Rhodococcus kroppenstedtii sp. nov., a novel actinobacterium isolated from a cold desert of the Himalayas, India

S. Mayilraj,¹ S. Krishnamurthi,¹ P. Saha¹ and H. S. Saini²

Correspondence

S. Mayilraj

mayil@imtech.res.in

¹Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh 160 036, India

²Department of Microbiology, Guru Nanak Dev University (GNDU), Amritsar 143 005, India

The taxonomic position of an actinomycete, strain K07-23^T, isolated from a cold desert of the Himalayas, India, was established by a polyphasic approach. The strain exhibited phenotypic characters that were typical of the genus *Rhodococcus*. 16S rRNA gene sequence (1467 bases) comparisons confirmed that strain K07-23^T belongs to the genus *Rhodococcus*. 16S rRNA sequence similarity studies showed that the isolate is very closely related to *Nocardia corynebacterioides* DSM 20151^T (98.6%), which has been recently reclassified as *Rhodococcus corynebacterioides*. It showed 94.4–96.6% sequence similarity with other species of the genus *Rhodococcus*. However, genomic relatedness between strain K07-23^T and *R. corynebacterioides* as revealed by DNA–DNA hybridization was low (62%). Based on polyphasic analysis, strain K07-23^T could be clearly distinguished from other species. It is proposed that strain K07-23^T (= MTCC 6634^T = DSM 44908^T = JCM 13011^T) represents a novel species of *Rhodococcus*, *Rhodococcus kroppenstedtii* sp. nov.

In this study, the taxonomic position of an actinobacterium, which was isolated during our study of microbial diversity of Lahaul-Spiti Valley, a cold desert of the Himalayas, India, was examined by a polyphasic approach. Analysis of strain K07-23^T showed that it belongs to the genus *Rhodococcus*, but is quite distinct from other species within the genus. Strain K07-23^T was isolated from a soil sample collected from the Lahaul-Spiti Valley by a dilution plating technique on tryptic soy agar (TSA; HiMedia) medium and maintained as glycerol stocks at –70 °C. The reference strain *Rhodococcus corynebacterioides* MTCC 699^T (= JCM 3376^T = DSM 20151^T) was taken from the Microbial Type Culture Collection (MTCC), Chandigarh, India.

Strain K07-23^T and *R. corynebacterioides* MTCC 699^T were tested for a range of phenotypic properties (Table 1). Gram reaction was determined using a HiMedia Gram Staining kit according to the manufacturer's instructions. Physiological tests such as growth at different temperatures, pH and NaCl concentrations were examined by growing the strain on basal tryptic soy broth (TSB) medium. Catalase activity and urea hydrolysis were determined as described by Cowan & Steel (1965). Hydrolysis of casein, gelatin, Tween 80 and starch, methyl red/Voges–Proskauer tests and oxidase

activity (using *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride) were determined as described by Smibert & Krieg (1994). Nitrate reduction was determined as described by Lanyi (1987). Growth on sole carbon sources was examined on carbon utilization agar (Shirling & Gottlieb, 1966). The ability to use sole nitrogen sources was studied according to Williams *et al.* (1983) with the modification that agarose (1.5%) was used instead of agar. Antibiotic susceptibility was examined as described by Groth *et al.* (2004) using antibiotic discs (HiMedia). For cellular fatty acid analysis, strains were grown on TSA medium at 30 °C for 36 h and fatty acid methyl ester analysis was performed using the Sherlock Microbial Identification System (MIDI) as described previously (Pandey *et al.*, 2002). Freeze-dried cells for other chemotaxonomic analyses were prepared following growth of the strains in TSB for 4 days at 30 °C. Standard procedures were used to determine the diagnostic cell wall sugars and amino acids (Staneck & Roberts, 1974). Phospholipids and menaquinones were extracted and analysed as described by Minnikin *et al.* (1984) and Kroppenstedt (1982). The presence of mycolic acids was demonstrated by TLC (Minnikin & Goodfellow, 1976) and the mycolic acid pattern was determined according to Klatte *et al.* (1994). The glycolic acid content of the bacterial cell wall was determined by the colour reaction method of Uchida & Aida (1984). Genomic DNA extraction, amplification, sequencing of the 16S rRNA gene and phylogenetic analysis were performed as described previously (Mayilraj *et al.*, 2005). DNA–DNA hybridization was

Published online ahead of print on 16 December 2005 as DOI 10.1099/ij.s.0.63831-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Rhodococcus kroppenstedtii* strain K07-23^T is AY726605.

