Rhodococcus kyotonensis sp. nov., a novel actinomycete isolated from soil

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A polyphasic study was undertaken to establish the taxonomic position of an isolate, strain DS472^T, from soil in Kyoto, Japan. Phylogenetic analysis, based on the 16S rRNA gene sequences, revealed that this strain constitutes a new subline within the genus *Rhodococcus*, with *Rhodococcus yunnanensis* YIM 70056^T and *Rhodococcus fascians* DSM 20669^T as its nearest phylogenetic neighbours (98.2 and 97.8 % sequence similarity, respectively). DNA–DNA hybridization experiments revealed 36 and 29 % relatedness between the isolate and its phylogenetic relatives, *R. yunnanensis* and *R. fascians*, respectively. Chemotaxonomic characteristics, including the major quinone MK-8(H₂), predominant fatty acids C_{16:0}, C_{18:1ω9c} and 10-methyl C_{18:0}, the presence of cell-wall chemotype IV and mycolic acids, were consistent with the properties of members of the genus *Rhodococcus*. The DNA G + C content was 64.5 mol%. On the basis of both phenotypic and genotypic evidence, strain DS472^T represents a novel species of the genus *Rhodococcus*, for which the name *Rhodococcus kyotonensis* sp. nov. is proposed. The type strain is strain DS472^T (=IAM 15415^T=CCTCC AB206088^T).

The improved classification of the genus *Rhodococcus* provides a sound framework for the recognition and description of additional *Rhodococcus* species (Goodfellow *et al.*, 1998, 1999). At the time of writing, the genus *Rhodococcus* was composed of 26 species with validly published names, including the recently described *Rhodococcus imtechensis* (Ghosh *et al.*, 2006) and *Rhodococcus kroppenstedtii* (Mayilraj *et al.*, 2006).

Strain DS472^T was isolated from a soil sample in Kyoto city, Japan and grown on nutrient agar (Difco) medium. The plates were incubated at 27 °C for 3 weeks. Single colonies from the plates were purified by transferring them onto new plates. Strain DS472^T was one of the isolated colonies and was used in this study. Strain DS472^T was maintained on a trypticase soy agar (TSA; BBL) slant at 4 °C and as a glycerol suspension sample (20 %, v/v) at –20 °C. Cell morphology was observed by light microscopy (BX60; Olympus). For phenotypic properties, the growth range for temperature and pH were tested on yeast extract-malt extract ISP2 medium (Shirling & Gottlieb, 1966), the pH range was tested with pH values ranging from 5.0 to 11.0. For pH 5.0–7.5, the pH was adjusted by the addition of 1 M HCl after adding 1 g NaHCO₃ l^{–1}. For

pH 8.0–11.0, different amounts of carbonate were used. Catalase activity was determined by 3 % H₂O₂, and bubble production was identified as a positive reaction. Oxidase was determined by cytochrome oxidase paper (Nissui Pharmaceutical). Tolerance of salinity was determined by inoculating the strains into ISP2 medium supplemented with 0–12 % (w/v) NaCl at 1 % intervals. API 50CH, API 20E and API ZYM strips (bioMérieux) were used to determine physiological and biochemical characteristics according to the manufacturer's instructions.

For cellular fatty acid analysis, the strain was grown on TSA (BBL) at 27 °C for 3 days, and fatty acid methyl esters were prepared and identified using the Microbial Identification System as described by Xie & Yokota (2003). For other chemotaxonomic analyses, freeze-dried cells were obtained from cultures grown in trypticase soy broth (TSB; BBL) at 27 °C for 5 days. The amino acid composition and isomers of diaminopimelic acid (DAP) in the cell walls were examined by two-dimensional TLC (Tokyo Kasei Co.) as described by Harper & Davis (1979), and by HPLC according to procedures described by Yokota *et al.* (1993). The alkaline methanolysis procedure was used to detect mycolic acids (Minnikin *et al.*, 1980) and whole-cell sugars were analysed by TLC (Yokota & Takashima, 2001). Respiratory quinones were extracted from dried cells (300 mg) using chloroform/methanol (2:1) and were purified by TLC. The purified respiratory quinones were analysed by reverse-phase HPLC (Komagata & Suzuki, 1987). The DNA G + C content was determined by HPLC as described by Mesbah *et al.* (1989).

Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DS472^T is AB269261.

The characteristics that distinguish strain DS472^T from the closely related species of the genus *Rhodococcus* are presented in a supplementary table available with the online version of this paper.

An approximately 1500 bp fragment of the 16S rRNA gene sequence was amplified by PCR and sequenced as described by Xie & Yokota (2003). The 16S rRNA gene sequence of strain DS472^T was compared with the sequences obtained from GenBank and aligned by using the CLUSTAL_X software package (Thompson *et al.*, 1997), evolutionary distances and the K_{nuc} value (Kimura, 1980) were calculated. Alignment gaps and ambiguous bases were not taken into consideration in the calculations. A phylogenetic tree based on a comparison of 1210 bases was constructed using the neighbour-joining method (Saitou & Nei, 1987). The topology of the tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates, whereas similarity values were calculated by using MEGA3 (Kumar *et al.*, 2004). DNA–DNA hybridization was performed by using the photobiotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989), and using a multi-well plate reader (CytoFluoR; PerSeptive Biosystems). The experiment was performed with two replications for each sample and the DNA relatedness values were calculated as the mean of the two values.

