Rhodococcus lactis sp. nov., an actinobacterium isolated from sludge of a dairy waste treatment plant

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A Gram-stain-positive, non-motile and aerobic bacterium, designated strain DW151B\textsuperscript{T}, was isolated from a sludge sample of a dairy industry effluent treatment plant. 16S rRNA gene sequence analysis of strain DW151B\textsuperscript{T} placed it within the genus Rhodococcus. It displayed significant similarity with recognized species of the genus: Rhodococcus pyridinivorans PDB9\textsuperscript{T} (98.8 %), Rhodococcus gordoniae W 4937\textsuperscript{T} (98.6 %), Rhodococcus rhodochrous DSM 43241\textsuperscript{T} (98.5 %) and Rhodococcus artemisiae YIM 65754\textsuperscript{T} (97.5 %). However, strain DW151B\textsuperscript{T} differed from phylogenetically closely related species in various phenotypic properties. The cellular polar lipid profile consisted of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) as major lipids, MK-8(H\textsubscript{2}) was the major menaquinone and meso-diaminopimelic acid was the cell-wall peptidoglycan. The fatty acid profile consisted of C\textsubscript{16:0}, C\textsubscript{18:1}\textsuperscript{9} and C\textsubscript{16:1}\textsuperscript{9} as main components. The presence of C\textsubscript{16:0} and diphosphatidylglycerol as major fatty acid and polar lipid, respectively, was in accordance with chemotaxonomic markers of the genus Rhodococcus. The DNA G\textsubscript{+}C content of strain DW151B\textsuperscript{T} was 69.9 mol\%, a value within the limits reported for the members of this genus. Furthermore, strain DW151B\textsuperscript{T} showed low similarity at the whole genome level in DNA–DNA hybridization experiments with phylogenetically closely related strains. Considering the low similarity at the genome level and differences in phenotypic properties, strain DW151B\textsuperscript{T} is considered to represent a novel species of the genus Rhodococcus, for which the name Rhodococcus lactis sp. nov. is proposed. The type strain is DW151B\textsuperscript{T} (=MTCC 12279\textsuperscript{T}=DSM 45625\textsuperscript{T}).

The genus Rhodococcus is one among an increasing number of genera of Gram-positive bacteria. Species of this genus are considered biotechnologically very important mainly due to their increasing use in applications such as bioremediation (Kuyukina & Ivshina, 2010), biodesulfurization (McLeod et al., 2006) and bioflocculant formation (Peng et al., 2014). The genus name Rhodococcus was first used by Zopf in 1891 (Bell et al., 1998) and the genus was classified in the family Nocardiaceae under suborder Corynebacterineae (Stackebrandt et al., 1997). On the basis of 16S rRNA gene sequence phylogenetic analysis, the genus was divided into nine subclades by Görtler et al. (2004). Species of the genus have been isolated from diverse ecological niches such as a limestone deposition site (Nimaichand et al., 2013), leaf surface (Kämpfer et al., 2013) and blood sucking bug (Yassin, 2005), and have been frequently isolated from industrial waste treatment plants (Apajalahti et al., 1986; Yoon et al., 2000; Wang et al., 2010; Kämpfer et al., 2014; Peng et al., 2014). There has been a significant increase in the number of species characterized within this genus in the last decade (http://www.bacterio.cict.fr/qg/rhodococcus.html) suggesting its importance in the environment. In the present study, we describe a novel Rhodococcus-like strain, DW151B\textsuperscript{T}, by comparing its phenotypic and genotypic characteristics with phylogenetically closely related species. Strain DW151B\textsuperscript{T} was isolated while studying the cultivable microbial diversity of a dairy industry effluent treatment plant, located at Hyderabad (17° 25′ 21.3″ N 78° 27′ 39.7″ E), India. A peach-coloured colony was isolated from a nutrient agar plate (NA; HiMedia) incubated at 30 °C and subsequently subcultured. Upon purity testing,

Abbreviations: ML, maximum-likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for 16S rRNA gene sequence of strain DW151B\textsuperscript{T} is KP342300.

One supplementary table and one supplementary figure are available with the online Supplementary Material.

