Rhodococcus antrifimi sp. nov., isolated from dried bat dung of a cave

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A Gram-reaction-positive, high DNA G + C content, non-motile actinobacterium, strain D7-21T, was isolated from dried bat dung inside a natural cave and its taxonomic status was examined by using a polyphasic approach. The 16S rRNA gene sequence study showed that the isolate belonged to the genus Rhodococcus and formed a cluster with Rhodococcus defluvii (98.98 % gene similarity), Rhodococcus equi (98.62 %) and Rhodococcus kunmingensis (97.66 %). Whole-cell hydrolysates contained meso-diaminopimelic acid, arabinose and galactose as the diagnostic diaminomono acid and sugars. MK-8(H2) was the predominant menaquinone. The major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside, an unknown phosphoglycolipid and an unknown glycolipid. Mycolic acids were present. The major fatty acids were C16 : 0, C18 : 1ω9c and 10-methyl C18 : 0. The DNA G + C content was 70.1 mol%. A battery of phenotypic features and DNA–DNA relatedness data support that strain D7-21T (= KCTC 29469T = DSM 46727T) represents a novel species of the genus Rhodococcus, for which Rhodococcus antrifimi sp. nov. is proposed.

The genus Rhodococcus, which was first proposed by Zopf (1891), is a member of the family Nocardiaceae (order Corynebacteriales), which also consists of the genera Nocardia as the type genus, Gordonia, Millisia, Skermania, Smaragdocroccus and Williamsia (Zhi et al., 2009; Adachi et al., 2007). At the time of writing, this genus contains 39 species with validly published names and most of the members have been isolated from a variety of environmental sources, such as soils, air, bioreactor sludge, industrial wastewater, animal dung, the gut of insects, limestone, marine sediments, cold deserts and animals and plants (Jones & Goodfellow, 2012; Li et al., 2012; Nimaichand et al., 2013; Kämpfer et al., 2013, 2014). During a study on bacterial diversity from a cave on Jeju Island, Republic of Korea, many novel strains have been isolated from soils and bat dung inside natural caves (Lee, 2006a, b, c, 2013). The aim of present study was to describe the isolation and classification of another actinobacterium recovered from a natural cave by using a polyphasic taxonomic approach.

Strain D7-21T was isolated from dried bat dung inside a natural cave on Jeju Island, Republic of Korea, using starch-casein agar as the selective medium, as described by Lee (2006c). After incubation for 14 days at 30 °C, a colony was transferred onto ISP (International Streptomycetes Project) 2 medium (Shirling & Gottlieb, 1966) two or three times. The purified isolate was maintained as a 20 % (v/v) glycerol suspension at −20 °C and −80 °C. For phenotypic comparison and DNA–DNA hybridization experiments, Rhodococcus defluvii DSM 45893T, Rhodococcus equi DSM 20307T, Rhodococcus kunmingensis DSM 45001T and Rhodococcus wratislaviensis KACC 20817T were grown on ISP 2 medium at 30 °C. For examining cultural and morphological characteristics, cells of strain D7-21T were grown aerobically at 30 °C on ISP 2 agar medium. Cell morphology was examined by light microscopy (LABOPHOT equipped with phase-contrast optics; Nikon) and scanning electron microscopy (JSM-6700F; JEOL), with the cultures grown in ISP 2 broth at 30 °C for 11–24 days. For electron microscopy, cells attached to coverslips coated with a solution of poly-L-lysine were treated as described previously (Lee, 2006c) before observation. Growth on various media was checked using ISP 2–4 media (Shirling & Gottlieb, 1966), trypticase soy agar (TSA; Difco) and nutrient agar (NA;
Difco). Colony colours were determined on these media after incubation for 21 days at 30 °C. Growth at different temperatures (4, 10, 20, 30, 35, 37, 42 and 45 °C), at pH 4–10 (at intervals of 1 pH unit) and in the presence of 0–10 % (w/v) NaCl (at intervals of 2 %) was observed on ISP 2 agar plates. The pH of ISP 2 agar was adjusted using several buffers (0.1 M final concentration): sodium acetate buffer for pH 4–6, potassium phosphate buffer for pH 7–8, Tris/HCl buffer for pH 9 and glycine/NaOH buffer for pH 10. The results for growth tests were recorded after incubation for 21 days. Gram staining was performed by using the Colour Gram 2 kit (bioMérieux) according to the instructions of the manufacturer. Oxidase and catalase activities were observed by examining the oxidation of 1 % (v/v) tetramethyl-p-phenylenediamine and bubble production with a 3 % (v/v) hydrogen peroxide solution, respectively. Hydrolysis of aesculin, starch and urea, nitrate reduction and gelatin liquefaction were examined as described previously (MacFaddin, 1980). Decomposition of hypoxanthine, DL-tyrosine and xanthine were tested following the methods of Gordon et al. (1974). Utilization of carbohydrates and organic acids was examined using ISP 9 medium (Shirling & Gottlieb, 1966) as described previously (Lee, 2006c).

