Pseudonocardia tropica sp. nov., an endophytic actinomycete isolated from the stem of *Maytenus austroyunnanensis*

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A novel strain, designated YIM 61452^T, was isolated from the stem of *Maytenus austroyunnanensis* collected from the tropical rainforest of Xishuangbanna, Yunnan Province, south-west China. The strain was characterized taxonomically by using a polyphasic approach. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain YIM 61452^T belonged to the genus *Pseudonocardia* and was most closely related to *Pseudonocardia alni* IMSNU 20049^T (99.5%), *Pseudonocardia antarctica* DVS 5a1^T (99.5%) and *Pseudonocardia carboxydivorans* Y8^T (99.4%). The chemotaxonomic properties of strain YIM 61452^T, for example MK-8(H₄) as the predominant quinone system and iso-C_{16:0} and iso-C_{16:1} H as the major fatty acids, were also consistent with its classification within the genus *Pseudonocardia*. Strain YIM 61452^T could be differentiated from closely related species of the genus *Pseudonocardia* by phenotypic and genotypic analysis. DNA–DNA hybridization experiments between strain YIM 61452^T and closely related reference strains further confirmed that strain YIM 61452^T represented a novel taxon of the genus *Pseudonocardia*. Therefore, strain YIM 61452^T represents a novel species, for which the name *Pseudonocardia tropica* sp. nov. is proposed. The type strain is YIM 61452^T (=DSM 45199^T=CCTCC AA 208018^T).

The genus *Pseudonocardia* was first proposed by Henssen (1957) to accommodate nocardioform actinomycetes that have a type IV cell wall and lack mycolic acids. The genus description was later emended by Warwick *et al.* (1994), McVeigh *et al.* (1994), Reichert *et al.* (1998), Huang *et al.* (2002) and Park *et al.* (2008). At the time of writing, the genus comprises 31 species with validly published names. Recently, three species of this genus, *Pseudonocardia oroxyli* (Gu *et al.*, 2006), *Pseudonocardia endophytica* (Chen *et al.*, 2009) and *Pseudonocardia acaciae* (Duangmal *et al.*, 2009) have been isolated from surface-sterilized endophytic

environments of higher plants. During the course of our study into the culturable actinobacterial community of different tropical rainforest medicinal plants in Xishuangbanna, Yunnan Province, PR China, a large number of novel actinobacterial strains were isolated (Qin *et al.*, 2009). In this study, another isolate, strain YIM 61452^T, was characterized. Based on the results of the polyphasic taxonomic analysis, this strain is considered to represent a novel species of the genus *Pseudonocardia*.

Strain YIM 61452^T was isolated from healthy stems of a traditional Chinese medicinal plant *Maytenus austroyunnanensis*. The stem samples were air-dried at room temperature after having been thoroughly washed under tap water and surface-sterilized according to a five-step sterilization procedure (Qin *et al.*, 2008). The surface-sterilized samples were pulverized in a ceramic mortar and spread onto sodium propionate agar (Qin *et al.*, 2009).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 61452^T is GQ906587.

A supplementary table detailing the fatty acid contents of strain YIM 61452^T and related species of the genus *Pseudonocardia* and scanning electron micrographs of strain YIM 61452^T are available with the online version of this paper.

Strain YIM 61452^T was isolated after incubation at 28 °C for 3 weeks and was maintained subsequently as mycelial fragments in a 20 % (v/v) glycerol suspension at –80 °C.

Cultural and morphological characteristics were observed when the organism was grown on yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4) and glycerol-asparagine agar (ISP 5) (Shirling & Gottlieb, 1966), as well as potato dextrose agar (PDA), Czapek's agar and nutrient agar (Waksman, 1967) for 20 days at 28 °C. Colony colour was determined with the use of ISCC–NBS colour charts (Kelly, 1964). Cell morphological characteristics were observed by using light microscopy (BH 2; Olympus) and scanning electron microscopy (JSM5600LV; JEOL) after 3 weeks growth on ISP 2 medium. Determination of temperature (0, 4, 10, 15, 20, 28, 37, 40, 45 and 55 °C) and pH (pH 4.0–11.0 at intervals of 0.5 pH units) growth ranges and tolerance of NaCl (0–15 %, w/v) (at intervals of 0.5 %) were performed using ISP 2 as the basal medium. Other physiological and biochemical characteristics of strain YIM 61452^T were examined by using the standard procedures described by Gordon *et al.* (1974).

