Rhodococcus canchipurensis sp. nov., an actinomycete isolated from a limestone deposit site

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A novel actinobacterial strain, MBRL 353T, was isolated from a sample collected from a limestone quarry at Hundung, Manipur, India. Comparison of 16S rRNA gene sequences of strain MBRL 353T and other members of the genus Rhodococcus showed sequence similarities ranging from 95.5 to 98.2 %, with strain MBRL 353T showing closest sequence similarity to Rhodococcus triatomae IMMIB RIV-085T (98.2 %) and Rhodococcus equi DSM 20307T (97.2 %). DNA–DNA hybridization results, however, revealed that DNA–DNA relatedness values between strain MBRL 353T and R. triatomae DSM 44892T (43.4 %) and R. equi DSM 20307T (33.4 %) were well below the 70 % limit for species identification. Strain MBRL 353T contained meso-diaminopimelic acid as the diagnostic diamino acid and galactose and arabinose in the cell wall. Mycolic acids were present. The major fatty acids were C16:0 (45.7 %), C18:1\(^v\)9c (18.2 %) and 10-methyl C18:0 (11.3 %). The only menaquinone detected was MK-8(H2), while the major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and one unknown phospholipid. The G+C content of the genomic DNA was 69.2 mol%. The phenotypic and genotypic data showed that strain MBRL 353T merits recognition as a representative of a novel species of the genus Rhodococcus for which the name Rhodococcus canchipurensis sp. nov. is proposed; the type strain is MBRL 353T (=KCTC 19851T=JCM 17578T).

The genus Rhodococcus was first proposed by Zopf (1891), but later elucidated by Tsukamura (1974) with Rhodococcus rhodochrous as the type species of the genus and emended subsequently by Rainey et al. (1995). The genus is placed in the family Nocardiaceae of the suborder Corynebacterineae (Stackebrandt et al., 1997). A special feature of this genus is the simple rod–coccus developmental cycle (Fuhrmann et al., 1997). Biotechnologically, the genus has acquired significant value because of its ability to degrade a diversity of organic compounds, including some of the most difficult compounds with regard to recalcitrance and toxicity (Larkin et al., 2005). At the time of writing, there are more than 36 species with validly published names in the genus, including the most recently described species Rhodococcus jialingiae, Rhodococcus artemisiae and Rhodococcus nanhaiensis (Wang et al., 2010; Zhao et al., 2012; Li et al., 2012).

The Microbial Biotechnology Research Laboratory (MBRL) has been exploring actinomycete diversity in various habitats of Manipur and also investigating their biotechnological potential for applications such as industrial enzyme production (Ningthoujam et al., 2009a), biodegradation of xenobiotics, and antimicrobial (Ningthoujam et al., 2009b; Sanasam & Ningthoujam, 2010) and biocontrol (Ningthoujam et al., 2009c) agents. As a part of our ongoing studies on actinomycete diversity in Manipur, strain MBRL 353T was isolated from a soil sample collected from a limestone quarry at Hundung, Manipur, India (25.05° N

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MBRL 353T is JN164649.

Four supplementary figures and one supplementary table are available with the online version of this paper.
94.33° E) using starch casein nitrate agar (SCNA; Küster & Williams, 1964) as the selective medium. Soil samples were air dried for a week, crushed and sieved. Sieved soil (1 g) was treated with 0.1 g CaCO₃ for a week to prevent the growth of fast-growing bacteria and was then suspended in 99 ml quarter-strength Ringer’s salt solution (pH 7.0) and kept in an orbital shaker at 150 r.p.m. for 30 min. The suspension was centrifuged, 0.1 ml of the supernatant was decimally diluted and 0.1 ml aliquots were spread on SCNA (pH 7.2) and incubated at 28°C for 3 weeks. The isolates were subcultured in yeast extract-malt extract agar (ISP 2) (Shirling & Gottlieb, 1966) to obtain pure cultures. Strain MRBL 353T, one of the Hundung isolates, was preserved in glycerol (20%, v/v) at −80°C.