Strain D7-21T exhibited good growth on ISP 2 medium, TSA and NA, and moderate growth on ISP 3 and ISP 4 media. Strain D7-21T exhibited a rod–coccus cycle during the growth phase, which is typical for members of the genus Rhodococcus. Scanning electron microscopy revealed that strain D7-21T formed filaments or preliminary branches at the early phase of growth (11 h) and fragmented into short rods after 48 h of growth; cells consisted of short rods, ovals and cocci during incubation for 6–12 days and most cells appeared as ovals and cocci after 17 days of incubation (Fig. S1 available in the online Supplementary Material). The colour of colonies was creamy to light yellow. Soluble pigments were not produced on any media tested. Strain D7-21T utilized a broad range of carbohydrates as sole carbon source and showed growth at 10–35 °C, pH 7.0–9.0 and in the presence of up to 6 % (v/v) NaCl.

For 16S rRNA gene sequencing, determination of DNA base composition and DNA–DNA hybridization experiments, chromosomal DNA was isolated according to the method of Hopwood et al. (1985). PCR amplification of the 16S rRNA gene and its sequencing was performed by Solgent (Korea). For determining the phylogenetic affiliation of strain D7-21T, the 16S rRNA gene sequence of the isolate was preliminarily compared with the corresponding sequences contained in the GenBank database using the BLAST search. The CLUSTAL X program (Thompson et al., 1997) was used for multiple alignments of the 16S rRNA gene sequences of strain D7-21T and related actinobacterial taxa. Pairwise 16S rRNA gene sequence similarities were calculated using the EzTaxon-e server (Kim et al., 2012). Phylogenetic analyses were performed using three tree-making algorithms, namely neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods contained in PHYLIP software package (Felsenstein, 2008). A neighbour-joining tree was reconstructed from evolutionary distances calculated by the model of Jukes & Cantor (1969). The topology of the tree was evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining dataset, using 1000 replicates.

The 16S rRNA gene sequence (1379 nt) of strain D7-21T determined in this study was compared with the corresponding sequences of strains representing the genus Rhodococcus retrieved from the public databases. In a neighbour-joining tree (Fig. 1) based on comparison of 16S rRNA gene sequences with continuous stretches of 1202 nt present in all the strains, strain D7-21T fell within a clade encompassing members of the genus Rhodococcus and was clustered with the type strains of R. defluvii, R. equi and R. kunmingensis, with a bootstrap support value of 76 %. This cluster was also found in the maximum-parsimony tree. Determination of pairwise 16S rRNA gene sequence similarity showed that strain D7-21T was most closely related to R. defluvii DSM 45893T (98.98 % gene similarity), R. equi DSM 20307T (98.62 %) and R. wratislaviensis NCIMB 13082T (98.40 %). The other member of the phylogenetic cluster, R. kunmingensis DSM 45001T shared 16S rRNA gene sequence similarity of 97.66 % with strain D7-21T. The 16S rRNA gene sequence similarity values of strain D7-21T with the remaining type strains of species of the genus Rhodococcus ranged from 98.04 to 95.51 %.