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it was preserved as glycerol stocks (10 %, v/v) at −70 °C for further characterization. Strain DW151B grew well on NA and tryptone soya agar (TSA; HiMedia). For studies of cell morphology it was grown on TSA medium at 30 °C. Colonies were observed at different time intervals for determination of morphology and also for determination of cell morphology. Characteristics of the cells were observed under a phase-contrast microscope (Zeiss Axioplan). An active culture was assigned for Gram staining using a Gram-staining kit (HiMedia) according to the manufacturer’s instructions. Growth of strain DW151BT at various physiological conditions was tested using NA medium as it provided clearer growth than TSA. Temperature-dependent growth was determined by incubating at 4, 10, 25, 28, 30, 37, 42, 55 and 60 °C. The growth pH range was tested at 30 °C using NA as basal medium and adjusting the pH between 4.0 and 12.0 (in 0.5 pH unit steps). The pH of broth medium was adjusted using 0.1 M citric acid/sodium citrate buffer (pH 4.0–6.0), 0.2 M Na₂HPO₄/NaH₂PO₄ buffer (pH 6.0–8.0), 0.1 M Na₂CO₃/NaHCO₃ buffer (pH 8.0–10.0) and 0.1 M Na₂CO₃/NaOH buffer (pH 10–12), with the addition of 1.5 % (w/v) agar, and the medium was then autoclaved. NA without NaCl was supplemented with different concentrations of NaCl (1–12 % w/v, at 1 % intervals) to determine the salt tolerance of the strain. Assimilation of different substrates and other biochemical characteristics for strain DW151B were tested as per standard methods using Biolog and Vitek 2 (bioMérieux) systems following the manufacturers’ instructions. Strain DW151B grown on TSA medium at 30 °C was used as inoculant to carry out these reactions. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). Tests for hydrolysis of aesculin, indole, Voges–Proskauer and methyl red tests, H₂S production and nitrate reduction were performed as described by Lányi (1988). Utilization of various carbon compounds (glucose, fructose, lactose, maltose, sucrose and xylose), and hydrolysis of casein, gelatin, starch and Tweens were determined as described by Smibert & Krieg (1994). Acid production from different sugars was assessed as described by Chittpurna et al. (2011).

Strain DW151B grew optimally at pH 7.0, at 30 °C and with 0.5 % NaCl, and these conditions were used to determine phenotypic properties. Colonies were opaque and raised with irregular configuration. Unlike pink colonies produced by Rhodococcus gordoniae DSM 44689T and Rhodococcus rhodochrous DSM 43241T, strain DW151B

### Table 1. Phenotypic characteristics that differentiate strain DW151B from closely related species of the genus Rhodococcus

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain 1</th>
<th>Strain 2</th>
<th>Strain 3</th>
<th>Strain 4</th>
<th>Strain 5*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigmentation</td>
<td>Peach</td>
<td>Peach</td>
<td>Pink</td>
<td>Light pink</td>
<td>Creamy pink</td>
</tr>
<tr>
<td>Growth with 6 % NaCl</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Aesculin</td>
<td>(+)</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Urea</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Tween 20</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Tween 40</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Tween 60</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>l-Ascorbic acid</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Inositol</td>
<td>–</td>
<td>–</td>
<td>(+)</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Maltose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Mannose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Salicin</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>69.9</td>
<td>66</td>
<td>68</td>
<td>67–70</td>
<td>66.2</td>
</tr>
<tr>
<td>Assimilation of substrate in Biolog/Vitek 2:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Proline</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>L-Lactate</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

