Rhodococcus maanshanensis sp. nov., a novel actinomycete from soil

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A polyphasic study was undertaken to establish the taxonomic position of a soil isolate that had provisionally been assigned to the genus Rhodococcus. The organism showed a combination of phenotypic properties typical of rhodococci and formed a distinct phyletic line within the Rhodococcus erythropolis 16S rDNA subclade. The organism was readily distinguished from representatives of validly described species classified in this subclade on the basis of DNA–DNA relatedness and phenotypic data. Consequently, it is proposed that the organism be recognized as a novel species of Rhodococcus, Rhodococcus maanshanensis sp. nov. The type strain is strain M712T (= AS 4.1720T = JCM 11374T).

Keywords: Rhodococcus maanshanensis, polyphasic taxonomy, novel soil actinomycete

The application of chemosystematic, numerical phenetic and molecular systematic methods was instrumental in clarifying relationships within the genus Rhodococcus and between this taxon and related genera (Rainey et al., 1995; Goodfellow et al., 1998a, 1999; McMinn et al., 2000). The genus Rhodococcus belongs to the suborder Corynebacterineae Stackebrandt et al. 1997, which encompasses the genera Corynebacterium, Dietzia, Gordonia, Mycobacterium, Nocardia, Skermania, Tsukamurella, Turicella and Williamsia. Members of these taxa form a distinct phyletic line in the 16S rDNA tree and can be distinguished from one another by a combination of biochemical, chemical and morphological features (Goodfellow et al., 1999).

The taxonomic integrity of most of the 16 validly described species of Rhodococcus is underpinned by a wealth of genotypic and phenotypic data (Goodfellow et al., 1998a; Yoon et al., 2000a, b), though there is evidence that the genus is underspeciated (Colquhoun et al., 1998, 2000). The improved classification of the genus provides a sound framework for the recognition and description of additional species of Rhodococcus, which can be expected to include strains of ecological and industrial significance. It is important to establish the species richness of rhodococci in natural habitats, notably soil, in order to determine the roles that members of specific taxa play in nutrient and energy recycling.

The aim of the present investigation was to determine the taxonomic position of a rhodococcus-like strain, isolated from a soil sample, using a polyphasic taxonomic approach. The resultant data show that strain M712T should be recognized as a novel species of Rhodococcus, for which the name Rhodococcus maanshanensis sp. nov. is proposed.

Strain M712T was isolated on a modified Sauton’s agar plate (Mordarska et al., 1972) that had been incubated at 28°C for 7 days following inoculation with a suspension of a soil sample that had been collected from Maanshan Mountain in Anhui Province, China. Both the isolate and the marker cultures used in the DNA–DNA relatedness studies (Rhodococcus erythropolis DSM 43066T, Rhodococcus globularus DSM 43954T, Rhodococcus koreensis JCM 10743T, Rhodococcus marinonascens JCM 6241T, Rhodococcus opacus DSM 43205T and Rhodococcus percolatus DSM 44240T) were maintained on TSB agar slants [3% trypticase soy broth (BBL), 1.5% Bacto agar (Difco),
Table 1. Characteristics that distinguish strain M712<sup>T</sup> from the type strains of species classified in the *R. erythropolis* 16S rDNA subclade

Strains are identified as: 1, strain M712<sup>T</sup>; 2, *R. erythropolis* DSM 43066<sup>T</sup>; 3, *R. fascians* DSM 20669<sup>T</sup>; 4, *R. globerulus* DSM 43954<sup>T</sup>; 5, *R. koreensis* JCM 10743<sup>T</sup>; 6, *R. marinonascens* JCM 6241<sup>T</sup>; 7, *R. opacus* DSM 43205<sup>T</sup>; 8, *R. percolatus* DSM 44240<sup>T</sup>; 9, *R. wratislaviensis* DSM 44107<sup>T</sup>. Data for all species except strain M712<sup>T</sup> are from previous studies (Helmke & Weyland, 1984; Klatte et al., 1994; Briglia et al., 1996; Yoon et al., 2000a; Goodfellow et al., 2002). Characteristics are scored as: +, positive; w, weakly positive; −, negative.

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

by weight] at 4 °C and as glycerol suspensions (20%, v/v) at −20 °C.

