Rhodococcus kunmingensis sp. nov., an actinobacterium isolated from a rhizosphere soil

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A Gram-positive, aerobic, non-motile actinobacterium strain, designated YIM 45607^T, was isolated from a rhizosphere soil sample in Kunming, south-west China. Chemotaxonomically, the isolate contained chemical markers that supported its assignment to the genus *Rhodococcus*. On the basis of 16S rRNA gene sequence similarity analysis, strain YIM 45607^T formed a new subline within the genus *Rhodococcus*, with *Rhodococcus equi* as its closest phylogenetic neighbour (98.2 % 16S rRNA gene sequence similarity to the type strain). However, DNA–DNA hybridization demonstrated that strain YIM 45607^T was different from *R. equi* DSM 20307^T (35.4 % relatedness). Based on polyphasic analysis, strain YIM 45607^T could be clearly distinguished from other species of the genus *Rhodococcus*. The isolate therefore represents a novel species of *Rhodococcus*, for which the name *Rhodococcus kunmingensis* sp. nov. is proposed. The type strain is strain YIM 45607^T (=KCTC 19149^T =DSM 45001^T).

The genus *Rhodococcus* was classified into the family *Nocardiaceae* of the suborder *Corynebacterineae* (Stackebrandt *et al.*, 1997). With the emergence of molecular identification methods, particularly 16S rRNA gene sequencing, the classification of the rhodococci has been greatly improved. For example, members of the genus *Rhodococcus* have been assigned to four 16S rRNA subclades, represented by *Rhodococcus equi*, *Rhodococcus rhodnii*, *Rhodococcus rhodochrous* and *Rhodococcus erythropolis* (McMinn *et al.*, 2000), and the discovery of novel *Rhodococcus* species has been greatly facilitated. At the time of writing, more than 40 species are recognized and these micro-organisms exhibit broad metabolic diversity. In this study, the taxonomic position of an actinobacterium was examined by using a polyphasic approach. On the basis of phenotypic, chemotaxonomic and genotypic characteristics, it is proposed that strain YIM 45607^T represents a novel species of the genus *Rhodococcus*.

Strain YIM 45607^T was isolated from a soil sample collected from the rhizosphere of *Taxus chinensis* in Kunming by the following method. Soil samples were air-dried for 7 days; 1 g soil was then suspended in 50 mM phosphate buffer (pH 7.0) containing 0.1 % sodium cholate and incubated at 45 °C for 1 h with vigorous shaking in order to disperse soil aggregates and restrain the growth of fast-growing bacteria. The soil–water suspension was centrifuged and 0.1 ml supernatant was resuspended in 9 ml of the same sterile buffer before being spread on humic acid-vitamins-gellan gum (HVG) medium (Suzuki *et al.*, 1999) and incubated at 28 °C for 30 days. The strain was cultivated on yeast extract-malt extract agar (ISP 2; Shirling & Gottlieb, 1966) and maintained as a glycerol suspension (20 %, w/v) at −70 °C.

To investigate its morphological properties, strain YIM 45607^T was cultivated aerobically at 28 °C on ISP 2. Cell morphology was examined by using light microscopy with a model BH-2 microscope (Olympus) and scanning electron microscopy (Philips XL30; ESEM-TMP). Growth on ISP 2, ISP 5 (Shirling & Gottlieb, 1966), trypticase soy agar (TSA; Difco) and nutrient agar was also evaluated. Colony colour was determined by comparison with colour chips from the ISCC-NBS colour chart standard samples (Kelly, 1964). The Gram reaction was tested using the non-staining method as described by Buck (1982).

Strain YIM 45607^T formed smooth, circular, convex, opaque and pink-pigmented colonies with entire margins

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that varied in diameter from 0.35 to 1.2 mm on ISP 2 after 7 days of incubation at 28 °C. The cells were Gram-positive, non-spore-forming and non-motile. Light microscopy revealed that cells of strain YIM 45607^T formed filaments or showed elementary branching at the early phase of growth (12 h) and fragmented into short rods during the exponential phase (24 h). Most cells appeared as cocci in stationary phase (64 h). Thus, the results confirmed that strain YIM 45607^T had a rod–coccus cycle during its growth phase. The strain grew well on ISP 2 and TSA and grew weakly on ISP 5, but did not grow on nutrient agar.

