Nocardia cavernae sp. nov., a novel actinobacterium isolated from a karst cave sample

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Abstract

A novel actinobacterial strain, designated YIM A1135T, was isolated from a soil sample collected from a karst cave in Xingyi county, Guizhou province, south-western China. The taxonomic position of the strain was investigated using a polyphasic approach. Cells of the strain were aerobic, Gram-stain-positive and partially acid-alcohol-fast. Strain YIM A1135T shared 98.3 % 16S rRNA gene sequence similarity with Nocardia jejuensis NBRC 103114T and 97.6 % with Nocardia alba YIM 30243T. DNA–DNA hybridization values between strain YIM A1135T and related type strains of the genus Nocardia were less than 70 %. In addition, meso-diaminopimelic acid was the diagnostic diamino acid in cell-wall peptidoglycan. The whole-cell sugars were fructose, mannose, galactose and glucose. The major isoprenoid quinone was MK-8(H4-cyclo), while the major fatty acids (>10 %) were C16 : 0, C18 : 2ω9c, C18 : 1ω7c and/or C18 : 1ω6c). The polar lipids contained diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannoside. Mycolic acids were present. The genomic DNA G+C content of strain YIM A1135T was 66.7 mol%. Based on the results of the molecular studies supported by its morphological, physiological, chemotaxonomic and other differential phenotypic characteristics, strain YIM A1135T is considered to represent a novel species within the genus Nocardia, for which the name Nocardia cavernae sp. nov. is proposed. The type strain is YIM A1135T (=KCTC 39595T=CCTCC AA 2017030T).

The genus Nocardia, proposed by Trevisan [1], is the type genus of the family Nocardiaceae within the order Corynebacteriales [2, 3]. The members of this genus are aerobic, Gram-stain-positive and contain mycolic acid. They usually form extensively branched substrate hyphae that fragment into rod-shaped, non-motile elements, and aerial hyphae that are sometimes visible only under microscope [4–6]. The whole-cell hydrolysates contain meso-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan, and arabinose and galactose as the diagnostic sugars. The predominant menaquinone is MK-8(H4-cyclo). Members of the genus Nocardia generally contain straight-chain, saturated, unsaturated and 10-methyl types of fatty acids as the major fatty acids. The G+C content of the genomic DNA ranges between 65.1 and 74.0 mol%. Strains of genus Nocardia originate from many kinds of niches such as soil, marine sediment, wastewater system, plants and animals, however, some species have been isolated from clinical samples as pathogenic agents [7–9]. At the time of writing, more than 100 species of the genus have been described with validly published names (www.bacterio.net/nocardia.html). During a study of the diversity of actinobacteria of the karst niche, strain YIM A1135T was isolated from a karst cave. The aim of the present study is to determine the taxonomic status of the isolate by using a polyphasic approach. The results of phenotypic, chemotaxonomic and phylogenetic analyses indicate that strain YIM A1135T represents a novel species in the genus Nocardia.

Strain YIM A1135T was isolated from a soil sample from a karst cave located in Xingyi county (25°09′ N, 104°46′ E), Guizhou province, south-western China. The sample was collected with a sterile spoon into a sterile sampling bag, and then transported to a laboratory under ambient conditions. Isolation of actinobacteria was done using the standard dilution plate method on humic acid–vitamin...
(HV) agar [10] supplemented with cycloheximide (50 mg l⁻¹) and nalidixic acid (20 mg l⁻¹) and incubated for 2 weeks at 28 °C. Selected colonies were further purified on International Streptomyces Project (ISP) medium 2 [11] and maintained on ISP medium 2 slants at 4 °C and as glycerol suspensions (20 %, w/v) at −80 °C. The reference type strains N. jejuensis NBRC 103114T and N. alba JCM 13373T were obtained from NITE Biological Resource Centre (NBRC, Japan) and the Japan Collection of Microorganisms (JCM, Japan), respectively. All strains were maintained routinely on ISP medium 2 (28 °C, 1 week). Biomass of strain YIM A1135T and the reference type strains for chemotaxonomical and molecular investigations were harvested from cultures grown on ISP medium 2 (28 °C, 1 week).