DNA–DNA hybridization experiments between strain D7-21T and R. defluvii DSM 45893T, R. equi DSM 20307T and R. wratislaviensis KACC 20817T were performed fluorometrically using photobiotin-labelled DNA probes and micro-dilution wells (Ezaki et al., 1989). Hybridization was performed with five replications for each DNA pair and DNA–DNA relatedness was determined as described by Lee (2012). For determination of DNA G+C content, genomic DNA of strain D7-21T was treated with nuclease P1 and then alkaline phosphatase, and the resultant deoxy-nucleoside mixture was separated using reverse-phase HPLC (Mesbah et al., 1989) with a SUPELCOSIL LC-18 S (150 × 4.6 mm) column.

The DNA G+C content of strain D7-21T was 70.1 mol% as determined by HPLC and slightly higher than those of the phylogenetic neighbours determined in this study: R. defluvii DSM 45893T (68.7 mol%), R. equi DSM 20307T (67.9 mol%) and R. wratislaviensis KACC 20817T (67.0 mol%). Strain D7-21T exhibited DNA–DNA relatedness values of 45.4 % (51.2 % in a reciprocal test) with R. defluvii DSM 45893T, 29.3 % (44.4 %) with R. equi DSM 20307T and 30.8 % (41.4 %) with R. wratislaviensis KACC 20817T, respectively.

For chemotaxonomic analyses of strain D7-21T, cell biomass was obtained from cultures grown in ISP 2 broth for 7 days at 30 °C with shaking. Cell-wall analyses were
**Fig. 1.** Neighbour-joining tree showing the phylogenetic relationship of strain D7-21T within the clade encompassing members of the genus *Rhodococcus*. Asterisks indicate branches that were also found in both maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) trees. Numbers at nodes indicate bootstrap support values (>50%). Bar, 1 nucleotide substitution per position.
performed as described previously (Lee et al., 2000): the
diaminopimelic acid isomers (Staneck & Roberts, 1974)
and the acyl type (Uchida & Aida, 1984) of cell-wall pepti-
doglycan, and whole-cell sugars (Saddler et al., 1991). Polar
lipids and isoprenoid quinones were extracted using the
integrated procedure of Minnikin et al. (1984). Polar
lipids were analysed by two-dimensional TLC (Minnikin
et al., 1977). Isoprenoid quinones were separated by one-
dimensional TLC using hexane/diethyl ether (85:15, v/v)
and purified by column chromatography as described by
Collins (1985). The resultant isoprenoid quinones were ana-
ysed by reverse-phase HPLC (Kroppenstedt, 1985). Mycolic
acids were extracted by alkaline methanolysis as described
by Minnikin (1988) and analysed by one-dimensional TLC
(Minnikin et al., 1980). For determining cellular fatty acids,
strain D7-21 T, R. defluvii DSM 45893 T, R. equi DSM
20307 T, R. kunningsensis DSM 45001 T and R. wratislaviensis
KACC 20817 T were grown on TSA for 6 days at 30 °C. Fatty
acid methyl esters were prepared and analysed according to
the instructions of the Sherlock Microbial Identification
System (version 4.5; MIDI), using the TSBA50 library for
identification of each peak on chromatograms.

The results obtained from chemotaxonomic analyses sup-
ported the assignment of strain D7-21 T to the genus Rhodococcus.
The major menaquinone was MK-8(H2) (85% of the
total), with minor amounts of MK-6(H2) and MK-7 (H2).
Strain D7-21 T contained meso-diaminopimelic acid in the
cell wall. The acyl type of muramic acid was glycolyl type.
Whole-cell sugars detected were arabinose, galactose, glucose,
cell wall. The acyl type of muramic acid was glycolyl type.