Utilization of carbohydrates and organic acids was determined by using the methods described by Shirling & Gottlieb (1966). Decomposition of adenine, casein, hypoxanthine, tyrosine, urea and xanthine was examined by using the methods of Gordon *et al.* (1974). Decomposition of gelatin, elastin, aesculin and starch was examined by using the methods of Goodfellow & Pirouz (1982). Enzyme activity tests were performed using API ZYM test kits (bioMérieux). The results were evaluated after incubation at 28 °C for 48 h. Antibiotic susceptibility was examined as described by Groth *et al.* (2004) using antibiotic discs (Himedia). Growth at different temperatures, pH and NaCl concentrations was determined on ISP 2. Catalase activity was determined by assessing bubble production in 3 % (v/v) H_2O_2 and oxidase activity was determined using a 1 % (w/v) solution of tetramethyl-*p*-phenylenediamine (Kovacs, 1956).

The isolate was positive for the enzymes alkaline phosphatase, esterase C4, esterase lipase C8, lipase C14, *N*-acetyl-β-glucosaminidase, leucine arylamidase, α-glucosidase, β-glucosidase, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, α-mannosidase and fucosidase and negative for acid phosphatase, cystine arylamidase, valine arylamidase, α-galactosidase, β-galactosidase, α-chymotrypsin and trypsin. The strain was resistant to clindamycin (2 μg), norfloxacin (10 μg) and trimethoprim (1.25 μg), but sensitive to amikacin (30 μg), amoxicillin (10 μg), ampicillin (10 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), netilmicin (30 μg), penicillin G (10 IU), rifampicin (5 μg), tetracycline (30 μg) (weak), tobramycin (10 μg) and vancomycin (30 μg). The detailed differential phenotypic properties are listed in Table 1 and other phenotypic characteristics are presented in the species description.

The amino acid and sugar contents of cell walls were determined according to the procedures described by Staneck & Roberts (1974). Mycolic acids were extracted and analysed according to the protocol of Minnikin *et al.* (1975) with *R. equi* DSM 20307^T as the reference strain. Polar lipids were extracted as described by Minnikin *et al.* (1979) and identified by two-dimensional TLC and

Table 1. Differentiating characteristics between strain YIM 45607^T and its closest phylogenetic neighbours

Strains: 1, YIM 45607^T; 2, *R. equi* DSM 20307^T; 3, *R. opacus* DSM 43205^T; 4, *R. wratislaviensis* DSM 44107^T; 5, *R. triatomae* DSM 44892^T. All results are from this study except those for *R. triatomae* DSM 44892^T (taken from Yassin, 2005). +, Positive; − negative; w, weakly positive. All of the strains are positive for utilization of sodium citrate, glucose and acetate. All of the strains are negative for hydrolysis of casein, elastin and hypoxanthine.

Characteristic	1	2	3	4	5
Growth cycle*	H–RC	H–RC	H–RC	EB–RC	H–RC
Hydrolysis of:					
Aesculin	+	−	+	−	−
Tyrosine	+	−	+	+	−
Urea	+	+	+	+	−
Utilization as sole carbon source of:					
Arabinose	+	−	+	+	−
Lactose	+	−	+	+	−
Mannitol	+	−	+	+	−
myo-Inositol	+	−	+	+	−
Raffinose	w	−	+	+	−
Rhamnose	+	−	+	+	−
Sodium gluconate	−	−	+	+	−
Sodium lactate	+	+	−	−	−
Sorbitol	+	−	+	+	−
Sucrose	+	−	+	+	−
Trehalose	w	−	+	+	−
Xylose	+	−	+	+	−
Growth at 37 °C	+	+	−	−	+