Cell morphology of strain YIM A1135T was observed under a light microscope and a scanning electron microscope (JEM-6330F, JEOL). For scanning electron microscopy, harvest cells of strain YIM A1135T were suspended in sterilized water and fixed on glass cover-slips with glutaraldehyde (2 %) for 2 h. Subsequently, the fixed cells were dehydrated through a gradient series of alcohol (30, 50, 70, 90 and 100 %, respectively). The cell specimens were sputter-coated with gold powder for 200 s and then examined. Gram’s reaction was determined by Solarbio’s Gram staining kit (China) as per the manufacturer’s instructions. The acid-alcohol fast staining of strain YIM A1135T was done according to a modified Kiyoun staining method [12]. Cultural characteristics were tested on ISP media 2–7 [11], Czapek’s agar [13], potato dextrose agar, Gause’s synthetic agar [14] and nutrient agar [13] for 3 weeks at 28 °C. The colony colour was determined using the ISCC-NBS colour chart [15]. Growth at various NaCl concentrations (0–8 %, w/v, at intervals of 0.5 % unit) and different temperatures (0, 4, 10, 15, 20, 28, 30, 35, 37, 40 and 45 °C) were examined on ISP 2 plates while the pH range for growth (4.0–12.0, at intervals of 1 pH unit) was determined using the buffer system as described by Xu et al. [16] were tested for 4 weeks by culturing the strains in ISP 2 broth. Activities of oxidase, catalase and urease, gelatin liquefaction, milk peptonization and coagulation, nitrate reduction, H₂S production, degradation of Tween 20, 40, 60 and 80, starch and cellulose were investigated according to the conventional procedures described by Gordon et al. [17] and Williams et al. [18]. Carbon-source utilization tests were performed according to the methods described by Shirling and Gottlieb [11] and Athalye et al. [19] using modified basal medium, as recommended by Pridham et al. [20]. Nitrogen source utilization was observed according to Nie et al. [21]. Other physiological and biochemical characteristics were assessed by using the media and methods described by Gordon et al. [17].

Genomic DNA was extracted, and the 16S rRNA gene was PCR-amplified and sequenced as described by Li et al. [22]. The amplicons were purified by using a PCR purification kit (Sangon Biotech, China). The 16S rRNA gene sequences obtained were assembled using SeqMan program (DNASTar software) and compared with the corresponding sequences of cultured species in the EzTaxon server and the NCBI database by using a BLAST search [23, 24]. To determine the phylogenetic relationships of strain YIM A1135T and the genus Nocardia, multiple alignments of their sequences with related type strains of the genus Nocardia were performed using the CLUSTAL_X software package [25]. Phylogenetic and molecular evolutionary analyses were performed using the software package MEGA version 7.0 [26]. Phylogenetic dendrograms were generated using neighbour-joining, maximum-parsimony and maximum-likelihood algorithms [27–29]. The Kimura two-parameter model [30, 31] was used to calculate the evolutionary distances in the neighbour-joining phylogenetic tree. Bootstrap analysis was used to evaluate the topology of each tree with 1000 replications [32]. The genomic DNA G+C content of strain YIM A1135T was determined by high-performance liquid chromatography (HPLC), with E. coli JM-109 as the reference strain [33]. DNA–DNA relatedness was carried out to determine the relationship between strains YIM A1135T, N. jejunensis NBRC 103114T and N. alba JCM 13373T according to the fluorometric micro-well method [34–36]. The hybridizations were performed by using DNA probes labelled with photobiotin (A1935; Sigma) and 96-well microdilution plates (Greiner BioOne). Each sample was set with eight replications.

Chemotaxonomical characteristics of strain YIM A1135T and the reference strains were observed using several standard methods under identical conditions. A purified cell-wall preparation was obtained and hydrolysed as described by Schleifer and Kandler [37], and then the diaminopimelic acid and whole-cell sugar were analysed by using the methods described by Tang et al. [38]. Menaquiones were extracted from lyophilized cells as described by Collins et al. [39] and Minnikin et al. [40], and the extracts were purified and analysed by HPLC [41, 42]. For analysis of fatty acids, strains YIM A1135T, N. jejunensis NBRC 103114T and N. alba JCM 13373T were grown on TSA at 28 °C for 1 week. The cellular fatty acids were extracted, methylated and analysed following the instructions of the Microbial Identification System (MIDI; Sherlock version 6.1; MIDI database TSBA6) [43]. Polar lipids were extracted and the individual polar lipids separated by two-dimensional thin-layer chromatography (TLC) on silica gel G 60 plates (Merck; Germany). The polar lipid profile was identified using the procedures described previously [40, 44, 45]. Mycolic acids were detected using the method described by Minnikin et al. [46].

