Rhodococcus artemisiae sp. nov., an endophytic actinobacterium isolated from the pharmaceutical plant Artemisia annua L.

Guo-Zhen Zhao,1 Jie Li,2 Wen-Yong Zhu,1 Shou-Zheng Tian,1 Li-Xing Zhao,1 Ling-Ling Yang,1 Li-Hua Xu1 and Wen-Jun Li1,2

The genus Rhodococcus is classified in the family Nocardiaceae of the suborder Corynebacterineae (Stackebrandt et al., 1997). Phylogenetic analysis based on the 16S rRNA gene shows that members of the genus Rhodococcus can be assigned to four (Rainey et al., 1995) or six subclades (Gürtler et al., 2004). The genus Rhodococcus has undergone considerable expansion in the past few years and, at the time of writing, more than 32 species were recognized. As these micro-organisms exhibit a broad metabolic diversity, particularly with respect to hydrophobic compounds, they have attracted interest with regard to the biochemical and genetic characterization of their metabolic capabilities and they have also been considered to be excellent candidates for use in bioremediation treatment (Finnerty, 1992; Warhurst & Fewson, 1994; Bell et al., 1998).

It now appears that endophytes are a relatively untapped source of novel natural products for exploitation in medicine, agriculture and industry (Strobel & Daisy, 2003). Moreover, it has been suggested that there are many opportunities to find novel and interesting endophytic micro-organisms from plants in different settings and ecosystems. During research on the endophytic actinobacteria of Artemisia annua L., a pharmaceutical plant, strain YIM 65754T was isolated from a stem collected from Yunnan province, south-west China.

Samples were thoroughly washed in running water, employing a sonication step (160 W), to remove all soil and organic matter, and dried at room temperature. Tissue segments were surface-sterilized by immersing in 0.1 % Tween 20 for 1 min, followed by 5 % (available Cl2) NaClO for 6 min. The samples were then rinsed in 2.5 % (w/v) Na2S2O3 for 10 min to remove the residual chlorine (Miché & Balandreau, 2001;
Qin et al., 2009) and washed with sterilized H₂O at least three times. Finally, the samples were immersed in 70 % (v/v) ethanol for 6 min, washed with sterile water at least three times, placed on sterile plates with filter paper in a laminar flow cabinet and left to dry. To confirm that the surface disinfection process had been successful, 0.2 ml final washing water was spread onto each of the isolation media and yeast extract-malt extract agar [International Streptomyces Project (ISP) medium 2] (Shirling & Gottlieb, 1966) and incubated at 28 °C for 1–3 weeks. To isolate strain YIM 65754T, 1 g sample was ground with a mortar and pestle, added to 9 ml sterile water, serially diluted to 10⁻³ and spread on sodium propionate-asparagine-salt agar (pH 7.2) supplemented with 3 % (w/v) NaCl, as described by Qin et al. (2009), containing (1⁻¹) 25 mg nalidixic acid and 50 mg nystatin. A pure culture was obtained by repeated streaking on half-strength ISP 2. Strain YIM 65754T was routinely cultivated on ISP 2 at 28 °C and stored in 20 % (v/v) glycerol at −80 °C.

Gram-staining was carried out using the standard Gram-reaction and cell motility was confirmed by the development of turbidity throughout a tube containing semisolid medium (Leifson, 1960). To investigate cell morphology, cells of strain YIM 65754T were cultivated aerobically at 28 °C on ISP 2 and observed by light microscopy (BH2; Olympus) and scanning electron microscopy (Quanta 200; FEI). Growth on ISP 3–5 (Shirling & Gottlieb, 1966), Czapek’s agar, potato-glucose agar and nutrient agar was also evaluated. Colony colour was determined by comparison with standard sample chips from the ISCC-NBS colour chart (Kelly, 1964).

The morphology of strain YIM 65754T was consistent with assignment to the genus Rhodococcus. Cells were aerobic, Gram-positive and non-motile. Colonies were smooth, creamy pink, opaque and convex with slightly irregular edges on ISP 2. Scanning electron microscopy revealed that strain YIM 65754T formed filaments or preliminary branches at an early phase of growth (18 h) and fragmented into short rods during the exponential phase (36–48 h); most cells appeared as cocci in the stationary phase (60 h) (Supplementary Fig. S1, available in IJSEM Online). Thus, the results confirmed that strain YIM 65754T had a rod-coccus cycle during its growth phase. The isolate grew well on potato-glucose agar and nutrient agar, but did not grow on ISP 3, ISP 4 or Czapek’s agar.