*H–RC, Hypha–rod/coccus; EB–RC, elementary branching–rod/coccus.

spraying with specific reagents (Collins & Jones, 1980). Menaquinones were extracted by using the method of Collins *et al.* (1977) and analysed by HPLC as described by Tamaoka *et al.* (1983). Biomass for quantitative fatty acid analysis of strain YIM 45607^T was prepared by scraping growth from TSA plates that had been incubated for 4 days at 28 °C. Analysis of the whole-cell fatty acid pattern followed the methods described by Sasser (1990) using the Microbial Identification System (MIDI).

Strain YIM 45607^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid and arabinose and galactose in cell-wall hydrolysates (cell-wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970). The non-diagnostic sugars ribose and glucose were also found in cell-wall hydrolysates. The polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannoside (phospholipid type II *sensu* Lechevalier *et al.*, 1977). MK-8(H_2) was the only menaquinone detected and mycolic acids were present which co-migrated with those of *R. equi* DSM 20307^T except for one band. The major fatty acids (>10 %) were $C_{16:0}$ (44.0 %), $C_{18:1}\omega9c$ (25.9 %) and $C_{16:1}\omega7c$ (10.2 %). All these chemotaxonomic markers support the assignment of strain YIM 45607^T to the genus *Rhodococcus*.

Genomic DNA extraction and PCR amplification of the 16S rRNA gene were performed as described by Li *et al.* (2007). An almost-complete 16S rRNA gene sequence (1421 bp) of strain YIM 45607^T was obtained and aligned with the 16S rRNA gene sequences of other *Rhodococcus* species (obtained from GenBank/EMBL/DDBJ) by using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic analysis was performed using the software package MEGA 3.1 (Kumar *et al.*, 2004). Distances (using distance options according to Kimura's two-parameter model; Kimura, 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).

Phylogenetic analysis showed that strain YIM 45607^T formed a distinct subclade within the genus *Rhodococcus* with *R. equi* DSM 20307^T (Fig. 1), and this result further confirmed the affiliation of strain YIM 45607^T to the genus *Rhodococcus*. The 16S rRNA gene sequence similarities of strain YIM 45607^T to type strains of species of the genus *Rhodococcus* with validly published names were below 97.0 % except those of *Rhodococcus opacus*, *R. wratislaviensis*, *R. triatomae* and *R. equi* (97.4, 97.6, 97.6 and 98.2 %, respectively). The G+C content of the genomic DNA was determined by HPLC according to Mesbah *et al.* (1989) and a value of 64.9 mol% was measured.

To determine whether strain YIM 45607^T represents a distinct species of the genus *Rhodococcus*, DNA–DNA hybridization was performed by applying the method of He *et al.* (2005) with five replications for each sample. Strain YIM 45607^T displayed low DNA–DNA reassociation with

