Nocardia artemisiae sp. nov., an endophytic actinobacterium isolated from a surface-sterilized stem of *Artemisia annua* L.

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A novel actinobacterium, designated YIM 65623^T, was isolated from a surface-sterilized stem of *Artemisia annua* L. Strain YIM 65623^T had morphological, biochemical, physiological and chemotaxonomic properties that were consistent with its classification in the genus *Nocardia*. Growth occurred with 0–7 % (w/v) NaCl (optimum 0–3 %), at pH 5.0–9.0 (optimum pH 6.0) and at 10–37 °C (optimum 20–28 °C). Comparative 16S rRNA gene sequence analysis showed that strain YIM 65623^T constituted a distinct sublineage within the genus *Nocardia* and displayed 94.1–98.2 % sequence similarity to members of established species in the genus *Nocardia*. However, DNA–DNA relatedness and physiological and biochemical characteristics showed that strain YIM 65623^T could be differentiated from its closest phylogenetic relatives. The G + C content of the genomic DNA was 69.6 mol%. It is proposed that strain YIM 65623^T be classified as a representative of a novel species, *Nocardia artemisiae* sp. nov. The type strain is YIM 65623^T (=DSM 45379^T =CCTCC AA 209038^T).

The genus *Nocardia* (Trevisan, 1889) belongs to the family *Nocardiaceae*, a member of the suborder *Corynebacterineae* (Stackebrandt *et al.*, 1997), and has undergone a revolution in its taxonomy in recent years (Kiska *et al.*, 2002; Roth *et al.*, 2003). Members of the genus are aerobic, Gram-positive, high-G + C-content (64–72 mol%) organisms that form extensively branched substrate mycelium and aerial hyphae that fragment into rod-shaped or coccoid non-motile elements. The genus *Nocardia* is also characterized by a number of chemical markers, including the presence of *meso*-diaminopimelic acid (DAP), arabinose, galactose and mycolic acids (Goodfellow & Lechevalier, 1989; Goodfellow, 1992). Some novel species have been described in recent years and, at the time of writing, the

genus encompasses 80 species with validly published names. Many *Nocardia* species have been shown to be agents of human disease, such as *Nocardia asteroides*, *Nocardia farcinica* and *Nocardia nova* (Schaal & Lee, 1992; Wallace *et al.*, 1991), although it has also been shown that some species produce secondary metabolites of potential industrial value (Isik *et al.*, 1999; Kinoshita *et al.*, 2001), e.g. *Nocardia uniformis* and *Nocardia jinanensis*, which produce nocardicin and amicoumacin B, respectively. The recently described species *Nocardia callitridis* (Kaewkla & Franco, 2010) and *Nocardia endophytica* (Xing *et al.*, 2011) were isolated from the endophytic environment. During the course of our research on new actinobacterial resources, we obtained a novel endophytic member of the genus *Nocardia*, strain YIM 65623^T.

Strain YIM 65623^T was isolated from the healthy inner tissue of a stem of *Artemisia annua* L., a traditional Chinese medicinal plant, which was collected in Yunnan Province, south-west China. Samples were washed thoroughly in running water to remove all soil and sonicated (160 W) to dislodge any soil and organic matter from the surface. After

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Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 65623^T is GU367157.

Two supplementary figures and two supplementary tables are available with the online version of this paper.