Table 1. Comparison of phenotypic characters and G+C content of strain K07-23^T and *Rhodococcus corynebacterioides* MTCC 699^T

Both strains are orange-red-pigmented, do not produce endospores and are catalase-positive. Both produce acid from D-fructose, mannitol, D-glucose and sucrose; acid is not produced from L-rhamnose, D-galactose, trehalose, D-lactose, inulin, D-maltose, adonitol, L-arabinose, D-melibiose or dulcitol. Both are positive for hydrolysis of adenine and testosterone and negative for urea, casein, elastin, aesculin, gelatin, guanine, hypoxanthine, tyrosine and xanthine. +, Positive; –, negative; W, weakly positive.

Characteristic	Strain K07-23 ^T	<i>R. corynebacterioides</i> MTCC 699 ^T
Nitrate reduction	+	–
Tolerance to:		
Sodium azide (0.01 %)	+	W
8 % NaCl	+	–
10 % NaCl	W	–
Growth at 45 °C	–	+
Acid production from:		
Raffinose	+	–
meso-Inositol	+	–
Salicin	–	+
D-Xylose	+	–
Cellobiose	–	+
D-Mannose	–	+
Utilization as sole source of nitrogen:		
L-Arginine	+	W
L-Serine	+	W
L-Methionine	+	–
L-Histidine	+	–
L-Phenylalanine	+	W
Potassium nitrate	+	–
Utilization as sole source of carbon:		
Citrate	–	–*
Sodium lactate	+	–
L-Rhamnose	+	–
Salicin	–	+
Susceptibility to polymyxin B (300 µg)	+	–
DNA G+C content (mol%)	69.7	71.4

*Result differs from data published previously by Yassin & Schaal (2005).

performed using the membrane filter method (Tourova & Antonov, 1987) as described by Reddy *et al.* (2000). The G+C content of genomic DNA was determined by HPLC (Groth *et al.*, 1996).

Strain K07-23^T had chemical and phenotypic properties consistent with its classification in the genus *Rhodococcus*. The detailed differential phenotypic properties and G+C content are listed in Table 1 and other characters are given in the species description. The diagnostic cell wall amino acid is meso-A₂pm; the diagnostic cell wall sugars are arabinose and galactose (cell wall chemotype IV; Lechevalier & Lechevalier, 1970). The non-diagnostic sugars glucose and ribose were also found in the whole-cell hydrolysate. The acyl type was acetyl. The isolate had phospholipid pattern type PII, according to Lechevalier *et al.* (1977). Mycolic acids

with a chain length of 42–50 carbon atoms were present. Under similar growth conditions, quantitative differences in fatty acid composition were found between strain K07-23^T and its closest relative. Like all rhodococci, strain K07-23^T had a fatty acid pattern of type 1b (Kroppenstedt, 1985). Compared to *R. corynebacterioides*, strain K07-23^T had higher proportions of C_{16:0} (42.1 vs 36.5 % for K07-23^T and *R. corynebacterioides*, respectively) and C_{18:1ω9c} (28.5 vs 22.7 %), and lower proportions of C_{16:1ω7c} (10.1 vs 13.8 %), 10-Me C_{18:0} (6.1 vs 9.9 %), C_{17:1ω8c} (6.8 vs 9.0 %) and C_{14:0} (5.8 vs 8.2 %).

To determine the phylogenetic position of strain K07-23^T, the 16S rRNA gene sequence (1467 nt) was compared with those of type strains of species of the genus *Rhodococcus* retrieved from GenBank. Strain K07-23^T is closely related

to *R. corynebacterioides* (98.6 %) and distantly related to all other species studied (94.4–96.6 %). It is evident from the phylogenetic tree (Fig. 1) based on the neighbour-joining method that strain K07-23^T and *R. corynebacterioides* formed a separate phylogenetic branch along with *Rhodococcus triatomae* and *Rhodococcus equi*. Genomic relatedness, as shown by DNA–DNA hybridization of strain K07-23^T with the closely related strain *R. corynebacterioides*, was only 62 %. Other species of the genus can be excluded from DNA–DNA hybridization studies because of the dissimilarity (3–4 %) in 16S rRNA gene sequences (Stackebrandt & Goebel, 1994). This is 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 the delineation of bacterial species (Wayne *et al.*, 1987). The DNA G + C content of strain K07-23^T is 69.7 mol%. Based on phenotypic and genotypic data, strain K07-23^T is very clearly distinguishable from its closest phylogenetic relative and other species of the genus *Rhodococcus*. It is therefore concluded that strain K07-23^T represents a novel species of the genus *Rhodococcus*; the name *Rhodococcus kroppenstedtii* sp. nov. is proposed.