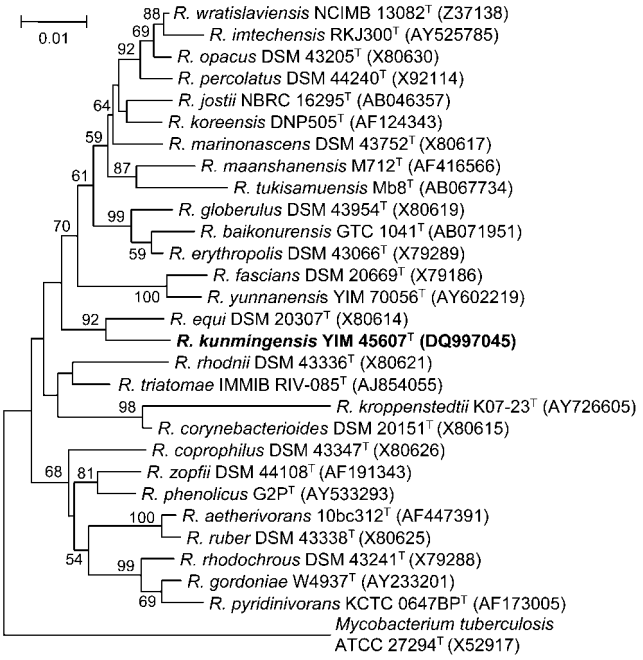

Fig. 1. Neighbour-joining tree showing the phylogenetic relationships between strain YIM 45607^T and related species of the genus *Rhodococcus*, based on 16S rRNA gene sequences. Numbers on branch nodes are bootstrap values (percentages of 1000 replicates). Bar, 1 substitution per 100 nucleotide positions.

R. opacus DSM 43205^T (46.5±4 %), *R. wratislaviensis* DSM 44107^T (40.3±7 %), *R. triatomae* DSM 44892^T (34.4±5 %) and *R. equi* DSM 20307^T (35.4±10 %) (means±SD). These results were consistent with the conclusion drawn by Yassin (2005) that representatives of *Rhodococcus* species with 16S rRNA gene sequence similarities greater than 98 % may share whole genomic DNA relatedness values well below the 70 % cut-off point recommended for delineation of bacterial genomic species (Wayne *et al.*, 1987).

The genotypic and phenotypic data (Table 1) described above suggest that strain YIM 45607^T could be distinguished from its closest phylogenetic neighbours. Therefore, strain YIM 45607^T represents a novel species of the genus *Rhodococcus*, for which the name *Rhodococcus kunmingensis* sp. nov. is proposed.

Description of *Rhodococcus kunmingensis* sp. nov.

Rhodococcus kunmingensis (kun.ming.en′sis. N.L. masc. adj. *kunmingensis* pertaining to Kunming, a city of Yunnan in south-west China).

Cells are Gram-positive, aerobic, acid-fast, non-spore-forming and non-motile. Forms filaments or shows elementary branching in the early growth phase and occurs mostly as cocci in stationary phase. Colonies are pink

pigmented (approx. 0.35–1.2 mm in diameter after 7 days of incubation at 28 °C), circular and convex with a smooth surface on ISP 2 and light-pink pigmented (approx. 0.23–1.35 mm in diameter after 7 days of incubation at 28 °C) on TSA. Growth occurs at 10–37 °C and pH 7.0–7.5; no growth below 10 °C or above 37 °C. Growth occurs in the presence of 7 % NaCl, but not above 7 % NaCl. Catalase-positive, oxidase-negative. H_2S is not produced and nitrate is reduced. Decomposes adenine, aesculin, L-arginine, L-asparagine, gelatin, L-histidine, L-proline, L-tyrosine and urea, but not casein, elastin, hypoxanthine or starch. Utilizes amygdalin, L-arabinose, butanediol, fructose, D-glucose, lactose, mannose, melibiose, sodium benzoate, sodium malate, sodium succinate, sucrose, tartrate, xylitol and L-xylose as sole carbon sources and utilizes raffinose and trehalose weakly, but does not utilize chitin, maltose, oxalate or propionate. Contains *meso*-diaminopimelic acid, and arabinose and galactose are present in cell-wall hydrolysates. MK-8(H_2) is the predominant menaquinone. The phospholipid pattern consists of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannoside. Mycolic acids are present. The fatty acid profile (>1 %) is as follows: $C_{16:0}$ (44.0 %), $C_{18:1}\omega9c$ (25.9 %), $C_{16:1}\omega7c$ (10.2 %), $C_{14:0}$ (6.9 %), 10-methyl $C_{18:0}$ (tuberculostearic acid) (4.8 %), $C_{18:0}$ (2.3 %), $C_{17:1}$ $\omega8c$ (1.5 %) and $C_{17:0}$ (1.4 %). The G+C content of genomic DNA of the type strain is 64.9 mol%.

The type strain, YIM 45607^T (=KCTC 19149^T =DSM 45001^T), was isolated from a rhizosphere soil sample collected in Kunming, south-west China.

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