For observing its morphological characteristics, strain MBRL 353T was cultivated aerobically at 28°C on ISP 2 medium. Cell morphology was observed by using light microscopy (Axioskop A1; Zeiss) and scanning electron microscopy (JSM-6360; Jeol). Growth on various ISP media, tryptic soy agar (Difco), SCNA, Czapek’s agar and nutrient agar were observed. The colony colour was determined using the ISCC-NBS colour chart (Kelly, 1964).

Strain MBRL 353T formed smooth, circular, convex, opaque and pink colonies after 7 days of incubation at 28°C. The cells were Gram-positive, non-spore-forming and non-motile. Observation by light and scanning electron microscopy revealed the typical rod–coccus developmental cycle for the genus Rhodococcus (Wang et al., 2008). The strain showed an elementary rod structure during the early growth phase (26 h), which started to disintegrate in shorter rods at 47–56 h, and finally formed cocci at around 109 h (Fig. S1, available in IJSEM Online). The strain grew well in most of the media tested, but showed poor growth on ISP 3, ISP 4 and ISP 5 media.

Utilization of sole carbon and nitrogen sources was determined as described by Shirling & Gottlieb (1966). Tests for decomposition of adenine, casein, tyrosine, urea and xanthine were performed following the methods of Gordon et al. (1974). Hydrolysis of starch, gelatin and Tweens 20, 40, 60 and 80 was determined as described by Gordon & Krieg (1994), and the esterase enzyme method, which observes the formation of fuzzy halos on solid medium containing 0.5% NaCl, 0.1% peptone, 0.01% CaCl₂, H₂O and 0.9% agar, was used. Nitrate reduction was monitored as described by Lányi (1987). Antibiotic susceptibility test was performed as described by Groth et al. (2004) using antibiotic discs (HiMedia). Growth at 5, 15, 25, 37, 40, 50 and 60°C, at pH 4, 5, 6, 7, 8, 9 and 10, and with 0, 2, 5, 7 and 10% NaCl was determined on TSA as described by Goodfellow (1986). Catalase activity was observed by assessing bubble production in 3% (v/v) H₂O₂ and oxidase activity was determined by using 1% (w/v) solution of tetramethyl-p-phenylenediamine (Kovacs, 1956). Other biochemical tests including Voges–Proskauer, methyl red and the production of H₂S and indole were performed as described by Goodfellow (1986).

The isolate could degrade adenine, but not guanine, tyrosine or xanthine. The strain could hydrolyse starch and Tweens 20, 40, 60 and 80, but not casein, gelatin or urea. The strain was positive for catalase and Voges–Proskauer tests, but negative for oxidase, methyl red, citrate utilization, indole production, nitrate reduction and H₂S production. The strain was resistant to (µg per disc, unless otherwise indicated) amikacin (30), ampicillin (10), amphotericin (100 U per disc), chloramphenicol (30), erythromycin (15), fluconazole (25), gentamicin (10), kanamycin (30), mettacin (5), nalidixic acid (30), neomycin (30), novobiocin (30), nystatin (100 U per disc), penicillin (10), streptomycin (10), trimethoprim (50) and vancomycin (30). However, strain MBRL 353T showed sensitivity to ampicillin/sulbactam (10), amoxicillin (10), carbamycin (10), ciproflaxacin (10), gentamicin (30), nitrofurantoin (300), rifampicin (5), tetracycline (30) and tobramycin (10). The detailed phenotypic properties of strain MBRL 353T are listed in Table 1 and other phenotypic characteristics are mentioned in the species description.