Description of *Rhodococcus kroppenstedtii* sp. nov.

Rhodococcus kroppenstedtii (krop.pen.sted'ti.i. N.L. gen. n. *kroppenstedtii* of Kroppenstedt, to honour Reiner Michael

Kroppenstedt, a German microbiologist, for his enormous contributions to the taxonomy of actinomycetes).

Cells are Gram-positive, acid-fast, non-motile, non-spore-forming, rod- and coccoid-like, occurring in groups. Colonies on TSA medium are orange-red-pigmented, small, smooth, glistening and convex. Catalase is produced, oxidase is not produced. Capable of growing in up to 9 % NaCl; grows weakly at 10 % NaCl, but not at 12 % NaCl. Temperature range for growth is 10–37 °C, with optimum growth at 30 °C; does not grow at 42 °C. pH range for growth is 5.0–11.0, with optimum growth at pH 8.0. Positive for hydrolysis of Tween 20, 40, 60, 80 and testosterone, but negative for hydrolysis of starch, casein, gelatin and urea. Acid is produced from raffinose, *meso*-inositol, D-lactose and L-rhamnose, but not from D-maltose, L-arabinose, D-galactose, D-glucose, D-xylose, sucrose, D-fructose or salicin. Negative for methyl red and Voges–Proskauer reactions and positive for nitrate reduction. Positive for utilization of L-arginine, L-serine, L-methionine, L-histidine, L-proline, L-phenylalanine and potassium nitrate as sole nitrogen sources. Positive for utilization of sodium lactate, sodium gluconate and *p*-hydroxybenzoate as sole carbon sources. Positive for tolerance to sodium azide (0.01 %) and negative for crystal violet (0.0001 %). Diagnostic cell wall amino acid is *meso*-A₂pm. Diagnostic cell wall sugars are arabinose and galactose, wall type IV; the non-diagnostic sugars ribose and glucose are also found. The acyl type is acetyl. Type II phospholipid. The strain synthesizes mycolic acids with a chain length of 42–50 carbon atoms showing

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences (1467 bases) showing the phylogenetic relationship between strain *Rhodococcus kroppenstedtii* K07-23^T and other species of the genus *Rhodococcus*. *Corynebacterium diphtheriae* was used as an outgroup. Bootstrap values (expressed as percentage of 1000 replications) greater than 50 % are given at the nodes. Bar, 1 % sequence variation.

1–3 double bonds in the chain. Tuberculostearic acid (10-methyloctadecanoic acid, 6.1 %) is present. Major fatty acids are C_{14:0} (5.8 %), C_{16:0} (42.07 %), C_{16:1ω7c} (10.1 %), C_{17:1ω8c} (6.8 %) and C_{18:1ω9c} (28.5 %). Major menaquinone is MK-8(H₂).

The type strain is K07-23^T (=MTCC 6634^T=DSM 44908^T=JCM 13011^T), isolated from soil, 1.5 feet below an ice glacier, 4200 m above sea-level, in Kibber village of the Spiti Valley, Himachal Pradesh, India. The DNA G+C content of strain K07-23^T is 69.7 mol%.

Acknowledgements

We would like to thank Dr Peter Schumann, DSMZ, for performing G+C analysis, Ms Gabi Poetter for the chemotaxonomic analysis and Mr Ganesan and Mr Malkit Singh for their excellent technical assistance. P.S. and S.K. are the recipients of CSIR research fellowships. Financial assistance from CSIR and DBT, Government of India is duly acknowledged. This is IMTECH communication number 15/2005.