Cells of strains YIM A1135T were Gram-stain-positive, aerobic and non-motile. Strain YIM A1135T showed good growth on ISP 2, ISP 3, ISP 4, ISP 5, Czapek’s agar, nutrient agar, potato dextrose agar and Gause’s synthetic agar, and moderate growth on ISP 6 and ISP 7 agar media (Table S1, available in the online Supplementary Material). Soluble pigments and melanin pigments were not produced on the tested media. Strain YIM A1135T formed well-developed and branched substrate mycelia that fragmented into
irregular rod-shaped elements with light aerial mycelia on the agar surface (Fig. S1). The cultural and morphological characteristics of strain YIM A1135T were typical of those of the genus Nocardia. Growth occurred at temperatures ranging from 4 to 35°C (optimum, 28°C). Strain YIM A1135T was able to grow at pH 6.0–8.0 (optimum, pH 7.0) and in the presence of NaCl concentrations of up to 3% (w/v). The strain grew well in medium without NaCl. The strain was positive for the coagulation and peptonization of milk but negative for catalase, oxidase, urease, reduction of nitrate and production of H2S. The strain could hydrolyse Tween 60, but not cellulose, starch or Tweens 20, 40 and 80. The physiological and biochemical characteristics of strain YIM A1135T are summarized in the species description and characteristics that differentiate strain YIM A1135T from its closest related type strains are listed in Table 1.

To determine the phylogenetic position, the almost-complete 16S rRNA gene sequence (1510 nt; accession number KY285257) of strain YIM A1135T was determined. Comparison of the sequence with the corresponding 16S rRNA gene sequences in the GenBank/EMBL/DDBJ databases clearly demonstrated that strain YIM A1135T is a member of the genus Nocardia. The strain shared 16S rRNA gene sequence similarities of 98.3% and 97.6% with N. jejuensis NBRC 103114T and N. alba YIM 30243T, respectively. The neighbour-joining tree based on 16S rRNA gene sequences showed that strain YIM A1135T forms a distant clade with N. jejuensis NBRC 103114T. An apparently distinct lineage was also supported from maximum-parsimony and maximum-likelihood phylogenetic trees (Figs S2 and S3). The clades common to all the three phylogenetic trees are marked with asterisks in each branch of Fig. 1. The genomic DNA G+C content of strain

### Table 1. Differential phenotypic characteristics between strain YIM A1135T and its closely related type strains of the genus Nocardia.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl tolerance (% w/v)</td>
<td>0–3</td>
<td>0–6</td>
<td>0–7</td>
</tr>
<tr>
<td>Degradation of:</td>
<td></td>
<td></td>
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<tr>
<td>Tween 60</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tween 80</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Milk coagulation</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Milk peptonization</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Carbon sources:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Arabinose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>d-Galactose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>d-Glucose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Inositol</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Lactose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>d-Mannose</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Raffinose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>d-Rhamanose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>d-Ribose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>d-Sorbitol</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Trehalose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>d-Xyitol</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>d-Xylose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Nitrogen source</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>l-Glutamic acid</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>l-Phenylalanine</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>l-Valine</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Major fatty acids</td>
<td>C16:0, C18:1ω7c, C18:0 10-methyl, summed feature 3</td>
<td>C16:0, C18:1ω9c, C18:0 10-methyl</td>
<td>C16:0, C18:1ω9c, C18:0 10-methyl, summed feature 3</td>
</tr>
<tr>
<td>The polar lipids</td>
<td>DPG, PE, PI, PIM</td>
<td>DPG, PE, PI†</td>
<td>DPG, PE, PI, PIM, GL†</td>
</tr>
<tr>
<td>DNA G+C content (mol %)</td>
<td>66.7</td>
<td>69.6†</td>
<td>74†</td>
</tr>
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</table>

*Data retrieved from Lee [48].
†Data retrieved from Li et al. [49].
YIM A1135\textsuperscript{T} was 66.7 mol\%, which is within the range for members of the genus \textit{Nocardia}. Based on phylogenetic trees and EzTaxon-e server results, strains \textit{N. jejuensis} NBRC 103114\textsuperscript{T} and \textit{N. alba} JCM 13373\textsuperscript{T} were selected as reference strains for DNA–DNA hybridization studies. DNA–DNA relatedness between strain YIM A1135\textsuperscript{T} and reference strains \textit{N. jejuensis} NBRC 103114\textsuperscript{T} and \textit{N. alba} JCM 13373\textsuperscript{T} were 45.3±1.0 and 39.7±1.1 \%, respectively, which were lower than the threshold value (70 \%) recommended for distinguishing novel species [47].