The amino acid content of the cell wall was determined according to Staneck & Roberts (1974) and the sugars of the whole-cell hydrolysate were analysed as described by Tang et al. (2009). For other chemotaxonomic analyses, strain MBRL 353T was grown in tryptic soy broth shake flasks for 1 week at 28°C. Cell biomass was harvested by centrifugation, washed with distilled water and lyophilized. Mycolic acids were extracted and analysed according to the protocol of Minnikin et al. (1980). Polar lipids were extracted and analysed by two-dimensional TLC as described by Minnikin et al. (1984). The extraction of

### Table 1. Phenotypic characteristics that differentiate strain MBRL 353T from its closest phylogenetic neighbours

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>3</th>
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<td>Utilization as sole C source</td>
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<td>Dulcitol</td>
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<td>D-Galactose</td>
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<td>D-Mannose</td>
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<td>D-Xylose</td>
<td>+</td>
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<td>Utilization as sole N source</td>
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<tr>
<td>L-Arginine</td>
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<td>L-Phenylalanine</td>
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<td>Potassium nitrate</td>
<td>+</td>
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<td>L-Serine</td>
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<td>L-Valine</td>
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<td>Hydrolysis of:</td>
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<tr>
<td>Starch</td>
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<td>Urea</td>
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<td>pH for growth</td>
<td>6–10</td>
<td>6–8</td>
<td>6–8</td>
<td>6–10</td>
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<td>NaCl for growth (%)</td>
<td>0–5</td>
<td>0–2</td>
<td>0–5</td>
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</table>
menaquinones was done as described by Collins et al. (1977) and menaquinones were analysed by HPLC (Tamaoka et al., 1983). The cellular fatty acids were extracted, methylated and analysed by using the Sherlock Microbial Identification System (MIDI) according to the method of Sasser (1990) and the manufacturer’s instructions. The fatty acid methyl esters were then analysed by GC (7890A GC; Agilent Technologies) by using the Microbial Identification software package (Sherlock version 6.1; MIDI database TSBA6).

Strain MBRL 353\textsuperscript{T} had \textit{meso}-diaminopimelic acid as the diagnostic diamino acid and galactose and arabinose in the cell-wall hydrolysate (cell-wall chemotype IV \textit{sensu} Lechevalier & Lechevalier, 1970). The non-diagnostic sugars mannose, ribose, rhamnose and glucose were also detected in the whole-cell hydrolysate. Mycolic acids were present (Fig. S2). The major polar lipids detected were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and one unknown phospholipid (Fig. S3; phospholipid type II \textit{sensu} Lechevalier et al., 1977). MK-8(H\textsubscript{2}) was the only menaquinone detected. The cellular fatty acid composition is shown in Table S1. The observed chemotaxonomic features support the assignment of strain MBRL 353\textsuperscript{T} to the genus \textit{Rhodococcus}.

Genomic DNA isolation and PCR amplification of the 16S rRNA gene was done as described by Li et al. (2007). The almost-complete 16S rRNA gene sequence (1522 bp) of the strain was submitted to the EzTaxon server (Kim et al., 2012) and aligned with the 16S rRNA gene sequences of other \textit{Rhodococcus} species using \textsc{clustal x} version 2.1 (Larkin et al., 2007). Phylogenetic analysis was done using the software package \textsc{mega} version 5.0 (Tamura et al., 2011). 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). To determine the support of each clade, bootstrap analysis was performed with 1000 resamplings (Felsenstein, 1985). The validity of the neighbour-joining tree was compared with the maximum-parsimony tree (Kluge & Farris, 1969) drawn using \textsc{mega} 5.0 (Fig. S4). The G+C content of the genomic DNA was determined by the HPLC method.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{tree.png}
\caption{Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain MBRL 353\textsuperscript{T} and type strains of species of the genus \textit{Rhodococcus}. \textit{Corynebacterium diphtheriae} NCTC 11397\textsuperscript{T} was used as an outgroup. Bootstrap values (>50\%) based on 1000 resamplings are shown at branch nodes. Bar, 1\% sequence divergence.}
\end{figure}
according to Mesbah et al. (1989). DNA–DNA relatedness was studied in triplicate by the thermal renaturation method (De Ley et al., 1970) using a Lambda 35 UV/Vis spectrophotometer (Perkin Elmer) equipped with a PTP 6 + 6 Peltier temperature programmer (Perkin Elmer) and a BG-chiller E15 (Baygene Biotech).