References

- Cowan, S. T. & Steel, K. J. (1965). *Manual for the Identification of Medical Bacteria*. London: Cambridge University Press.
- Groth, I., Schumann, P., Weiss, N., Martin, K. & Rainey, F. A. (1996). *Agrococcus jenensis* gen. nov., sp. nov., a new genus of actinomycetes with diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 234–239.
- Groth, I., Rodriguez, C., Schutze, B., Schmitz, P., Leistner, E. & Goodfellow, M. (2004). Five novel *Kitasatospora* species from soil: *Kitasatospora arboriphila* sp. nov., *K. gansuensis* sp. nov., *K. nipponensis* sp. nov., *K. paranensis* sp. nov. and *K. terrestris* sp. nov. *Int J Syst Evol Microbiol* **54**, 2121–2129.
- Klatte, S., Kroppenstedt, R. M. & Rainey, F. A. (1994). *Rhodococcus opacus* sp. nov., an unusual nutritionally versatile *Rhodococcus*-species. *Syst Appl Microbiol* **17**, 355–360.
- Kroppenstedt, R. M. (1982). Separation of bacterial menaquinones by HPLC using reverse phase (RP-18) and a silver loaded ion exchanger. *J Liq Chromatogr* **5**, 2359–2367.
- Kroppenstedt, R. M. (1985). Fatty acid and menaquinone analysis of actinomycetes and related organisms. In *Chemical Methods in Bacterial Systematics*, pp. 173–179. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.
- Lanyi, B. (1987). Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* **19**, 1–67.
- Lechevalier, M. P. & Lechevalier, H. (1970). Chemical composition as a criterion in the classification of aerobic actinomycetes. *Int J Syst Bacteriol* **20**, 435–443.
- Lechevalier, M. P., De Bièvre, C. & Lechevalier, H. A. (1977). Chemotaxonomy of aerobic actinomycetes: phospholipid composition. *Biochem Syst Ecol* **5**, 249–260.
- Mayilraj, S., Prasad, G. S., Suresh, K., Saini, H. S., Shivaji, S. & Chakrabarti, T. (2005). *Planococcus stackebrandtii* sp. nov., isolated from a cold desert of the Himalayas, India. *Int J Syst Evol Microbiol* **55**, 91–94.
- Minnikin, D. E. & Goodfellow, M. (1976). Lipid composition in the classification and identification of *Nocardia* and related taxa. In *Biology of the Nocardaceae*, pp. 160–219. Edited by M. Goodfellow, G. H. Brownell & J. A. Serrano. London: Academic Press.
- Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, K. & Parlett, J. H. (1984). An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* **2**, 233–241.
- Pandey, K. K., Mayilraj, S. & Chakrabarti, T. (2002). *Pseudomonas indica* sp. nov., a novel butane-utilizing species. *Int J Syst Evol Microbiol* **52**, 1559–1567.
- Reddy, G. S. N., Aggarwal, R. K., Matsumoto, G. I. & Shivaji, S. (2000). *Arthrobacter flavus* sp. nov., a psychrophilic bacterium isolated from a pond in McMurdo Dry Valley, Antarctica. *Int J Syst Evol Microbiol* **50**, 1553–1561.
- Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.
- Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Staneck, J. L. & Roberts, G. D. (1974). Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* **28**, 226–231.
- Tourova, T. P. & Antonov, A. S. (1987). Identification of microorganisms by rapid DNA-DNA hybridization. *Methods Microbiol* **19**, 333–355.
- Uchida, K. & Aida, K. (1984). An improved method for the glycolate test for simple identification of the acyl type of bacterial cell walls. *J Gen Appl Microbiol* **30**, 131–134.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Williams, S. T., Goodfellow, M., Alderson, G., Wellington, E. M. H., Sneath, P. H. A. & Sackin, M. J. (1983). Numerical classification of *Streptomyces* and related genera. *J Gen Microbiol* **129**, 1743–1813.
- Yassin, A. F. (2005). *Rhodococcus triatoma* sp. nov., isolated from a blood-sucking bug. *Int J Syst Evol Microbiol* **55**, 1575–1579.
- Yassin, A. F. & Schaal, K. P. (2005). Reclassification of *Nocardia corynebacterioides* Serrano *et al.* 1972 (Approved Lists 1980) as *Rhodococcus corynebacterioides* comb. nov. *Int J Syst Evol Microbiol* **55**, 1345–1348.