Growth at 4, 10, 15, 20, 28, 37, 40, 42, 45 and 50 °C was tested on tryptic soy agar (TSA) for 21 days. Growth at pH 4.0–10.0 (at intervals of one pH unit) was tested in tryptic soy broth (TSB) using the buffer system described by Xu et al. (2005). Growth with 0, 1, 3, 5, 7, 9, 10, 12, 15 and 20 % (w/v) NaCl was tested at 28 °C for 14–21 days on TSA. Hydrolysis of starch, gelatin, and Tweens 20, 40 and 80 was determined as described by Smibert & Krieg (1994). Oxidase activity was determined from the oxidation of tetramethylp-phenylenediamine. Catalase activity was detected by the production of bubbles after the addition of a drop of 3 % H₂O₂. Nitrate reduction was determined as described by Lányi (1987). For determination of other physiological characteristics, carbon source utilization and acid production from carbohydrates, the media and procedures described by Gordon et al. (1974) were used.

Strain YIM 65754T grew optimally at 20–37 °C and grew slightly at 10, 15 and 40 °C, but did not grow at 4, 42, 45 or 50 °C. The isolate grew at pH 6.0–9.0 (optimum pH 7.0–8.0). The isolate grew with 0–7 % (w/v) NaCl, although growth was very weak with 7 % NaCl, but did not grow with 9 % (w/v) NaCl. Strain YIM 65754T was catalase-positive and oxidase-negative and hydrolysed Tweens 20 and 40, but did not hydrolyse Tween 80, starch, casein, gelatin or urea. H₂S was not produced and nitrate was not reduced. L-Arabinose, cellobiose, dulcitol, D-fructose, D-galactose, glucose, glyceral, maltose, D-mannitol, D-mannose, L-rhamnose, ribose, sucrose and xylose were utilized as sole carbon sources and acid was produced from L-arabinose, glucose and ribose. Further details and differential characteristics of the isolate and related strains are given in Table 1.

For chemotaxonomic analysis, biomass was obtained from cultures grown in TSB in shake flasks (about 200 r.p.m.) for 7 days at 28 °C. The diaminopimelic acid isomer and the sugars of whole-cell hydrolysates were analysed 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 described procedures (Minnikin et al., 1979; Collins & Jones, 1980). Menaquinones were extracted (Collins et al., 1977) and separated by HPLC (Tamaoka et al., 1983). Mycolic acids were extracted and analysed according to the protocol of 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 the Microbial Identification software Sherlock version 4.0 (MIDI database TSBA40). The G+C content of the genomic DNA was determined by HPLC according to Mesbah et al. (1989), using Escherichia coli JM-109 as the reference strain.

Strain YIM 65754T contained meso-diaminopimelic acid as the diagnostic diamino acid and the whole-cell hydrolysates were rich in arabinose, galactose, mannose and glucose (cell-wall chemotype IV sensu Lechevalier & Lechevalier, 1970). The predominant menaquinone was MK-8(H₂) (97.8 %); the minor component was MK-8 (2.2 %). Mycolic acids that co-migrated with those of DSM 44553T were present. The fatty acid composition contained a mixture of straight-chain saturated, unsaturated, branched and 10-methyl fatty acids, including C₁₆:0 (27.83 %), iso-C₁₅:0 2-OH and/or C₁₆:1ω7c (20.21 %), 10-methyl C₁₈:0 (17.50 %), C₁₈:1ω9c (8.82 %), 10-methyl C₁₆:0 (7.98 %), C₁₇:1ω5c (5.50 %), C₁₉:1ω9c and/or C₁₉:1ω11c (1.99 %), C₁₄:0 (1.95 %), C₁₈:0 (1.71 %), C₁₇:0 (1.28 %), C₁₈:1ω7c (1.26 %), C₁₇:1ω7c (1.11 %), 10-methyl C₁₇:0 (0.80 %) and C₁₉:0 (0.45 %), which is very
similar to those described for recognized members of the genus *Rhodococcus*. The phospholipids were diposphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositolmannoside and traces of some unknown phospholipids (Supplementary Fig. S2, available in IJSEM Online; phospholipid type II sensu Lechevalier et al., 1977). The DNA G+C content of strain YIM 65754<sup>T</sup> was 66.2 mol%, which is in accordance with values for the genus *Rhodococcus*. All these chemotaxonomic properties of strain YIM 65754<sup>T</sup> were consistent with its classification in the genus *Rhodococcus*.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were performed as described previously (Li et al., 2007). The 16S rRNA gene sequence of strain YIM 65754<sup>T</sup> was compared against those of cultured species using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the EzTaxon database (http://www.eztaxon.org; Chun et al., 2007) to retrieve the most similar sequences of recognized bacteria. Multiple alignment and sequence similarity calculation were carried out using CLUSTAL X (Thompson et al., 1997). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) tree-making algorithms in MEGA version 4.0 (Tamura et al., 2007), PHYLIP version 3.6 (Felsenstein, 2002) and PhyML (Guindon & Gascuel, 2003), respectively. The topologies of the phylogenetic trees were evaluated using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. DNA–DNA relatedness was studied according to the fluorometric micro-well method (Ezaki et al., 1989; Christensen et al., 2000; He et al., 2005) with six replications.