drying at room temperature, tissue segments were surface sterilized by immersing in 0.1 % Tween 20 for 1 min, followed by 5 % (available Cl^-) NaClO for 4 min (leaf) or 6 min (stem and root). The samples were then rinsed in 2.5 % (w/v) $\text{Na}_2\text{S}_2\text{O}_3$ for 10 min to remove residual chlorine (Miché & Balandreau, 2001; Qin *et al.*, 2009) and washed at least three times with sterilized water. The last disinfection step was immersion in 70 % (v/v) ethanol for 4 min (leaf) or 6 min (stem and root), which was followed by a minimum of three washes with sterile water. Samples were dried on sterile filter paper in a laminar flow cabinet. To confirm that the sterilization process was successful, 0.2 ml water from the final washing was spread onto both the isolation medium and yeast extract-malt extract agar [International *Streptomyces* Project (ISP) 2; Shirling & Gottlieb, 1966] and incubated at 28 °C for 2–4 weeks. The isolation method involved processing 1 g sample in a commercial blender, followed by grinding with a mortar and pestle, suspending in 9 ml sterile water and then serially diluting 1 ml tissue suspension to 10^{-2} before plating. The isolation medium was sodium propionate-asparagine-salt agar (Qin *et al.*, 2009) supplemented with 3 % (w/v) NaCl and (I^{-1}) 25 mg nalidixic acid and 50 mg nystatin. A pure culture was obtained by repeated streaking on half-strength ISP 2 agar. Strain YIM 65623^T was routinely cultivated on ISP 2 medium at 28 °C and stored as a glycerol suspension (20 %, v/v) at –80 °C.

The morphological characteristics of strain YIM 65623^T were assessed by light microscopy (BH-2; Olympus) and scanning electron microscopy (Philips XL30; ESEM-TMP) using 14-day-old cultures grown on ISP 2 agar. Cultural characteristics were recorded on 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 ISCC–NBS colour charts (standard samples, no. 2106; Kelly, 1964). Growth at 4, 10, 20, 28, 37, 40, 45, 50 and 55 °C was tested on tryptose soy agar for 21 days. Growth at pH 4–10 (in increments of one pH unit) was tested using the buffer system described by Xu *et al.* (2005). Growth with 0, 1, 3, 5, 7, 10, 15 and 20 % (w/v) NaCl was tested at 28 °C for 14–21 days in tryptose soy broth (TSB). Catalase, oxidase, urease and gelatinase activities, starch hydrolysis and nitrate reduction were assessed as described by Smibert & Krieg (1994). Carbon-source utilization and other physiological and biochemical properties were analysed as described by Gordon *et al.* (1974).

Biomass for chemical and molecular studies was obtained by cultivation in shake flasks (about 200 r.p.m.) in TSB at 28 °C for 1 week. The isomer of diaminopimelic acid and the sugars of the whole-cell hydrolysate were determined according to the procedures described by Hasegawa *et al.* (1983), Lechevalier & 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 & Jones, 1980). Menaquinones were isolated according to Collins *et al.*

(1977) and separated by HPLC (Tamaoka *et al.*, 1983). Mycolic acids were extracted and analysed by one-dimensional TLC as described by Minnikin *et al.* (1980). Cellular fatty acids were extracted, methylated and analysed using the Sherlock Microbial Identification System (MIDI) according to the manufacturer's instructions. The fatty acid methyl esters were analysed using Microbial Identification software (version 4.0, TSBA40 database; MIDI). The G + C content of the genomic DNA was determined using the HPLC method (Mesbah *et al.*, 1989).

Extraction of genomic DNA and PCR amplification and sequencing of the 16S rRNA gene were performed as described by Li *et al.* (2007). 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 using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) algorithms in MEGA version 4.0 (Tamura *et al.*, 2007). PHYLIP version 3.6 (Felsenstein, 2002) and PHYML (Guindon & Gascuel, 2003) were used to construct the maximum-likelihood tree (Felsenstein, 1981). The tree topologies were evaluated using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. DNA–DNA relatedness was studied according to the fluorometric microwell method (Ezaki *et al.*, 1989; He *et al.*, 2005).

Strain YIM 65623^T was an aerobic, Gram-stain-positive organism that had morphological properties consistent with its assignment to the genus *Nocardia*. The well-developed substrate mycelium was white, brown, orange or yellowish orange, with irregular branches penetrating the agar, while the aerial hyphae were white or brown (Supplementary Table S1, available in IJSEM Online). At a late stage of growth, the hyphae fragmented into rod-shaped elements with smooth surfaces (Supplementary Fig. S1). The physiological properties of strain YIM 65623^T are given in the species description and differences between strain YIM 65623^T and its closest phylogenetic neighbour, *Nocardia mexicana* DSM 44952^T, are shown in Table 1. The isolate and the reference strain had different profiles for utilization and hydrolysis of some substances. Also, the temperature and pH for growth were different between the two strains.

