Rhodococcus yunnanensis sp. nov., a mesophilic actinobacterium isolated from forest soil

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A Gram-positive, aerobic, non-motile, mesophilic strain, designated YIM 70056^T, was isolated from a forest soil sample in Yunnan Province, China. Phylogenetic analysis based on 16S rRNA gene sequences revealed that this isolate had less than 97·0 % similarity to any *Rhodococcus* species with validly published names, with the exception of *Rhodococcus fascians* (DSM 20669^T), which was found to be its closest neighbour (98·9 % similarity). Chemotaxonomic data, including peptidoglycan type, diagnostic sugar compositions, fatty acid profiles, menaquinones, polar lipids and mycolic acids, were determined for this isolate; the results supported the affiliation of strain YIM 70056^T to the genus *Rhodococcus*. The DNA G+C content was 63·5 mol%. The results of DNA–DNA hybridization with *R. fascians* DSM 20669^T, in combination with chemotaxonomic and physiological data, demonstrated that isolate YIM 70056^T represents a novel *Rhodococcus* species, for which the name *Rhodococcus yunnanensis* sp. nov. is proposed, with YIM 70056^T (=CCTCC AA 204007^T=KCTC 19021^T=DSM 44837^T) as the type strain.

Since the generic name *Rhodococcus* was first proposed by Zopf (1891), this genus has undergone numerous changes (Goodfellow *et al.*, 1998). The emergence of molecular systematics and numerical phenetic taxonomy has been instrumental in the assignment of species of the genus *Rhodococcus* to the following four 16S rDNA subclades: *Rhodococcus equi*, *Rhodococcus rhodnii*, *Rhodococcus rhodochrous* and *Rhodococcus erythropolis* (McMinn *et al.*, 2000).

During the study of the microbial flora of Yunnan Province in south-west China, several *Rhodococcus*-like actinobacteria strains were isolated and identified from forest soil samples. On the basis of partial 16S rRNA gene sequence data, most of these isolates, with the exception of strain YIM 70056^T, had 98·0–99·0 % similarity to *Rhodococcus* species with validly published names. Further study of strain YIM 70056^T, based on a polyphasic approach including analysis of complete 16S rRNA gene sequences, DNA–DNA hybridization, morphological observation and physiological and chemotaxonomic analysis, showed that this isolate represents a novel species of the genus *Rhodococcus*, for which the name *Rhodococcus yunnanensis* sp. nov. is proposed.

Strain YIM 70056^T was isolated on glycerol/asparagine agar (ISP5 medium; Shirling & Gottlieb, 1966) medium at 28 °C. Good growth was recorded for YIM 70056^T on almost all of the tested media, such as ISP2 (Shirling & Gottleib, 1966), trypticase soy agar (TSA; Difco) and peptone/yeast extract/glucose medium (Holdeman *et al.*, 1977). The strain was maintained on ISP5 and TSA agar slants at 4 °C and as glycerol suspensions (20 %, v/v) at −20 °C. Biomass for chemical and molecular systematic studies was obtained by cultivation in flasks of trypticase soy broth (Difco) (pH 7·0) at 28 °C for 1 week.

Extraction of genomic DNA and amplification of 23S rRNA and 16S rRNA genes were done as described by Xu *et al.* (2003). The product of amplification of the 23S rRNA gene sequence of strain YIM 70056^T, comprising 380 bp, indicated that this strain is a high-G+C-DNA Gram-positive bacterium, i.e. an actinobacterium (Stackebrandt *et al.*, 1997; Yu *et al.*, 2001). An almost-complete 16S rRNA

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 70056^T is AY602219.

An extended phylogenetic tree showing the phylogenetic relationships between strain YIM 70056^T and related strains is available as a supplementary figure in IJSEM Online.

gene sequence of strain YIM 70056^T, comprising 1466 bp, was obtained and compared with those of type strains within the genus *Rhodococcus* (downloaded from the GenBank/EMBL/DDBJ database). Phylogenetic analysis was performed using the software packages PHYLIP (Felsenstein, 1993) and MEGA version 2.1 (Kumar *et al.*, 2001) after multiple alignment of the data using CLUSTAL X (Thompson *et al.*, 1997). Distances (using distance options according to the Kimura two-parameter model; Kimura, 1980, 1983) were calculated and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis (1000 resamplings) was used to evaluate the tree topology of the neighbour-joining data (Felsenstein, 1985).