An almost-complete 16S rRNA gene sequence (1516 bp) was determined in this study. Sequence comparison showed clearly that strain YIM 65754<sup>T</sup> was a member of the family Nocardiaceae. High 16S rRNA gene sequence similarity between the isolate and described members of the genus *Rhodococcus* (94.3–98.4 %) supported the addition of strain YIM 65754<sup>T</sup> to the genus *Rhodococcus*. The highest sequence similarities were shown with *R. pyridinivorans* PDB<sup>T</sup>, *Rhodococcus zopfii* DSM 44108<sup>T</sup>, *Rhodococcus gordoniae* W 4937<sup>T</sup>, *Rhodococcus phenolicus* G2P<sup>T</sup> and *Rhodococcus rhodochrous* DSM 43241<sup>T</sup> (98.4, 98.3, 97.9, 97.9 and 97.8 %, respectively). Sequence similarities between strain YIM 65754<sup>T</sup> and other members of the genus *Rhodococcus* ranged from 97.4 % (*Rhodococcus coprophilus* DSM 43347<sup>T</sup>) to 94.3 % (*Rhodococcus ceridiphylli* YIM 6003<sup>T</sup>). Moreover, the neighbour-joining, maximum-parsimony and maximum-likelihood tree-making algorithms showed that strain YIM 65754<sup>T</sup> formed a coherent cluster with *R. pyridinivorans* PDB<sup>T</sup>, *R. gordoniae* W 4937<sup>T</sup> and *R. rhodochrous* DSM 43241<sup>T</sup>, with bootstrap values of 86, 79 and 78.3 %, respectively (Fig. 1).

To establish whether strain YIM 65754<sup>T</sup> represented a distinct genomic species, chromosomal DNA–DNA hybridization was performed between strain YIM 65754<sup>T</sup> and *R. pyridinivorans* DSM 44555<sup>T</sup> at the optimal hybridization temperature (46 °C). DNA–DNA hybridization with *R. zopfii* DSM 44108<sup>T</sup> and *R. phenolicus* DSM 44812<sup>T</sup> was not carried out because they were placed in different phylogenetic clusters. We also did not investigate DNA–DNA relatedness between strain YIM 65754<sup>T</sup> and type strains that showed <98.0 % 16S rRNA gene sequence similarity with the reference strains. Therefore, YIM 65754<sup>T</sup> was not classified as a genomic species of *Rhodococcus* but was described as *Rhodococcus artemisiae* sp. nov. Strain YIM 65754<sup>T</sup> is available from the China General Microbiological Culture Collection Centre (CGMCC) with the collection number CGMCC 1.40212T.

**Table 1. Different physiological characteristics of strain YIM 65754<sup>T</sup> and its closest phylogenetic neighbours**

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<th>DSM 44555&lt;sup&gt;T&lt;/sup&gt;</th>
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<th>W 4937&lt;sup&gt;T&lt;/sup&gt;</th>
<th>Rhodococcus coprophilus DSM 43347&lt;sup&gt;T&lt;/sup&gt;</th>
<th>Rhodococcus ceridiphylli YIM 6003&lt;sup&gt;T&lt;/sup&gt;</th>
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*Acid produced.