Strain YIM 65623^T exhibited chemotaxonomic characteristics that were consistent for the genus *Nocardia*. Whole-cell hydrolysates of strain YIM 65623^T were rich in *meso*-DAP, arabinose, galactose, glucose and mannose. The polar lipid profile contained phosphatidylethanolamine, phosphatidylinositol, diphosphatidylglycerol and phosphatidylinositol mannosides. The major menaquinone was MK-8(H_{40} cyclo), which is very similar to those described for recognized *Nocardia* species. In addition, TLC analysis revealed that the isolate contained mycolic acids with an R_f value of 0.47, which was identical to that of *N. mexicana* DSM 44952^T used as a control. The fatty acid profile contained a mixture of straight-chain saturated, unsaturated and 10-methyl

Table 1. Different characteristics of strain YIM 65623^T and its closest phylogenetic neighbour

All data were obtained in this study. Both strains are positive for utilization of cellobiose, dulcitol, D-fructose, D-galactose, glucose, glycerol, lactose, maltose, D-mannitol, D-mannose, L-rhamnose, sucrose, ribose and xylose and growth with 5% (w/v) NaCl, at pH 5.0–9.0 and at 10–37 °C. Both strains are negative for utilization of *myo*-inositol. +, Positive; w, weakly positive; –, negative.

Characteristic	YIM 65623 ^T	<i>N. mexicana</i> DSM 44952 ^T
Use of sole carbon sources		
L-Arabinose	–	+
Raffinose	–	+
Sodium acetate	–	+
D-Sorbitol	–	+
Use of L-tyrosine as a sole nitrogen source	+	–
Hydrolysis of Tweens 20, 40 and 80	–	+
Growth at/with:		
7% (w/v) NaCl	w	–
40 °C	–	+
45 °C	–	w
pH 10.0	–	w

branched fatty acids, including C_{16:0} (34.35%), 10-methyl C_{18:0} (16.60%), C_{17:1}ω8c (15.92%), C_{18:1}ω9c (12.29%), C_{16:1}ω7c and/or iso-C_{15:0} 2-OH (8.90%), C_{15:0} (4.80%), C_{18:0} (1.81%), C_{15:1}ω6c (1.77%), C_{17:0} (1.61%), C_{14:0} (0.86%), iso-C_{16:0} (0.38%) and anteiso-C_{17:1}ω9c (0.22%) (Supplementary Table S2). This profile most closely matches those of members of the genus *Nocardia*. However, strain YIM 65623^T showed remarkable differences in its fatty acid profile from *N. mexicana* DSM 44952^T, such as quantitative

differences in the proportions of 10-methyl C_{18:0}, C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, C_{18:1}ω9c, C_{17:1}ω8c, C_{15:1}ω6c and C_{15:0}. The G + C content of the DNA of strain YIM 65623^T was 69.6 mol%, which is in accordance with the range for the genus *Nocardia*.

The 16S rRNA gene sequence (1511 bp) of strain YIM 65623^T was determined in this study. Phylogenetic analysis showed that strain YIM 65623^T was closely related to members of the genus *Nocardia*. Sequence similarity calculations obtained by pairwise comparisons indicated that the closest relatives of strain YIM 65623^T were *Nocardia lijiangensis* YIM 33378^T (98.2% 16S rRNA gene sequence similarity) and *N. mexicana* OFN 785-81^T (98.1%). Lower similarities (<98.0%) were found with the other members of the genus *Nocardia*. This relationship between strain YIM 65623^T and other members of the genus *Nocardia* was also evident in the phylogenetic trees based on neighbour joining, maximum parsimony and maximum likelihood (Fig. 1 and Supplementary Fig. S2), in which strain YIM 65623^T formed a distinct sublineage with *N. mexicana* OFN 785-81^T. To establish the precise taxonomic position of strain YIM 65623^T, DNA–DNA hybridization was performed between strain YIM 65623^T and *N. mexicana* DSM 44952^T, which revealed 36.0 ± 2.0% DNA–DNA relatedness, which is far below the threshold value of 70% recommended by Stackebrandt & Goebel (1994) for assigning strains to different genomic species. DNA–DNA hybridization between strain YIM 65623^T and *N. lijiangensis* YIM 33378^T was not carried out because they were positioned in different clusters in the phylogenetic trees. Furthermore, 16S rRNA gene sequence similarities much higher than 98.1% have been found between type strains of species of the genus *Nocardia* that have DNA–DNA relatedness much lower than the 70% threshold recommended for the delineation of species (Yassin &