The colonial properties of isolate M712<sup>T</sup> were recorded from modified Bennett’s agar [1% d-glucose, 0.1% beef extract, 0.2% casein enzymic hydrolysate (Sigma), 0.1% yeast extract, by weight; pH 7.2] and TSB agar plates that had been incubated for up to 6 days at 28 °C. The micromorphological properties of the isolate were recorded, on samples taken from a TSB plate, using light and scanning electron microscopy; in the latter case, gold-coated dehydrated preparations from a 6 day culture were examined using a Hitachi S-570 scanning electron microscope. Gram (Hucker’s modification; Society for American Bacteriologists, 1957) and Ziehl–Neelsen (Gordon, 1967) preparations were also examined by light microscopy.

Strain M712<sup>T</sup> was also examined for a range of phenotypic properties (see Table 1 and species description), using standard procedures (Goodfellow, 1971; Williams et al., 1983). In addition, acid production from carbohydrates was examined after Gordon et al. (1974) and the utilization of sole carbon and sole carbon and nitrogen sources was examined after Gordon & Mihn (1957) and Tsukamura (1966), respectively. Determination of tolerance to pH, temperature and NaCl regimes was carried out on TSB agar plates incubated for up to 7 days. Resistance to lysozyme was determined after Gordon et al. (1974).
Biomass for most of the chemosystematic studies was prepared following growth of the isolate and marker strains in shake flasks of TSB for 4 days at 28 °C; after checking for purity, the biomass was harvested by centrifugation, washed twice in distilled water and freeze-dried. Established HPLC and TLC procedures were used to determine the predominant isoprenoid quinones (Collins et al., 1977; Wu et al., 1989), the diagnostic isomers of diaminopimelic acid (A(pm; Lechevalier & Lechevalier, 1980), whole-organism sugars (Lechevalier & Lechevalier, 1980) and polar lipids (Minnikin et al., 1984). The acyl type of the peptidoglycan was determined by the method of Uchida & Aida (1977). The acid methanolsysis procedure was used to detect mycolic acids (Minnikin et al., 1984). Biomass for quantitative fatty acid analysis of strain M712T was prepared by scraping growth from TSB agar plates that had been incubated for 6 days at 28 °C; fatty acids were extracted, methylated and analysed using the MIDI (Microbial Identification) system.

Genomic DNA extraction, PCR amplification of 16S rDNA and purification of the PCR product from strain M712T were carried out using procedures described by Rainey et al. (1996). The purified product was sequenced directly using a Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) and universal primers as described previously (Lu et al., 2001). Sequence gel electrophoresis was performed and the nucleotide sequences were obtained automatically by using an Applied Biosystems DNA sequencer (model 377) and software provided by the manufacturer. The resultant 16S rDNA sequence was aligned manually with corresponding sequences of the type strains of Rhodococcus species and representatives of other constituent taxa of the suborder Corynebacterineae retrieved from the DDBJ/EMBL/GenBank databases. Evolutionary trees were inferred using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou & Nei, 1987) treeing algorithms. Evolutionary distance matrices were generated as described by Kimura (1980). The resultant unrooted tree topologies were evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings using the SEQBOOT and CONSENSE options from the PHYLIP suite of programs (Felsenstein, 1993).

Chromosomal DNA, which was extracted from the biomass of strain M712T following growth of the organism in TSB broth supplemented with glucose (0·75%, w/v) for 3 days at 28 °C, was purified using procedures described previously (Saito & Miura, 1963). The G+C content of the DNA was determined using the thermal denaturation (Tm) method (Marmur & Doty, 1962) using Escherichia coli AS 1.365 as the control. DNA–DNA relatedness values between strain M712T and the five marker strains of Rhodococcus were determined spectrophotometrically from renaturation values using established procedures (De Ley et al., 1970; Huß et al., 1983) and software (Jahnke, 1992).