**Strains: 1, Rhodococcus artemisiae** sp. nov. YIM 65754<sup>T</sup>; 2, *R. pyridinivorans* DSM 44555<sup>T</sup>; 3, *R. rhodochrous* DSM 43241<sup>T</sup>; 4, *R. gordoniae* W 4937<sup>T</sup>. Data for columns 1–3 were taken from this study and for column 4 from Jones et al. (2004). All strains are Gram-positive, non-motile and catalase-positive, grow under aerobic conditions, utilize L-arabinose, cellobiose, D-fructose, D-galactose, glucose, glycerol, maltose, D-mannitol, D-mannose, L-rhamnose, ribose, D-sorbitol, sucrose and xylose as sole carbon sources, and produce acid from L-arabinose and glucose. +, Positive; w, weakly positive; −, negative; ND, no data available.
similarity, as it has been shown that some Rhodococcus
cpecies share high 16S rRNA gene sequence similarity (98.6–
99.8 %) but low DNA–DNA relatedness (Ghosh et al., 2006;
Li et al., 2008; Mayilraj et al., 2006; Wang et al., 2010; Xu et al., 2007; Zhang et al., 2005). For example, Wang et al.
(2010) reported 10.6–27.7 % as the highest DNA–DNA
relatedness value among R. qingshengii djl-6T, R. baikonuresis
GTC 1041 T, R. erythropolis DSM 43066 T, R. globerulus 
DSM 43954 T and R. jialingiae djl-6-2 T (DG90961) 
with 98.75–99.80 % 16S rRNA gene sequence
similarity. Similarly, Rhodococcus kroppenstedtii K07-23 T 
and Rhodococcus corynebacterioides share 62 % DNA–DNA
relatedness but 98.6 % 16S rRNA gene sequence similarity
(Mayilraj et al., 2006). DNA–DNA relatedness between
strain YIM 65754 T and R. pyridinivorans DSM 44555 T was
determined to be 50.3 ± 3.6 %, which is below the cut-off
point recommended by Stackebrandt & Goebel (1994) for
the circumscription of bacterial genomic species and
confirms the separation of strain YIM 65754 T from its
nearest phylogenetic neighbour. The data further supported
the conclusion drawn by Yassin (2005) that representatives
of the genus Rhodococcus with >98 % 16S rRNA gene
sequence similarity share whole-genome relatedness well
below the 70 % cut-off point recommended for the
delineation of bacterial species. The phylogenetic distinct-
eness and DNA–DNA hybridization data were sufficient
to categorize strain YIM 65754 T as distinct from recognized
species of the genus Rhodococcus.

As well as the genotypic evidence, strain YIM 65754 T could
be distinguished from its closest phylogenetic neighbours
by various physiological characteristics (Table 1). Most characteristics
of strain YIM 65754 T were consistent with those of its closest relatives; however, there were some
differences in the utilization of dulcitol, myo-inositol, lactose, raffinose and sodium acetate, degradation of
gelatin, nitrate, starch and urea, and conditions for growth.

On the basis of the data described above, strain YIM
65754 T represents a novel species of the genus Rhodococcus,
for which the name Rhodococcus artemisiae sp. nov. is
proposed.

Description of Rhodococcus artemisiae sp. nov.
Rhodococcus artemisiae (ar.te.mi.si’a.e. L. n. artemisia
mugwort, also a plant genus; L. gen. n. artemisiae of
Artemisia, isolated from Artemisia annua L.).
Gram-positive, aerobic, non-motile and mesophilic actinobacterium. Grows well on ISP 2 and potato-glucose agar and weakly on ISP 5 and nutrient agar, but not on ISP 3, ISP 4 or Czapek's agar. Colonies are smooth, creamy pink, opaque and convex with slightly irregular edges on ISP 2 after 3–7 days at 28 °C. Exhibits a rod–coccus cycle. Grows at 10–40 °C (optimum 20–37 °C), at pH 6.0–9.0 (optimum pH 7.0–8.0) and with 0–7% NaCl. Positive for catalase and hydrolysis of Tween 20 and 40 but negative for oxidase, milk coagulation and peptonization, and hydrolysis of Tween 80, starch, casein, gelatin and urea. H2S is not produced and nitrate is not reduced. Utilizes L-arabinose, cellobiose, dulcitol, D-fructose, D-galactose, glucose, glycerol, maltose, D-mannitol, D-mannose, L-rhamnose, ribose, D-sorbitol, sucrose and xylose as sole carbon sources, but not myo-inositol, lactose, rafinose or sodium acetate. Acid is produced from L-arabinose, glucose and ribose. Utilizes L-alanine, L-arginine, L-asparagine, glycine, L-hydroxyproline, L-lysine, L-phenylalanine, L-serine, L-tyrosine and L-valine as sole nitrogen sources, but not hypoxanthine or xanthine. Whole-cell hydrolysates are rich in meso-diaminopimelic acid, arabinose, galactose, mannose and glucose (cell-wall chemotype IV). The predominant menaquinone is MK-8(H2) and mycolic acids are present. The major fatty acids (>5%) are C16:0, iso-C15:0 2-OH and/or C16:1ω7c, 10-methyl C18:0, C18:1ω9c, 10-methyl C16:0 and C17:0ω5c. The phospholipids are diphasphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositolmannoside and traces of some unknown phospholipids (phospholipid type II).

The type strain, YIM 65754^T (=CCTCC AA 209042^T =DSM 45380^T), was isolated from surface-sterilized stem of *Artemisia annua* L., collected from Yunnan province, south-west China. The DNA G+C content of the type strain is 66.2 mol%.

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**References**


Rhodococcus artemisiae sp. nov.


