Pseudonocardia endophytica sp. nov., isolated from the pharmaceutical plant *Lobelia clavata*

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An endophytic actinomycete strain, designated YIM 56035^T, was isolated from the inner tissue of the traditional Chinese medicinal plant *Lobelia clavata*. The strain stained Gram-positive, was aerobic and exhibited branching, white aerial mycelium and yellowish-brown substrate mycelium. The strain formed spore chains on aerial hyphae. The G+C content of the genomic DNA was 70.3 mol%. On the basis of morphological, physiological, chemotaxonomic and phylogenetic characteristics, strain YIM 56035^T was assigned to the genus *Pseudonocardia*. Analysis of 16S rRNA gene sequences showed 98.5, 97.3, 97.3 and 97.1 % similarity to the closely related type strains *Pseudonocardia kongjuensis* LM 157^T, *Pseudonocardia autotrophica* IMSNU 20050^T, *Pseudonocardia ammonioxydans* H9^T and *Pseudonocardia compacta* IMSNU 20111^T, respectively. The results of DNA–DNA hybridizations and comparison of some phenotypic characteristics revealed that the strain represents a novel species of the genus *Pseudonocardia*. The name *Pseudonocardia endophytica* sp. nov. is proposed, with the type strain YIM 56035^T (=DSM 44969^T =CCTCC AA 206026^T =KCTC 19150^T).

There are many endophytic micro-organisms inside healthy tissues of nearly all living plants, and endophytes are important sources of bioactive metabolites (Strobel *et al.*, 2004; Sardi *et al.*, 1992). Actinomycetes have been isolated from the stem and root interior of many plants species (de Araújo *et al.*, 2000; Coombs & Franco, 2003; Okazaki *et al.*, 1995). The genus *Pseudonocardia* was originally proposed by Henssen (1957) for mycolateless, nocardioform actinomycetes with a type IV cell wall (Lechevalier & Lechevalier, 1970), and its description has been emended by Warwick *et al.* (1994), McVeigh *et al.* (1994), Reichert *et al.* (1998) and Huang *et al.* (2002). In the present study, an endophytic strain YIM 56035^T was isolated from the inner tissue of *Lobelia clavata*. Polyphasic analysis demonstrated that the strain should be recognized

as a novel species of the genus *Pseudonocardia*, for which the name *Pseudonocardia endophytica* sp. nov. is proposed.

Strain YIM 56035^T was isolated from the healthy inner tissue of *L. clavata* collected from Yunnan Province, south-western China. *L. clavata*, a traditional Chinese medicinal plant, is usually used to treat parotitis and rheumatoid arthritis. Isolation of the strain was performed as follows: plant tissues (stems, leaves and roots) were washed thoroughly in running tap water to remove all soil, and these samples were surface sterilized by the method described by Shimizu *et al.* (2000). The surface-sterilization process was checked by rolling the surface-sterilized plant material aseptically onto the isolation medium, tap water-yeast extract agar (TWYE; Crawford *et al.*, 1993), and a control medium, tryptic soy agar (TSA), and then incubating the plates at 28 °C for 7 days. No contamination was found. Surface-sterilized stems, leaves and roots were cut and placed in a sterilized commercial blender and then ground into small pieces and plated onto TWYE containing nalidixic acid (10 mg ml⁻¹), nystatin and cycloheximide (each at 50 mg ml⁻¹) to repress growth of

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 56035^T is DQ887489.

An electron micrograph showing substrate mycelium of strain YIM 56035^T is available as supplementary material with the online version of this paper.

bacteria and fungi. Petri dishes were incubated at 28 °C for 4 weeks. Pure cultures were obtained by repeated streaking on plates that contained TWYE medium. Peptone-yeast extract-glycerol agar (PYGA; Martí *et al.*, 1999) and TSA were used for cultivating the isolates. The selected isolate, YIM 56035^T, was maintained on slopes of TSA at 4 °C or room temperature. For long-term preservation, cells were stored as suspensions of mycelium fragments in 30 % (v/v) glycerol at -20 and -80 °C. Cell mass for chemical and molecular systematic studies was grown in shaken flasks (shaken at about 150 r.p.m.) of TSB (tryptic soy broth) at 28 °C for 10 days.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were performed as described by Li *et al.* (2007) and an almost-complete 16S rRNA gene sequence of strain YIM 56035^T (1452 bp) was determined. Multiple sequence alignments were performed using CLUSTAL_X software (Thompson *et al.*, 1997) and phylogenetic analysis was done with MEGA 3.1 (Kumar *et al.*, 2004). A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap values were determined using the method of Felsenstein (1985) with 1000 bootstrap resamplings. The 16S rRNA gene sequences analysis indicated that strain YIM 56035^T belongs to the genus *Pseudonocardia*, and it was most closely related to *Pseudonocardia kongjuensis* LM 157^T (98.5 % similarity), *Pseudonocardia autotrophica* IMSNU 20050^T (97.3 %), *Pseudonocardia ammonioxydans* H9^T (97.3 %) and *Pseudonocardia compacta* IMSNU 20111^T (97.1 %) (Fig. 1); sequence similarities between strain YIM 56035^T and other type strains of the genus *Pseudonocardia* were less than 97 %.

