.7%) and 10-methyl-$C_{17:0}$ (2.3%). The detailed cellular fatty acid profiles of strain YIM 63235^T and *P. parietis* are given in Supplementary Table S2. Strain YIM 63235^T could be distinguished easily from the type strain of *P. parietis* 04-St-002^T based on the presence/absence and amount of $C_{16:0}$, $C_{18:0}$, $C_{18:1}\omega 9c$, iso-$C_{16:0}$, iso-$C_{16:1}$ H, $C_{16:0}$ 10-methyl, $C_{17:0}$ 10-methyl and $C_{16:1}\omega 7c$ and $C_{15:0}$ iso 2-OH. The DNA G + C content of strain YIM 65235^T was 71.0 mol%.

An almost-complete 16S rRNA gene sequence of strain YIM 63235^T (1399 nucleotide) was determined. Comparison of the sequence with those stored in GenBank indicated that strain YIM 63235^T was a member of the genus *Pseudonocardia*, with which it shared 94.8–99.1% 16S rRNA gene sequence similarity. Strain YIM 63235^T shared a 16S rRNA similarity of 99.1%, 97.9%, 97.9%, 97.9%, 97.8% and 97.8% with the type strains of *P. parietis* 04-St-002^T, *P. carboxydivorans* Y8^T, *P. tropica* YIM 61452^T, *P. ammonioxydans* H9^T, *P. antarctica* DVS 5a1^T and *P. alni* DSM 44104^T, respectively. Levels of the 16S rRNA gene sequence similarity between strain YIM 63235^T and the other *Pseudonocardia* species were <97.0%. The phylogenetic tree constructed with 16S rRNA gene sequence data by neighbor-joining method (Figure 1 and Supplementary Figure S3) showed that strain YIM 63235^T formed a monophyletic clade with *P. parietis* 04-St-002^T, and which was supported by the maximum-parsimony (Supplementary Figure S4), minimum-evolution (Supplementary Figure S5) and maximum-likelihood method (Supplementary Figure S6). However, strain YIM 63235^T did not form a cluster with strains *P. carboxydivorans* Y8^T, *P. tropica* YIM 61452^T, *P. ammonioxydans* H9^T, *P. antarctica* DVS 5a1^T and *P. alni* DSM 44104^T in any of the four tree-making algorithms.

DNA–DNA relatedness value between strain YIM 63235^T and the most closely type strain *P. parietis* 04-St-002^T was determined using the fluorometric micro-well method under optimal hybridization conditions. The DNA–DNA relatedness study was not carried out between strain YIM 63235^T and other phylogenetic relatives with 16S rRNA gene sequence similarities that were <98.0%. Strain YIM 63235^T exhibited relatively low levels of DNA–DNA relatedness with respect to *P. parietis* 04-St-002^T (32.3 ± 2.0%), which is well below the 70% cutoff point recommended for the assignment of bacterial strains to the same genomic species.[42] These data suggest that strain YIM 63235^T represent a novel species of the genus *Pseudonocardia*.

The phenotypic properties of strain YIM 63235^T and the 16S rRNA gene sequence comparison supported the classification of the isolate in the genus *Pseudonocardia*. Differentiating characteristics (Table 1), phylogenetic analysis of the 16S rRNA gene sequence and DNA–DNA relatedness distinguished strain YIM 63235^T from other members of the genus *Pseudonocardia*. Therefore, strain YIM 63235^T is proposed to represent a hitherto unrecognized species of the genus *Pseudonocardia*, with the name *Pseudonocardia antimicrobica* sp. nov.

Description of *Pseudonocardia antimicrobica* sp. nov.

Pseudonocardia antimicrobica (an.ti.mi.cro′bi.ca. Gr. prep. *anti* against; N.L. n. *microbium* microbe; L. adj. suff.-*cus* -a -um suffix used with various meanings; N.L.fem. adj. *antimicrobica* antimicrobial) is an aerobic, non-motile, Gram-positive actinomycete that forms extensively branched substrate mycelia (orange–yellow/yellow–brown) and aerial mycelia (white). It produces yellowish-brown soluble pigment on the potato–glucose agar. The temperature range for growth is 10–42 °C, with optimal growth occurring at 20–28 °C, and the pH range for growth is 5.0–9.0 (optimum, pH 7.0–8.0). The NaCl concentration range for growth is 0–10% (optimum, 0–5% NaCl, w/v). It is 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. Tweens 20, 40 and 80 are hydrolyzed by it and it utilizes L-arabinose, D-cellobiose, D-fructose, D-galactose, glucose, maltose, D-mannitol, D-mannose, D-raffinose, L-rhamnose, ribose, D-sorbitol, sucrose and xylose as the sole carbon sources, whereas Dulcitol, glycerol, lactose,

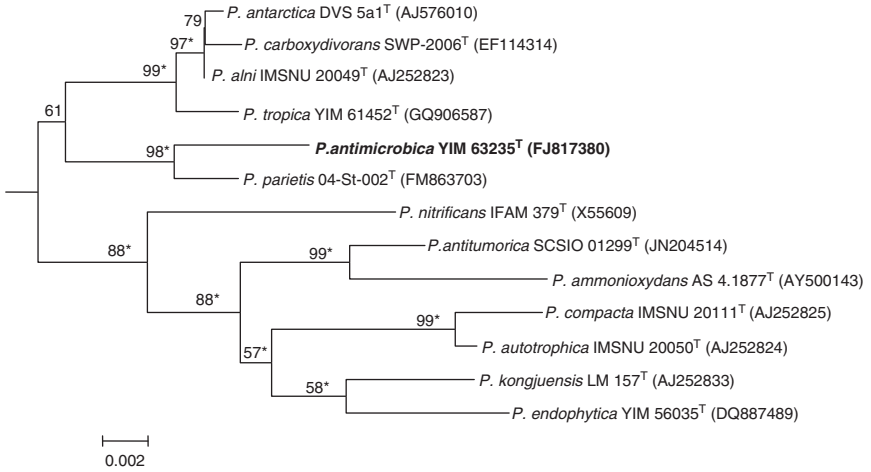

Figure 1 Neighbor-joining tree of *Pseudonocardia antimicrobica* YIM 63235^T sp. nov. and related species based on 16S rRNA gene sequences. Bar, 0.002 substitutions per nucleotide position. Asterisks indicate branches that were also recovered using the maximum-parsimony, minimum-evolution and maximum-likelihood methods.

myo-inositol and sodium acetate are not utilized. L-alanine, L-arginine, L-asparagine, glycine, L-hydroxyproline, hypoxanthine, L-phenylalanine, L-serine, L-tyrosine, L-valine and xanthine can be used as sole nitrogen sources, but not L-lysine. Acid is produced from D-galactose and glucose and it shows antimicrobial activities against *B. subtilis* and *E. coli*. The cell wall of strain YIM 63235^T contains *meso*-DAP. The whole-cell sugar pattern consists of glucose, arabinose, galactose and mannose (type IV cell wall). MK-8(H_4) is the predominant menaquinone. Mycolic acids are absent. The phospholipids consist of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylinositol mannoside and four unknown polar lipids (type PIII phospholipid). The major fatty acids are iso-$C_{16:0}$ (60.9%) and iso-$C_{16:1}$ H (22.7%). The G + C content of genomic DNA is 71.0 mol%.

The type strain, YIM 63235^T (= CCTCC AA 208080^T = DSM 45303^T), was isolated from surface-sterilized stems of *Artemisia annua* L. collected from Yunnan province, Southwest China.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)