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain YIM 65623^T and closely related members of the genus *Nocardia*. Bootstrap values (>50%) based on 1000 replicates are shown at branch nodes. Asterisks indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony and maximum-likelihood methods. Bar, 0.005 substitutions per nucleotide position.

Brenner, 2005; Kämpfer *et al.*, 2007; Sun *et al.*, 2009). Conville *et al.* (2000) also reported it reasonable to consider *Nocardia* isolates as belonging to different species if they share $\leq 98.5\%$ 16S rRNA gene sequence similarity. Strains belonging to the same species generally have at least 99.2% sequence similarity. By these criteria, strain YIM 65623^T represents a novel species of the genus *Nocardia*.

The chemical and morphological data clearly indicate that strain YIM 65623^T belongs to the genus *Nocardia*. However, 16S rRNA gene sequence analysis, DNA–DNA relatedness and biochemical characteristics indicated that strain YIM 65623^T represents a species separate from those in the genus *Nocardia* with validly published names. Thus, we consider that strain YIM 65623^T should be placed in a novel species, for which we propose the name *Nocardia artemisiae* sp. nov.

Description of *Nocardia artemisiae* sp. nov.

Nocardia artemisiae (ar.te.mi'si.æ. L. n. *artemisia* mugwort, and also a plant genus; L. gen. n. *artemisiae* of mugwort, of *Artemisia*, referring to the isolation of the type strain from *Artemisia annua* L.).

Aerobic, non-motile, Gram-stain-positive actinomycete that forms extensively branched substrate mycelium and aerial mycelium that bears smooth-surfaced rod-shaped spores. Good growth occurs on ISP media 2–5, Czapek's agar, potato-glucose agar and nutrient agar. Aerial mycelium is white or brown; substrate mycelium is white, brown, orange or yellowish orange on media tested. Diffusible pigments are observed on ISP 3 agar (grey–yellow) and potato-glucose agar (yellowish orange). Grows at 10–37 °C (optimum 20–28 °C), at pH 5.0–9.0 (optimum pH 6.0) and with 0–7% NaCl (optimum 0–3% NaCl). Positive for catalase and urease, but negative for milk coagulation, milk peptonization, nitrate reduction, oxidase, gelatin liquefaction, hydrolysis of cellulose and starch and production of H₂S. As sole carbon sources, utilizes cellobiose, dulcitol, D-fructose, D-galactose, glucose, glycerol, lactose, maltose, D-mannitol, D-mannose, L-rhamnose, sucrose, ribose and xylose, but not L-arabinose, *myo*-inositol, raffinose, sodium acetate or D-sorbitol. Acid is produced from glycerol and ribose. As sole nitrogen sources, utilizes L-alanine, L-arginine, L-asparagine, glycine, L-hydroxyproline, hypoxanthine, L-lysine, L-phenylalanine, L-serine, L-tyrosine, L-valine and xanthine. Whole-cell hydrolysates are rich in *meso*-DAP, arabinose, galactose, glucose and mannose. The phospholipids are phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. The major menaquinone is MK-8(H₄_{cyclo}). Mycolic acids are present. The major fatty acids are C_{16:0}, C_{18:0} 10-methyl, C_{17:1}ω8c, C_{18:1}ω9c, C_{16:1}ω7c and/or iso-C_{15:0} 2-OH and C_{15:0}. The DNA G + C content of the type strain is 69.6 mol%.

The type strain, YIM 65623^T (=DSM 45379^T =CCTCC AA 209038^T), was isolated from a surface-sterilized stem of *Artemisia annua* L., collected from Yunnan province, south-west China.

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