DNA-DNA hybridization was performed by using the fluorometric microwell method (Ezaki *et al.*, 1989; Christensen *et al.*, 2000; He *et al.*, 2005). DNA-DNA relatedness values (means $\pm$ SD, $n=3$) between strain YIM 56035^T and *P. kongjuensis* DSM 44525^T, *P. autotrophica* DSM 535^T, *P. ammonioxydans* H9^T and *P. compacta* DSM 43592^T were 62.2 ± 4.6 , 52.1 ± 2.7 , 45 ± 2.5 and

42.6 ± 3.2 %, respectively. These values indicated clearly that strain YIM 56035^T represents a distinct genomic species (Stackebrandt & Goebel, 1994).

Amino acids and sugars of whole-cell hydrolysates were analysed as described previously (Hasegawa *et al.*, 1983). Menaquinones were extracted according to the method of Collins (1985) and analysed by HPLC (Groth *et al.*, 1997). Polar lipids were extracted and identified following the method of Minnikin *et al.* (1979) and Collins & Jones (1980). The cellular fatty acids were determined according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The G + C content of genomic DNA was determined by the HPLC method (Mesbah *et al.*, 1989). The chemotaxonomic data match the description of the genus *Pseudonocardia* (Huang *et al.*, 2002). Strain YIM 56035^T had a type-IV cell wall composition, containing *meso*-diaminopimelic acid, arabinose and galactose (Lechevalier & Lechevalier, 1970), which are common to members of the genus *Pseudonocardia*. The menaquinones were MK-8(H₄), MK-7(H₆) and MK-9 (ratio 97.5:1.8:0.7); MK-8(H₄) is the predominant menaquinone generally found in this genus. The phospholipids were phosphatidylethanolamine, phosphatidylcholine, phosphatidylmethylethanolamine and diphosphatidylglycerol (type PIII; Lechevalier *et al.*, 1977; Lechevalier & Lechevalier, 1980). The major fatty acids were i-C_{16:0} (34.03 %), C_{17:1} *cis*9 (14.69 %) and i-C_{15:0} (9.62 %) (Table 1). Phospholipid type III and the presence of i-C_{16:0} as the major fatty acid are in agreement with the description of the genus *Pseudonocardia* (Huang *et al.*, 2002). The DNA G + C content of strain YIM 56035^T was 70.3 mol%, which is within the range of values for members of the genus *Pseudonocardia* (Huang *et al.*, 2002).

Cultural characteristics were examined by incubating strain YIM 56035^T at 28 °C on ISP media (Shirling & Gottlieb, 1966) and the media described by Waksman (1961). Morphological characteristics of the strain were observed on ISP 2 medium (yeast extract/malt extract agar), PYGA and TSA after 7–14 days at 28 °C. Spores and mycelium

Fig. 1. Neighbour-joining phylogenetic dendrogram based on 16S rRNA gene sequences showing relationships between strain YIM 56035^T and related representatives of *Pseudonocardia*. Numbers on branch nodes are bootstrap values (expressed as percentages of 1000 resamplings). The sequences of *Amycolatopsis azurea* IMSNU 20053^T and *Nocardia puris* IMMIB R-145^T were used as the outgroup. Bar, 0.005 substitutions per nucleotide position.

Table 1. Fatty acid contents (%) of strain YIM 56035^T and type strains of related *Pseudonocardia* species

Strains: 1, strain YIM 56035^T; 2, *P. kongjuensis* DSM 44525^T; 3, *P. ammonioxydans* H9^T; 4, *P. autotrophica* IMSNU20050^T; 5, *P. compacta* IMSNU 20111^T. Data were obtained from this study under identical growth conditions. Only fatty acids making up more than 0.5% of the total are given. ND, Not detected.