The 16S rRNA gene sequence of strain YIM 70056^T contained the signature nucleotides that are specific for the genus *Rhodococcus* (Goodfellow *et al.*, 1998) and showed the highest similarity (98·9 %) to that of *Rhodococcus fascians* DSM 20669^T (Fig. 1), but less than 97·0 % similarity to any other *Rhodococcus* species with validly published names. DNA–DNA hybridization experiments between strain YIM 70056^T and the marker strain *R. fascians* DSM 20669^T were performed using the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992). The hybridization value of the two strains was 40·1 %. The G+C content of the DNA was determined to be 63·5 mol% using the thermal denaturation (T_m) method (Marmur & Doty, 1962).

The morphological properties of strain YIM 70056^T were examined by using light microscopy (BH 2; Olympus) and electron microscopy (JEM-1010; JEOL). Cells were stained according to the classical Gram procedure after incubation for 24 h on ISP5 agar medium at 28 °C; their morphology was then checked. Strain YIM 70056^T was Gram-positive, with hyphae that fragmented into short rods/coccobacilli. Colony colour was determined by comparison with colour chips from the ISCC-NBS colour chart standard samples (Kelly, 1964); colonies were pale yellow to orange. The maximum diameter of colonies was approximately 0·5–1·0 mm after 3 days growth.

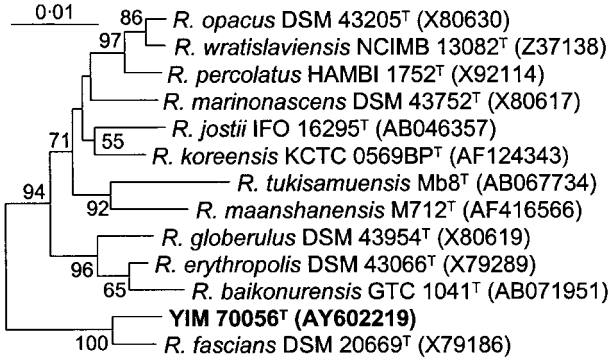

Fig. 1. Neighbour-joining tree showing the phylogenetic relationships among strain YIM 70056^T and related strains, based on 16S rRNA gene sequences. Numbers on branch nodes are bootstrap values (percentages of 1000 replicates). Bar, 1 nt substitution per 100 nt. An extended version of this tree is available as supplementary material in IJSEM Online.

All physiological and biochemical tests were performed at 28 °C. Carbon-source utilization tests, sugar fermentation analyses and qualitative enzyme tests were carried out using API ID 32E and API 50 CH test kits (bioMérieux). Catalase activity was detected from the production of bubbles after the addition of a drop of 3 % H_2O_2. Oxidase activity was determined from the oxidation of 1 % *p*-aminodimethylaniline oxalate. Growth at various NaCl concentrations was investigated in ISP 5 broth: growth of strain YIM 70056^T was observed in the presence of 0–10 % NaCl.

The amino acid and sugar contents of cell walls were determined according to procedures described by Stanek & Roberts (1974). Polar lipids were extracted, examined by two-dimensional TLC and identified using published procedures (Minnikin *et al.*, 1984). Menaquinones were isolated using the methods of Minnikin *et al.* (1984) and separated by HPLC (Kroppenstedt, 1982). The cellular fatty acid composition was determined as described by Sasser (1990), using the Microbial Identification System (MIDI). Mycolic acids were extracted and analysed as described by Klatte *et al.* (1994a).

Strain YIM 70056^T contained *meso*-LL-diaminopimelic acid as the diamino acid. Whole-cell hydrolysates were rich in arabinose and galactose (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970a, b). The phospholipids contained phosphatidylcholine, diphosphatidylglycerol and phosphatidylinositol mannoside. The major menaquinone was MK-8(H_2). The fatty acid and mycolic acid profiles of strain YIM 70056^T are described in detail in the species description.

