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 65623T, was isolated from a surface-sterilized stem of Artemisia annua L. Strain YIM 65623T 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 65623T 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 65623T 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 65623T be classified as a representative of a novel species, Nocardia artemisiae sp. nov. The type strain is YIM 65623T (=DSM 45379T = CCTCC AA 209038T).

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 65623T.

Strain YIM 65623T 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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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) Na₂S₂O₃ 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 (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 65623ᵀ 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 65623ᵀ 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 dainomipimelic 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 65623ᵀ 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 65623ᵀ are given in the species description and differences between strain YIM 65623ᵀ and its closest phylogenetic neighbour, Nocardia mexicana DSM 44952ᵀ, 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 65623ᵀ exhibited chemotaxonomic characteristics that were consistent for the genus Nocardia. Whole-cell hydrolysates of strain YIM 65623ᵀ 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(H₄cyclo), which is very similar to those described for recognized Nocardia species. In addition, TLC analysis revealed that the isolate contained mycolic acids with an Rₜ value of 0.47, which was identical to that of N. mexicana DSM 44952ᵀ used as a control. The fatty acid profile contained a mixture of straight-chain saturated, unsaturated and 10-methyl...
branched fatty acids, including C_{16:0} (34.35 %), 10-methyl C_{18:0} (16.60 %), C_{17:1} \text{v}_{8c} (15.92 %), C_{18:1} \text{v}_{9c} (12.29 %), C_{16:1} \text{v}_{7c} and/or iso-C_{15:0} \text{2-OH} (8.90 %), C_{15:0} (4.80 %), C_{18:0} (1.81 %), C_{15:1} \text{v}_{6c} (1.77 %), C_{17:0} (1.61 %), C_{14:0} (0.86 %), iso-C_{16:0} (0.38 %) and anteiso-C_{17:1} \text{v}_{9c} (0.22 %) (Supplementary Table S2). This profile most closely matches those of members of the genus *Nocardia*. However, strain YIM 65623\textsuperscript{T} showed remarkable differences in its fatty acid profile from *N. mexicana* DSM 44952\textsuperscript{T}, such as quantitative differences in the proportions of 10-methyl C_{18:0}, C_{16:1} \text{v}_{7c} and/or iso-C_{15:0}, 2-OH, C_{18:1} \text{v}_{9c}, C_{17:1} \text{v}_{8c}, C_{15:1} \text{v}_{6c} and C_{15:0}. The G+C content of the DNA of strain YIM 65623\textsuperscript{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\textsuperscript{T} was determined in this study. Phylogenetic analysis showed that strain YIM 65623\textsuperscript{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\textsuperscript{T} were *Nocardia lijiangensis* YIM 33378\textsuperscript{T} (98.2 % 16S rRNA gene sequence similarity) and *N. mexicana* OFN 785-81\textsuperscript{T} (98.1 %). Lower similarities (<98.0 %) were found with the other members of the genus *Nocardia*. This relationship between strain YIM 65623\textsuperscript{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\textsuperscript{T} formed a distinct sublineage with *N. mexicana* OFN 785-81\textsuperscript{T}. To establish the precise taxonomic position of strain YIM 65623\textsuperscript{T}, DNA–DNA hybridization was performed between strain YIM 65623\textsuperscript{T} and *N. mexicana* DSM 44952\textsuperscript{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\textsuperscript{T} and *N. lijiangensis* YIM 33378\textsuperscript{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 &

### Table 1. Different characteristics of strain YIM 65623\textsuperscript{T} and its closest phylogenetic neighbour

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

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain YIM 65623\textsuperscript{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.
The chemical and morphological data clearly indicate that strain YIM 65623T belongs to the genus *Nocardia*. However, 16S rRNA gene sequence analysis, DNA–DNA relatedness and biochemical characteristics indicated that strain YIM 65623T represents a species separate from those in the genus *Nocardia* with validly published names. Thus, we consider that strain YIM 65623T 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.ae. 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-glucose, D-mannose, L-rhamnose, sucrose, ribose and xylose, but not 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 phosphatidylglycerol. The major menaquinones are MK-8(H4 cyclo). Mycolic acids are present. The major fatty acids are C16:0, C18:0 10-methyl, C17:1ω8c, C18:1ω9c, C16:1ω7c and/or iso-C15:0 2-0H and C15:0. The DNA G+C content of the type strain is 69.6 mol%.

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

**Acknowledgements**

This research was supported by the National Basic Research Program of China (grant no. 2010CB833801) and the National Natural Science Foundation of China (grant no. U0932601).

**References**