Component	1	2	3	4	5
i-C _{14:0}	1.20	ND	ND	ND	ND
i-C _{15:0}	9.62	4.93	4.27	5.93	4.6
i-C _{16:0}	34.03	28.39	50.01	38.42	34.22
C _{16:1} <i>cis</i> 9	4.19	17.23	4.58	6.49	3.8
i-C _{16:1} H	4.19	3.72	13.46	16.23	4.2
C _{16:0}	2.00	6.19	1.63	ND	ND
C _{16:0} 10-methyl	4.68	4.63	5.63	9.35	4.81
i-C _{17:0}	5.11	8.87	6.45	5.95	5.21
C _{17:0} 10-methyl	1.85	2.67	3.25	6.60	4.45
ai-C _{17:0}	4.18	5.92	3.84	2.82	3.62
C _{17:1} <i>cis</i> 9	14.69	12.91	5.10	8.21	18.6
C _{17:0}	3.89	2.41	1.78	ND	ND
i-C _{18:0}	1.51	ND	ND	ND	ND
C _{18:1} <i>cis</i> 9	2.19	2.14	ND	ND	ND
C _{18:0}	0.98	ND	ND	ND	ND

were observed by light microscopy (Olympus microscope BH-2) and scanning electron microscopy (Philip XL30 ESEM-TMP). Growth at different temperatures (4, 10, 15, 20, 28, 37, 40, 45 and 55 °C), NaCl concentrations (0, 1, 3, 5, 7, 10, 15 and 20%) and pH values (pH 5.0–10.0) was assessed on TSA, with reference type strains in parallel. Other physiological and biochemical tests were carried out as described previously (Gordon *et al.*, 1974; Reichert *et al.*, 1998; Liu *et al.*, 2006).

Strain YIM 56035^T had morphological properties typical of the genus *Pseudonocardia*. The aerial mycelium was white and the substrate mycelium was yellowish brown on TSA and ISP2 medium. No diffusible pigment was produced. Spore chains were borne at the ends of branched aerial hyphae, and the spore surface was smooth. The substrate mycelium fragmented into rod-shaped elements on TSA (Supplementary Fig. S1, available in IJSEM Online). Growth was observed at 15–37 °C, 0–5% NaCl (w/v) and pH 6–8. The optimal growth pH, temperature and NaCl concentration were pH 7.0, 28 °C and 1%.

A range of phenotypic properties separated strain YIM 56035^T from the type strains of species of *Pseudonocardia* with validly published names (Tables 1 and 2). Strain YIM 56035^T can be differentiated from related *Pseudonocardia* species by the absence of phosphatidylglycerol and phosphatidylinositol. The isolate was distinguished from previously described species by the proportions of the major fatty acids (i-C_{16:0}, C_{16:1} *cis*9, i-C_{16:1} H and C_{17:1} *cis*9) and by the presence or absence of several fatty acids, including i-C_{14:0}, i-C_{18:0}, C_{18:1} *cis*9 and C_{18:0} (Table 1).

Table 2. Differential characteristics of strain YIM 56035^T and type strains of closely related *Pseudonocardia* species

Strains: 1, strain YIM 56035^T; 2, *P. kongjuensis* DSM 44525^T (data from this study); 3, *P. ammonioxydans* H9^T (unless indicated otherwise, data from Liu *et al.*, 2006); 4, *P. autotrophica* IMSNU 20050^T (Lee *et al.*, 2001); 5, *P. compacta* IMSNU 20111^T (Lee *et al.*, 2001); +, Positive or present; w, weakly positive; –, negative or absent; ND, no data available/not done. Data obtained during this study were obtained under identical growth conditions.