Strain YIM 61452^T showed good growth on ISP 2, 3 and 4, Czapek's and PDA media, and moderate growth on ISP 5 and nutrient agar. On most media, white aerial mycelia and

yellowish white to orange–yellow or brown substrate mycelia were formed. The aerial mycelium fragmented into rod-shaped spores. The spore surface was smooth (see Supplementary Fig. S1, available in IJSEM Online). Growth was observed at 15–37 °C, with 0–10 % NaCl (w/v) and at pH 6.0–9.0. The optimal growth pH, temperature and NaCl concentration were pH 7.0, 28 °C and 1 %, respectively. Detailed phenotypic characteristics are presented in the species description and in Table 1.

Amino acid and sugar analysis of whole-cell hydrolysates were performed according to the procedures described by Hasegawa *et al.* (1983). Polar lipids were extracted, examined using two-dimensional TLC and identified using published procedures (Minnikin *et al.*, 1979; Collins & Jones, 1980). Menaquinones were extracted and purified as described by Collins *et al.* (1977) and analysed by HPLC (Groth *et al.*, 1997). The fatty acids were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification system (Sasser, 1990; Kämpfer & Kroppenstedt, 1996).

The cell-wall diamino acid in the peptidoglycan layer of strain YIM 61452^T was *meso*-diaminopimelic acid and the whole-cell sugars were arabinose, galactose and glucose. The phospholipid profile comprised diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine,

Table 1. Characteristics that differentiate strain YIM 61452^T from closely related species of the genus *Pseudonocardia*

Strains: 1, YIM 61452^T; 2, *P. alni* IMSNU 20049^T; 3, *P. antarctica* DVS 5a1^T; 4, *P. carboxydivorans* Y8^T; 5, *P. parietis* 04-St-002^T. +, Positive or present; w, weakly positive; –, negative or absent; ND, not determined; PME, phosphatidylmonomethylethanolamine.

Characteristic	1	2	3	4	5*
Utilization of:					
Cellulose	w	+	+	+	–
D-Galactose	+	+	+	+	w
myo-Inositol	+	–	w	–	–
D-Mannitol	+	w	+	+	+
D-Mannose	+	+	+	+	w
L-Rhamnose	–	+	+	+	–
D-Ribose	+	+	w	+	–
Trehalose	+	+	+	+	–
D-Xylose	+	+	+	+	–
Hydrolysis of:					
Gelatin	–	+	+	–	ND
Starch	–	+	+	+	ND
Reduction of nitrate	+	+	+	–	ND
Oxidase activity	–	–	–	–	+
Growth at/on:					
10 °C	–	+	+	–	ND
37 °C	+	+	–	+	ND
5 % (w/v) NaCl	+	+	+	–	ND
Major menaquinone	MK-8(H ₄) (91.3 %)	MK-8(H ₄) (98.6 %)	MK-8(H ₄) (97.9 %)	MK-9*	MK-8(H ₄) (73 %)
Lipid PME	–	+	+	ND*	+
DNA G + C content (mol%)	72.4	71–74*	71*	77*	ND

*Data are taken from Prabakar *et al.* (2004), Park *et al.* (2008) and Schäfer *et al.* (2009). All other data are taken from this study.

phosphatidylglycerol, phosphatidylinositol, and phosphatidylinositol mannosides (phospholipid III; Lechevalier *et al.*, 1977). The predominant menaquinone was MK-8(H₄) (91.3%), minor components were also detected as MK-7(H₄) (5.5%) and MK-8(H₂) (3.2%). The major fatty acids were iso-C_{16:0} (33.9%), C_{16:1} iso H (27.3%), iso-C_{15:0} (8.7%), C_{16:0} 9 methyl (8.6%) and C_{16:1} *cis* 9 (6.6%). Detailed fatty acid data for the novel strain and related species of the genus *Pseudonocardia* are given in Supplementary Table S1 in IJSEM Online. The results of chemical analysis indicated that the new organism had chemotaxonomic markers typical of the genus *Pseudonocardia*.

Extraction of genomic DNA and amplification of the 16S rRNA gene were performed as described by Li *et al.* (2007). An almost full-length 16S rRNA gene sequence (1461 bp) was aligned with selected sequences obtained from the GenBank/EMBL/DBJ databases using the CLUSTAL_X 1.8 program (Thompson *et al.*, 1997). A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) using the MEGA version 3.1 software package (Kumar *et al.*, 2004). Distances between sequences were calculated using Kimura's two-parameter model (Kimura, 1980). Bootstrap values were determined according to the method of Felsenstein (1985) with 1000 bootstrap resamplings. The G+C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989). Levels of DNA–DNA,

relatedness were determined according to the fluorometric micro-well method (Ezaki *et al.*, 1989; He *et al.*, 2005).