Strain YIM A1135\textsuperscript{T} contained \textit{meso}-2,6 diaminopimelic acid as the cell-wall diamino acid, which was similar to the other members of the genus \textit{Nocardia}. The whole-cell sugars were fructose, mannose, galactose and glucose. The

**Fig. 1.** Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship of strain YIM A1135\textsuperscript{T} with the members of the genus \textit{Nocardia}. Asterisks indicate branches that were also recovered in the maximum-parsimony and maximum-likelihood dendrograms. Bootstrap values (expressed as percentages of 1000 replications) of above 50 \% are shown at branch points. \textit{Pseudonocardia thermophila} strain NRRL B-1978\textsuperscript{T} was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.
predominant menaquinone of strain YIM A1135<sup>T</sup> was MK-8(H<sub>4</sub>, ω<sub>7</sub>-cyclo) (88.6 %), which is common to the genus Nocardia. MK-9 (H<sub>4</sub>) was also detected as minor components (8.4 %). The predominant fatty acids profile of strain YIM A1135<sup>T</sup> (>10%) was C<sub>16:0</sub> (29.7 %), C<sub>18:1ω9c</sub> (20.9 %), C<sub>18:0 10-methyl</sub> (14.7 %) and summed feature 3 (C<sub>16:1ω7c</sub> and/or C<sub>16:1ω6c</sub>) (14.9 %). Detailed fatty acid compositions are shown in Table S2. The polar lipid of strain YIM A1135<sup>T</sup> comprised phosphatidylinositol, phosphatidylinositol mannoside, diphostatidylglycerol and phosphatidylethanolamine. The DNA G+C content is about 66.7 mol%.

The type strain YIM A1135<sup>T</sup> (=KCTC 39595<sup>T</sup>=CCTCC AA 2017030<sup>T</sup>) was isolated from a soil sample in a karst cave in Xingyi county, Guizhou province, south-western China.

**DESCRIPTION OF NOCARDIA CAVERNAE SP. NOV.**

*Nocardia cavernae* (ca.ver’ nae. L. gen. fem. n. cavernae of a cave, referring to the habitat from which the type strain was isolated).

Cells are Gram-stain-positive, partially acid-alcohol-fast, aerobic and non-motile. Substrate mycelia fragment into irregular rod-shaped elements. Good growth on ISP 2–5 agar, Czapek’s agar, nutrient agar, potato dextrose agar and Gause’s synthetic agar, and moderate growth on ISP 6 and ISP 7 agar. No soluble pigments and melanin pigments are produced on the above media. Growth occurs at 4–35 °C (optimum 28 °C), pH 6.0–8.0 and in the presence of up to 3 % (w/v) NaCl. Positive for coagulation and peptonization of milk, but negative for catalase, oxidase, urease, reduction of nitrate and production of H<sub>2</sub>S. Tween 60 is hydrolysed, but no cellulose, starch or Tween 20, 40 and 80. Utilizes L-arabinoose, D-fructose, D-glucose, glycerol, inositol, L-rhamnose, D-ribose, D-sorbitol, trehalose and D-xyllose as sole carbon sources but not cellobiose, D-galactose, lactose, maltose, D-mannitol, D-mannose, raffinose, D-ribose, sucrose or xylose. Utilizes L-alanine, L-arginine, L-aspartic acid, L-histidine, L-glutamine, glycine, L-lysine, L-methionine, L-serine, L-threonine, L-tryptophan and L-tyrosine as sole nitrogen sources but not L-cysteine, L-glutamic acid, L-phenylalanine or L-valine. Meso-diaminopimelic acid is the diagnostic diamino acid in cell-wall peptidoglycan. The whole-cell sugars are fructose, mannose, galactose and glucose. The predominant menaquinone is MK-8(H<sub>4</sub>, ω<sub>7</sub>-cyclo). The major fatty acids (>10%) are C<sub>16:0</sub>, C<sub>18:1ω9c</sub>, C<sub>18:0 10-methyl</sub> and summed feature 3 (C<sub>16:1ω7c</sub> and/or C<sub>16:1ω6c</sub>). Mycolic acids are present. The polar lipid profile consists of phosphatidylinositol, phosphatidylinositol mannosid, phosphatidylglycerol and phosphatidylethanolamine. The DNA G+C content is about 66.7 mol%.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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