Characteristic	1	2	3	4	5
Acid production from:					
Adonitol	–	+	+	+	–
L-Arabinose	–	–	+	+	–
Cellobiose	–	–	–	+	+
meso-Erythritol	+	–	ND	–	–
D-Fructose	w	+	+	ND	ND
D-Galactose	+	+	+	+	–
myo-Inositol	–	+	–	+	–
Maltose	+	+	+	+	–
D-Mannitol	–	+	ND	+	+
D-Mannose	–	+	+	+	–
Melezitose	+	+	–	+	–
D-Ribose	+	–	+	ND	ND
D-Sorbitol	+	–	–	+	–
Sucrose	+	+	–	+	–
Trehalose	+	+	+	+	–
D-Xylose	–	+	w	+	–
Decomposition of:					
Hypoxanthine	+	+	–	–	–
Tyrosine	+	+	+	–	–
Hydrolysis of:					
Gelatin	–	–	w	–	–
Starch	–	–	w	–	–
Casein	–	+	+	–	–
Urease activity	–	+	+	+	–
Nitrate reduction to nitrite	–	–	+	–	+
H ₂ S production	–	+	+	+	–
Oxidation of ammonia	–	–	+	–	+
Growth at/in:					
4 °C	–	+	+	–	+
37 °C	w	+	+	+	–
7% NaCl	–	+	+	+	–
pH 9.0	–	+	–	+	–

*Data from this study.

Strain YIM 56035^T differed from its closest phylogenetic neighbour, *P. kongjuensis* DSM 44525^T, in several phenotypic properties, including acid production from some substrates, hydrolysis of casein, urease activity, H₂S production and growth at 4 °C, 7% NaCl and pH 9.0 (Table 2). The isolate is distinct from other the reference strains examined in most of the tested physiological properties (Table 2).

The genotypic and phenotypic data mentioned above show that strain YIM 56035^T merits recognition as a novel

species of the genus *Pseudonocardia*, for which the name *Pseudonocardia endophytica* sp. nov. is proposed.

Description of *Pseudonocardia endophytica* sp. nov.

Pseudonocardia endophytica (en.do.phy'ti.ca. Gr. *endo* within; Gr. *phyton* plant; L. fem. suff. *-ica* adjectival suffix used with the sense of belonging to; N.L. fem. adj. *endophytica* within plant, endophytic, pertaining to the original isolation from plant tissues).

Cells stain Gram-positive and are aerobic. Forms yellowish-brown substrate mycelium and white aerial mycelium on TSA and ISP2. The substrate mycelium fragments into rod-shaped elements. Spore chains form on aerial mycelium. No pigment is produced. Growth occurs at 15–37 °C, pH 6.0–8.0 and 0–5 % NaCl. Acid is produced from arbutin, aesculin, fructose, galactose, glucose, glycerol, maltose, melezitose, D-ribose, sucrose, salicin, sorbitol, trehalose and turanose, but not from adonitol, amygdalin, arabinose, arabit, cellobiose, dulcitol, erythritol, fucose, gentiobiose, gluconate, glycogen, inositol, inulin, 2-keto-gluconate, lactose, lyxose, mannitol, mannose, melibiose, methyl α -glucoside, methyl α -mannoside, methyl β -xylo-side, *N*-acetylglucosamine, raffinose, rhamnose, sorbose, starch, tagatose, xylitol or xylose. Utilizes L-asparagine, L-glutamic acid, *p*-hydroxyphenylacetic acid, α -ketoglutaric acid, α -ketovaleric acid, D-malic acid, *N*-acetyl-L-glutamic acid, propionic acid, putrescine, L-pyrogutamic acid, pyruvic acid, succinamic acid and succinic acid, but not acetic acid, L-alaninamide, D- or L-alanine, L-alanyl glycine, glycyl L-glutamic acid, α -, β - or γ -hydroxybutyric acid, lactamide, L-lactic acid, L-malic acid, methyl pyruvate, monomethyl succinate, pyruvic acid or L-serine. Ornithine decarboxylase and malonate tests give positive reactions; negative for nitrate reduction, milk peptonization and coagulation, gelatin liquefaction, growth in cellulose, gluconate test, H₂S, melanin and urease production and starch hydrolysis. Catalase-positive and oxidase-negative. Other physiological properties are listed in Table 2. The cell-wall chemistry type is type IV. The predominant menaquinone is MK-8(H₄). The phospholipids contain phosphatidylethanolamine, phosphatidylcholine, phosphatidylmethylethanolamine and diphosphatidylglycerol (phospholipid type PIII pattern). Major fatty acids are i-C_{16:0}, C_{17:1} *cis*9 and i-C_{15:0}. The DNA G+C content of the type strain is 70.3 mol%.

The type strain, YIM 56035^T (=DSM 44969^T =CCTCC AA 206026^T =KCTC 19150^T), was isolated from a plant sample collected from Xishuangbanna, Yunnan Province, south-western China.

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