Phylogenetic analysis suggests that the isolate belongs within the genus *Rhodococcus* and clustered with *Rhodococcus fascians* and *Rhodococcus yunnanensis* (Fig. 1). Comparison of almost-complete 16S rRNA gene sequence (1420 nt) showed that strain DS472^T shared the highest similarities with the sequences of *R. yunnanensis* YIM

70056^T (98.2 %) and *R. fascians* DSM 20669^T (97.8 %). DNA–DNA hybridization experiments revealed 36 and 29 % relatedness between the isolate and its phylogenetic relatives, *R. yunnanensis* and *R. fascians*, respectively, which is below the 70 % cut-off point recommended for the delineation of bacterial species by Wayne *et al.* (1987).

Chemotaxonomic analysis further supported the phylogenetic results. Cell wall analysis by HPLC and TLC indicated that glutamic acid, alanine and *meso*-DAP occur in a molar ratio of 1:2:0.6; whole-cell hydrolysates were rich in arabinose and galactose (cell wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970). These results are consistent with the properties of the genus *Rhodococcus* (Goodfellow, 1986). Moreover, strain DS472^T could be distinguished from the other species of the genus *Rhodococcus* by various phenotypic data, as shown in Supplementary Table S1 (available in IJSEM Online) and Table 1. Other characteristics are given in the species description. On the basis of phenotypic, chemotaxonomic and genotypic characteristics, it is proposed that strain DS472^T represents a novel species of the genus *Rhodococcus*, for which the name *Rhodococcus kyotonensis* sp. nov. is proposed.

Description of *Rhodococcus kyotonensis* sp. nov.

Rhodococcus kyotonensis (kyo.to.nen'sis. N.L. masc. adj. *kyotonensis* pertaining to Kyoto, the source of soil from which the organism was isolated).

Fig. 1. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences showing the relationship between strain DS472^T and representatives of the genus *Rhodococcus*. Numbers at nodes are bootstrap values based on 1000 resamplings; only values greater than 50 % are shown. Bar, 0.01 K_{nuc} .

Table 1. Comparison of phenotypic characteristics between strain DS472^T and its most closely related species of the genus *Rhodococcus*

+, Positive; –, negative.

Characteristic	DS472 ^T	<i>R. yunnanensis</i> IAM 15239 ^T	<i>R. fascians</i> IAM 12427 ^T
Growth temperature (°C)	10–37	10–40	15–37
Highest NaCl tolerance (%; w/v)	9	10	9
Enzyme assay (API ZYM)			
Esterase lipase	+	–	–
Valine arylamidase	+	–	+
Cystine arylamidase	+	–	–
Acid phosphatase	+	–	+
β-Glucuronidase	–	+	–
α-Galactosidase	–	+	–
β-Galactosidase	–	+	–
β-Glucosidase	–	+	–
N-Acetyl-β-glucosaminidase	–	+	–
Naphthol-AS-BI-phosphohydrolase	+	–	+

Gram-positive, non-motile, non-spore-forming and acid-fast actinomycete that forms non-branching hyphae which fragment into short rod-to-coccus elements. Colonies on TSA medium are yellow–orange-pigmented, smooth and opaque. Growth occurs well over a pH range (6.0–8.0) on the ISP2 medium; the optimal pH for growth is around 7.0. The temperature range for growth is 10–37 °C; with optimal growth temperature of 27–30 °C, growth cannot occur at 40 °C. The NaCl tolerance range is up to 9%. Catalase activity is positive, whereas oxidase activity is negative. Hydrolyses Tweens 20, 40, 60 and 80. Gelatin hydrolysis, citrate utilization, activities of ONPG, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, production of hydrogen sulfide and indole, and reduction of nitrate and nitrite are negative. Positive results for the activities of esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, α-glucosidase, urease, tryptophan deaminase, hydrolysis of aesculin and Voges–Proskauer test, but negative results for alkaline phosphatase, esterase (C4), lipase (C4), trypsin, chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosamidase, α-mannosidase and α-fucosidase. Utilizes glycerol, glucose, ribose, galactose, fructose, sorbose, rhamnose, myo-inositol, mannitol, sorbitol, sucrose, xylitol, D-arabitol, and gluconate as the sole carbon and energy sources, but erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl β-D-xyloside, mannose, dulcitol, methyl α-D-mannoside, methyl α-D-glucoside, N-acetyl-D-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, trehalose, inulin, melezitose, raffinose, starch, glycogen, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, 2-keto-gluconate and 5-keto-gluconate are not utilized. Diamino acid in the peptidoglycan is *meso*-DAP; arabinose and galactose dominate in the whole-cell hydrolysates (wall type IV). The percentage of major

menaquinone MK-8(H₂) is 94.6 %, minor menaquinones MK-7(H₂) and MK-9(H₂) is 2.8 and 2.6 %, respectively. Mycolic acids are present, which co-migrate with those of the type strain of *R. rhodochrous*. The fatty acids are C_{16:0} (40.8 %), C_{18:1ω9c} (18.1 %), 10-methyl C_{18:0} (17.5 %), C_{14:0} (7.2 %), iso-C_{15:0} 2-OH/C_{16:1ω7c} (5.5 %) and C_{15:0} (2.1 %). The DNA G + C content is 64.5 mol%.

The type strain, DS472^T (=IAM 15415^T=CCTCC AB206088^T), was isolated from the soil in Kyoto city, Japan.

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