The 16S rRNA gene sequence similarities between strain YIM 70056^T and the type strains of species with validly published names were below 97·0 %, except in the case of *R. fascians* DSM 20669^T, which showed 98·9 % similarity (Fig. 1); this indicates that strain YIM 70056^T represents a different species with respect to *Rhodococcus* species apart from *R. fascians* DSM 20669^T. A full phylogenetic tree is available as a supplementary figure in IJSEM Online. The 16S rRNA gene sequence of strain YIM 70056^T, containing the signature nucleotides that were specific to the *R. erythropolis* 16S rDNA subclade, confirms the view that the tested strain should be classified in that subclade. This assignment is supported by chemotaxonomic data, including peptidoglycan type, cell-wall sugar, menaquinone and polar lipid compositions and fatty acid and mycolic acid profiles.

Most of the physiological characteristics of strain YIM 70056^T are also consistent with those of *R. fascians* DSM 20669^T. However, strain YIM 70056^T could utilize maltose, acetamide, lactose and *N*-acetylglucosamine as sole carbon

Table 1. Characteristics that distinguish strain YIM 70056^T from the most closely related species of the genus *Rhodococcus*

Strains: 1, YIM 70056^T; 2, *R. fascians* DSM 20669^T; 3, *Rhodococcus maanshanensis* M712^T; 4, *Rhodococcus erythropolis* DSM 43066^T; 5, *Rhodococcus globerulus* DSM 43954^T; 6, *Rhodococcus koreensis* KCTC 0569BP^T; 7, *Rhodococcus marinonascens* DSM 43752^T; 8, *Rhodococcus opacus* DSM 43205^T; 9, *Rhodococcus percolatus* HAMBI 1752^T; 10, *Rhodococcus wratislaviensis* NCIMB 13082^T; 11, *Rhodococcus jostii* IFO 16295^T; 12, *Rhodococcus tukisamuensis* Mb8^T; 13, *Rhodococcus baikonurensis* GTC 1041^T. Data for all species except strain YIM 70056^T are from previous studies (Helmke & Weyland, 1984; Zhang *et al.*, 2002; Klatte *et al.*, 1994b; Briglia *et al.*, 1996; Yoon *et al.*, 2000; Goodfellow *et al.*, 2002; Takeuchi *et al.*, 2002; Matsuyama *et al.*, 2003; Li *et al.*, 2004). Characteristics are scored as follows: +, positive; W, weakly positive; −, negative; ND, undetermined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Morphogenetic sequence*	H–R–C	H–R–C	R–C	EB-R–C	EB-R–C	EB-R–C	H–R–C	H–R–C	H–R–C	EB-R–C	H–R–C	H–R–C	ND
Hydrolysis of:													
Aesculin	+	−	+	+	+	−	+	−	−	+	ND	+	+
Urea	−	+	+	+	+	+	−	+	+	+	−	−	+
Utilization as sole carbon source													
L-Arabinose	+	+	−	−	−	W	−	−	−	+	ND	ND	−
Arabitol	+	+	−	−	+	+	−	+	+	+	ND	−	−
D-Cellobiose	−	−	−	−	−	−	−	+	−	−	ND	+	ND
D-Galactose	+	+	+	−	−	+	−	+	+	+	−	+	−
myo-Inositol	−	−	−	+	−	+	+	+	+	+	−	−	−
D-Lactose	+	−	−	−	−	+	−	+	−	−	+	−	−
D-Maltose	+	−	+	+	+	+	−	+	−	−	+	+	−
D-Mannitol	+	+	−	+	+	+	−	+	+	+	+	−	ND
D-Mannose	+	+	+	−	+	+	+	+	+	+	ND		ND
L-Rhamnose	−	−	W	−	−	+	−	−	−	+	−	+	ND
D-Ribose	+	+	+	+	+	+	−	+	+	−	−	W	ND
D-Sorbitol	+	+	−	+	+	+	W	+	+	+	−	−	ND
D-Sucrose	+	+	+	+	+	+	−	+	+	+	−	+	ND
D-Trehalose	+	+	W	+	+	+	−	+	+	+	−		ND
D-Xylose	+	+	−	−	+	+	W	−	+	−	+	−	−
Mycolic acid (no. of carbons)	44–52	38–52	ND	34–38	ND	ND	ND	48–53	46–54	ND	ND	44–52	32–42