The G + C content of the genomic DNA was 69.2 mol%. EzTaxon analysis and the phylogenetic tree showed that strain MBRL 353T formed a distinct lineage most closely related to R. triatoma DSM 44892T and R. equi DSM 20307T (Fig. 1) (16S rRNA gene sequence similarities of 98.2 and 97.2%, respectively). However DNA–DNA hybridization experiments showed that strain MBRL 353T displayed low DNA–DNA reassociation values with R. triatoma DSM 44892T (43.4 ± 6.2%) and R. equi DSM 20307T (33.4 ± 2.3%) (mean ± standard deviation), thereby indicating that the whole-genome DNA–DNA relatedness values with the isolate’s closest phylogenetic neighbours are well below the delineating 70% cut-off point for species identification (Wayne et al., 1987).

The genotypic and phenotypic features described above suggest that strain MBRL 353T could be clearly distinguished from its closest phylogenetic relatives. Besides low DNA–DNA relatedness to its closest phylogenetic neighbours, the strain was also distinguished by several phenotypic properties: for example, dulcitol was utilized by R. equi DSM 20307T but not by strain MBRL 353T; mannose and xylose were utilized by strain MBRL 353T but not by R. triatoma DSM 44892T; and, starch hydrolysis was positive for strain MBRL 353T but negative for the three reference strains (Table 1). Therefore, strain MBRL 353T represents a novel species of the genus Rhodococcus, for which the name Rhodococcus canchipurensis sp. nov. is proposed.

**Description of Rhodococcus canchipurensis sp. nov.**

*Rhodococcus canchipurensis* (can.chi.pur.en’sis. N.L. masc. adj. canchipurensis of or belonging to Canchipur, Manipur University, India, where the MBRL research group which isolated the type strain is located).

Gram-positive, aerobic, non-spore-forming and non-motile. Cells are mostly rods in the early growth phase. Growth occurs at 15–37 °C and pH 6–10, with optimum growth at 28 °C and pH 7. Growth occurs in the presence of up to 5% NaCl. Utilizes fructose, D-mannose, sodium malate, L-sorbosiv, succinic acid and D-xylose as sole carbon sources and L-arginine, glycine, L-histidine, L-hydroxyproline, DL-methionine, L-phenylalanine, potassium nitrate, L-serine and L-valine as sole nitrogen sources, but does not utilize cellobiose, dulcitol, D-galactose, myo-inositol, lactose, maltose, mannitol, raffinose, rhamnose, trehalose, xylitol, L-alanine, cysteine, L-ornithine, proline or threonine. Hydrolyses starch and Tweens 20, 40, 60 and 80, but not casein or urea. Decomposes adenine, but not guanine, tyrosine or xanthine. Positive for catalase and Voges-Proskauer test, but negative for oxidase, methyl red test, citrate utilization, indole production, nitrate reduction and H2S production. Contains meso-diaminopimelic acid, arabinose and galactose in the cell wall. Mycolic acids are present. MK-8(H2) is the only menaquinone, while the polar lipids consist of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylglycerol and one unknown phospholipid. The fatty acid composition (>1%) is C16:0, C18:0, 10-methyl C18:0 (tuberculostearic acid), summed feature 3 (containing C16:0,07c and/or C16:0,06c), C18:0, C14:0, C17:0, C17:1ω9c, iso-C19:0, and summed feature 9 (containing C19:1ω11c and/or C19:1ω9c).

The type strain, MBRL 353T (=KCTC 19851T=JCM 17578T), was isolated from a limestone quarry at Hundung, Manipur, India. The genomic DNA G + C content of the type strain is 69.2 mol%.

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**References**