It was evident from the polyphasic taxonomic study that strain M712T had properties typical of members of the genus Rhodococcus (Goodfellow et al., 1998a). The organism is an aerobic, Gram-positive, non-acid-fast, non-motile actinomycete, which forms a primary mycelium that fragments into rod- and coccoid-like elements (Fig. 1), produces cream-coloured colonies and contains meso-A(pm, arabinose and galactose in whole-organism hydrolysates (wall chemotype IV sensu Lechevalier & Lechevalier, 1970), N-glycolated muramic acid residues, predominant amounts of dihydrogenated menaquinones with eight isoprene units [MK-8(H8)], mycolic acids that co-migrate on TLC plates with those of the type strain of R. marinonascens, major amounts of diphosphatidylglycerol, phosphatidylyethanolamine and phosphatidylinositol mannosides (phospholipid type II sensu Lechevalier et al., 1977), DNA rich in G+C (G+C content 66·2 mol%) and mainly straight-chain saturated, unsaturated and 10-methyl-branched fatty acids (the predominant components as a proportion of the total fatty acids were C14:0, 4·5%; C15:0, 5·6%; C16:0, 29·7%; C17:0, 4·1%; C17:1ω9c, 5·3%; C18:1ω9c, 10·9%; 10-methyl-C17:0, 3·3%; and 10-methyl-C18:0, 16·7%). The lipid profile of the strain serves to distinguish it from members of all of the genera classified in the suborder Corynebacterineae, apart from Rhodococcus (Goodfellow et al., 1999). In addition, phylogenetic comparison of the almost complete 16S rDNA sequence (1411 nt of strain M712T)

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Fig. 1. Scanning electron micrograph of strain M712T showing rod and coccoid elements taken from TSB agar plates incubated at 28 °C for 6 days. Bar, 4·3 μm.
and corresponding sequences from representatives of the suborder *Corynebacterineae* showed that the organism belongs to the genus *Rhodococcus* (Fig. 2). The 16S rDNA sequence similarities between the tested strain and the type strains of *Rhodococcus* species ranged from 95.1 to 97.9%.

It is apparent from Fig. 2 that strain M712\(^T\) forms a distinct evolutionary line in the *R. erythropolis* subclade (Rainey *et al*., 1995; McMinn *et al*., 2000). The strain is most closely related to the type strain of *R. marinonascens*; the two organisms share 97.9–9% 16S rDNA similarity, which corresponds to 29 nt differences over 1411 positions. The organism is also closely related to the type strains of *R. erythropolis* (97.6% similarity, 34 nt differences), *R. fascians* (96.7%, 45 nt), *R. globerulus* (97.0%, 42 nt), *R. koreensis* (97.8%, 31 nt), *R. opacus* (97.6%, 33 nt), *R. percolatus* (97.7%, 31 nt) and *R. wratislaviensis* (97.4%, 37 nt). It is also interesting that, like other members of the *R. erythropolis* subclade, strain M712\(^T\) shows a unique nucleotide signature between positions 76 and 85 (Goodfellow *et al*., 1998a).

DNA–DNA relatedness studies provide a reliable way of distinguishing between representatives of species that share high 16S rDNA similarity (Goodfellow *et al*., 1998b). In the present study, strain M712\(^T\) showed relatively low levels of DNA–DNA relatedness with the type strains of *R. erythropolis* (33%), *R. globerulus* (21%), *R. koreensis* (35%), *R. marinonascens* (32%), *R. opacus* (30%) and *R. percolatus* (27%); all of these values are well below the 70% cut-off point recommended for the assignment of organisms to the same genomic species (Wayne *et al*., 1987). Strain M712\(^T\) can also be distinguished from the type strains of the constituent species of the *R. erythropolis* subclade by a range of phenotypic properties (Table 1).

It is evident from the genotypic and phenotypic data that strain M712\(^T\) merits recognition as a novel species of the genus *Rhodococcus*. It is, therefore, proposed that the organism be classified in the genus *Rhodococcus* as *Rhodococcus maanshanensis* sp. nov.

**Description of Rhodococcus maanshanensis** sp. nov.

*Rhodococcus maanshanensis* (ma.an.shan.en’sis. N.L. adj. *maanshanensis* referring to Maanshan, the source of the soil from which the organism was isolated).

Aerobic, Gram-positive, non-acid-fast, non-motile actinomycete that forms a branched substrate mycelium that fragments into rod- and coccoid-like elements. Convex, cream-coloured colonies with slightly irregular edges are formed on Bennett’s and TSB agars. Neither aerial hyphae nor diffusible pigments are formed. Nitrate is reduced to nitrite and catalase and urease are produced but the organism is oxidase-negative. Does not metabolize 2,4-dinitrophenol, 2,4,6-trichlorophenol or phenol. Adenine, aesculin, arbutin and Tweens 20, 60 and 80 are degraded but not casein, elastin, guanine, hypoxanthine, starch, tyrosine or xanthine. Acid is formed from D-fructose, D-glucose and glycerol but not from...
Acknowledgements

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