*Growth cycles: EB-R–C, elementary branching rod–coccus; H–R–C, hypha–rod–coccus; R–C, rod–coccus.

sources and could not hydrolyse starch, all of which distinguish it from *R. fascians* DSM 20669^T (Table 1). Additionally, the fatty acid and mycolic acid profiles of strain YIM 70056^T are distinguishable from those of *R. fascians* DSM 20669^T. The fatty acid profile is presented in the species description given below. In YIM 70056^T, the mycolic acids contain 44–52 carbon atoms, while those in *R. fascians* DSM 20669^T contain 38–52 carbon atoms. The DNA–DNA relatedness (40·1 %) between YIM 70056^T and *R. fascians* DSM 20669^T is below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the recognition of genomic species. This confirms that strain YIM 70056^T represents a novel species of the genus *Rhodococcus*, for which we propose the name *Rhodococcus yunnanensis* sp. nov.

Description of *Rhodococcus yunnanensis* sp. nov.

Rhodococcus yunnanensis (yun.nan.en′sis. N.L. masc. adj. *yunnanensis* pertaining to Yunnan, a province of south-west China).

Gram-positive, aerobic, non-motile, catalase-positive, oxidase-negative and mesophilic actinobacterium with hyphae that fragment into short rods/coccobacilli. Grows well on almost all media tested, such as ISP2, TSA and peptone/yeast extract/glucose medium. Colonies are pale yellow to orange, smooth, opaque and 0·5–1·0 mm in diameter. Strain YIM 70056^T is positive for lipase and alkaline phosphatase. It can hydrolyse Tweens 20, 40 and 80, while milk coagulation, nitrate reduction, β-glucuronidase, α-galactosidase, β-galactosidase, *N*-acetylglucosaminidase, β-glucosidase, urease and gelatin liquefaction tests are negative. Temperature range for growth is 10–40 °C, with an optimum temperature of 28–30 °C. Optimal pH for growth is 7·0–8·0. NaCl tolerance range is 0–10 %. Good growth occurs on almost all carbon sources tested, including glucose, D-ribose, acetamide, galactose, arabinose, lactose, mannose, mannitol, fructose, sucrose, arabitol, sorbitol, maltose and xylose, but acid is produced only from acetamide. It contains *meso*-LL-diaminopimelic acid, arabinose and galactose in whole-organism hydrolysates.

Predominant phospholipids are phosphatidylcholine, diphosphatidylglycerol and phosphatidylinositol mannoside. The major menaquinone is MK-8(H_2). The fatty acid profile is as follows: $C_{16:0}$, 18·3 %; $C_{18:1}\omega9c$, 11·4 %; 10-methyl $C_{18:0}$ (tuberculostearic acid), 15·6 %; $C_{14:0}$, 6·3 %; $C_{19:0}$, 3·2 %; $C_{15:0}$, 2·8 %; $C_{17:1}\omega8c$, 2·4 %; $C_{15:1}\omega5c$, 2 %; $C_{20:4}$, 1·8 %; 10-methyl $C_{16:0}$, 1·6 %; $C_{20:0}$, 1·3 %; and $C_{17:1}\omega5c$, 1·2 %. The mycolic acids range from C44 to C52: C48 (26 %), C50 (20 %), C46 (13 %), C49 (12 %), C51 (9 %), C52 (9 %), C44 (4 %), C45 (4 %) and C47 (4 %). The DNA G+C content is 63·5 mol%.

The type strain, strain YIM 70056^T (=CCTCC AA 204007^T=KCTC 19021^T=DSM 44837^T), was isolated from a forest soil sample collected in Yunnan Province in south-west China.

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