The DNA G+C content of strain YIM 61452^T was 72.4 mol%. The phylogenetic analysis (Fig. 1) confirmed that this isolate was a member of the genus *Pseudonocardia* and it formed a distinct clade with *Pseudonocardia alni* IMSNU 20049^T (99.5% 16S rRNA gene sequence similarity), *Pseudonocardia antarctica* DVS 5a1^T (99.5%) and *Pseudonocardia carboxydivorans* Y8^T (99.4%) with a high bootstrap value. Strain YIM 61452^T showed a relatively lower sequence similarity, 98.0%, with *Pseudonocardia parietis* 04-St-002^T. Much lower sequence similarities were found with all other recognized species of the genus *Pseudonocardia*. To establish the precise taxonomic position of strain YIM 61452^T, DNA–DNA hybridization studies were performed. The DNA–DNA relatedness values between strain YIM 61452^T and *P. alni* IMSNU 20049^T, *P. antarctica* DVS 5a1^T and *P. carboxydivorans* Y8^T were 35, 51 and 42%, respectively. When the recommendation of a threshold value of 70% DNA–DNA relatedness for the definition of bacterial species (Stackebrandt & Goebel, 1994) is considered, these results suggest strongly that strain YIM 61452^T represents a novel species of the genus *Pseudonocardia*. It has been shown that some species 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

Fig. 1. Phylogenetic relationships of strain YIM 61452^T and other closely related species of the genus *Pseudonocardia* based on 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method. Bootstrap values (expressed as percentages of 1000 replications) >40% are shown at branch points. Asterisks indicate the clades that were conserved when neighbour-joining and maximum-parsimony methods were used to construct phylogenetic trees. Bar, 0.005 substitutions per nucleotide position.

et al., 2006; Park *et al.*, 2008). For example, *P. antarctica* DVS 5a1^T and *P. alni* IMSNU 20049^T share a 16S rRNA gene sequence similarity of 99.7 %, but have a DNA–DNA relatedness of 50 % (Prabahar *et al.*, 2004). Similarly, *P. parietis* 04-St-002^T showed 16S rRNA gene sequence similarities of 99.2, 99.1 and 99.1 % respectively to *P. antarctica* DVS 5a1^T, *P. alni* IMSNU 20049^T and *P. carboxydivorans* Y8^T, but shared relatively low DNA–DNA values of 30.5–44.5 % (Schäfer *et al.*, 2009). For this reason, DNA–DNA relatedness experiments between strain YIM 61452^T and *P. parietis* 04-St-002^T and other species were performed due to the relatively lower sequence similarities.

On the basis of morphological and physiological characteristics, fatty acid profile and DNA–DNA relatedness, strain YIM 61452^T can be considered to be a member of the genus *Pseudonocardia*. However, strain YIM 61452^T can be clearly differentiated from the most closely related strains by its menaquinone and DNA G + C contents, the absence of phosphatidylmonomethylethanolamine and other physiological properties. Thus, based on the phylogenetic evidence together with the phenotypic characteristics presented in this study, strain YIM 61452^T represents a novel species within the genus *Pseudonocardia*, for which the name *Pseudonocardia tropica* sp. nov. is proposed.

Description of *Pseudonocardia tropica* sp. nov.

Pseudonocardia tropica (tro'pi.ca. L. fem. adj. *tropica* tropical, referring to the tropical region where the type strain was isolated).

Gram-positive and aerobic. Forms white aerial mycelia and yellowish white to orange–yellow or brown substrate mycelia on the media tested. The aerial mycelia fragment into rod-shaped elements. No pigment is produced. The temperature range for growth is 15–37 °C, with optimal growth occurring at 28 °C. The pH range for growth is 6.0–9.0. The NaCl concentration range for growth is 0–10 % (w/v). Positive in tests for catalase and nitrate reduction, but negative in tests for milk coagulation and milk peptonization, gelatin liquefaction, cellulose and starch hydrolysis and H₂S production. Acid is produced from cellobiose, fructose, glucose, maltose, mannitol, sucrose and xylose. The cell wall contains *meso*-diaminopimelic acid. The characteristic sugars are arabinose and galactose. MK-8(H₄) is the predominant menaquinone. The phospholipids are diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, and phosphatidylinositol mannosides (type PIII phospholipid). The major fatty acids are iso-C_{16:0} (33.9 %) and C_{16:1} iso H (27.3 %). The G + C content of the genomic DNA of the type strain is 72.4 mol%.

The type strain, YIM 61452^T (=DSM 45199^T=CCTCC AA 208018^T), was isolated from surface-sterilized stem of *Maytenus austroyunnanensis* collected from the tropical rainforest in Xishuangbanna, Yunnan Province, south-west China.

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