Table 1. Differential characteristics of strain D7-21 T from the
type strains of closely related species of the genus Rhodococcus

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<td>ND</td>
<td>58.5</td>
<td>64.9</td>
<td>66–68</td>
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</table>

*EB-RC, elementary branching rod/coccus; RC, rod/coccus; H-RC, hypha-rod/coccus.
†Different from the result reported previously (Kampfer et al., 2014).
‡Different from the result reported previously (Wang et al., 2008).
§Data from Jones & Goodfellow (2012), Wang et al. (2008) and
Goodfellow et al. (2002).

Combined data obtained from polyphasic taxonomic ana-
yses support that strain D7-21 T should be assigned to a
novel species of the genus Rhodococcus, for which Rhodo-
coccus antrifimi sp. nov. is proposed.

Description of Rhodococcus antrifimi sp. nov.

Rhodococcus antrifimi (L. neut. n. antrum cave; L. masc. n.
fimus dung; N.L. gen. n. antrifimi of dung inside a cave,
referring to the sample from which the type strain was
isolated).

Cells are aerobic, Gram-reaction-positive, catalase-positive,
oxidase-negative. Good growth occurs on ISP 2 medium,
TSA and NA. Moderate growth occurs on ISP 3 and 4
media. A rod–coccus cycle during growth is observed.

Strains: 1, D7-21 T; 2, R. defluvii DSM 45893 T; 3, R. equi DSM 20307 T;
4, R. kunningsensis DSM 45001 T; 5, R. wratislaviensis KACC 20817 T.
All data were taken from this study, except for DNA G+C contents.
All strains utilized d-glucose and malate as carbon and energy sources
but did not utilize d-arabinose, cellobiose, d-galactitol, melibiose,
methyl 2,3-d-mannoside, raffinose, l-sorbose or d-xylitol. They
showed a positive response in aesculin hydrolysis but a negative
response in hydrolysis of casein, gelatin, hypoxanthine, starch and
"xanthine.
Colony colour is creamy (ISP 3 and 4 media, TSA and NA) to light yellow (ISP 2 medium). Growth temperature is between 10 and 35 °C. The pH range for growth is pH 7.0–9.0 (optimum, pH 7.0). Growth occurs in the presence of up to 6 % (w/v). Nitrate reduction occurs. Aesculin hydrolysis is observed but hydrolysis of casein, gelatin, hypoxanthine, starch, L-tyrosine, urea and xanthine is not observed. Acetate, D-glucose, malate and succinate are utilized as sole carbon and energy sources but adonitol, D-arabinose, L-arabinose, benzoate, cellobiose, citrate, dextrin, formate, D-fructose, galactitol, D-galactose, glycerol, myo-inositol, lactose, maltose, D-mannitol, D-mannose, melezitose, melibiose, methyl α-D-glucoside, methyl α-D-mannoside, raffinose, L-rhamnose, salicin, L-sorbitose, sucrose, L-tartrate, trehalose, D-xylitol and D-xylose are not utilized as sole carbon and energy sources. meso-Diaminopimelic acid, arabinose and galactose are present in whole-cell hydrolysates. The acyl type of muramic acid is glycolyl type. MK-8(H2) is the predominant menaquinone. The major polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidyl-inositol, phosphatidylinositol mannoside, an unknown phosphoglycolipid and an unknown glycolipid. Mycolic acids are present. The major fatty acids are C16:0, C18:1ω9c and 10-methyl C18:0.

The type strain, D7-21T (=KCTC 29469T=DSM 46727T), was isolated from dry bat dung inside a natural cave on Jeju Island, Republic of Korea. The G+C content of the genomic DNA of the type strain is 70.1 mol%.

Acknowledgement

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References