*Data from Zhao et al. (2012).
†Data differ from those of Jones et al. (2004).
‡Data differ from those of Yoon et al. (2000).
produced peach-coloured colonies when grown under identical conditions. Microscopic examination revealed that cells of strain DW151T were Gram-stain-positive, non-spor-forming and non-motile with a rod–coccus morphology and scattered arrangement. Cells were short rods during the exponential growth phase and converted to cocci during the stationary growth phase. The strain grew at 4–42 °C and no growth was observed at pH values below 7 or above 9. The strain could tolerate a salt concentration up to 6 % (w/v). Strain DW151T showed a weak positive reaction for aesculin hydrolysis as compared with Rhodococcus pyridinivorans DSM 44555T, but this phenotypic feature was highly variable among type strains of related species. Unlike R. pyridinivorans DSM 44555T, R. rhodochrous DSM 43241T and R. gordonae DSM 44689T, acrid production was not observed for strain DW151T from inositol, salcin, maltose or mannose sugars (Table 1). Vitek 2 analysis showed significant differences in phenotypic properties of strain DW151T from other closely related strains. For instance, strain DW151T was positive for the utilization of D-xylose whereas it was negative for the other type strains. Similarly, R. pyridinivorans DSM 44555T and R. gordonae DSM 44689T were positive for tyrosine arylamidase and L-lactate alkalization, whereas strain DW151T and R. rhodochrous DSM 43241T displayed negative reactions. The results of Biolog and Vitek 2 phenotypic characterization of strain DW151T and its closest relatives are given in Table S1 (available in the online Supplementary Material).

To determine the total cellular fatty acid composition, cells of strain DW151T, R. pyridinivorans DSM 44555T, R. gordonae DSM 44689T and R. rhodochrous DSM 43241T were grown on TSA plates for 48 h at 30 °C. Methyl esters of fatty acids were extracted using the protocol described by Sato & Murata (1988). These fatty acid methyl esters were analysed by GC (Agilent 6890 series) and quantified using the Microbial Identification System (MIDI ChemStation version 4.0 using aerobe TSBA50 method) according to the MIDI instructions (Sasser, 1999). Mycolic acids were extracted and analysed as described by Minnikin et al. (1980). Polar lipids and menaquinones were extracted as described by Minnikin et al. (1984). Polar lipids were separated by two-dimensional TLC and sprayed with molybdophosphoric acid, molybdenum blue, ninhydrin and 2-naphthol spray reagents for the detection of total lipids, phospholipids, aminolipids and glycolipids, respectively. The peptidoglycan type was determined using hydrolysat of purified cell walls by TLC (Schumann, 2011). Genomic DNA was extracted and purified according to the method of Sambrook et al. (2001) and used to determine the G + C content with the thermal denaturation method (Mandel & Marmur, 1968) using a Lambda 35 spectrophotometer (Perkin Elmer). The DNA was also used for DNA–DNA hybridization experiments with closely related species using the method described by Tourova & Antonov (1988) in three individual experiments (Tindall et al., 2010).

The profiles of total cellular fatty acids of strain DW151T and phylogenetically related strains were compared (Table 2). Total fatty acid composition analysis revealed that strain DW151T contained C16 : 0 (33.5 %), C18 : 1 cis9 (21.8 %) and C16 : 1 cis9 (6.8 %) as major fatty acids, a profile similar to other members of the genus Rhodococcus. The mycolic acids of strain DW151T were co-migrated with those of R. pyridinivorans DSM 44555T, R. gordonae DSM 44689T and R. rhodochrous DSM 43241T. The total polar lipid profile of strain DW151T contained diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) as major lipids of the cell membrane. An aminolipid (AL) and two unknown lipids (UL1 and UL4) were also found in significant quantities (Fig. S1). A similar profile of major lipids was observed for R. pyridinivorans DSM 44555T. However, an additional amino lipid was present and two unknown lipids were absent in strain DW151T as compared with R. pyridinivorans DSM 44555T. The cell-wall peptidoglycan contained meso-diaminopimellic acid as the characteristic diamino acid and menaquinone MK-8(H2) as the major quinone. The total genomic DNA G + C content of strain DW151T was 69.9 mol%, as estimated by the Tm method, a value within the range reported for members of the genus Rhodococcus.

For phylogenetic analysis, the 16S rRNA gene was PCR amplified using universal primers 27f (5′-AGAGTTTGATC
The amplicon was purified, using a QIAquick PCR purification kit (Qiagen), and sequenced in an ABI Prism 3700 automatic DNA sequencer (Applied Biosystems) by the use of a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) as described by Suresh et al. (2006). The almost-complete sequence of the 16S rRNA gene (1426 bp) was obtained and used for the initial BLAST search. The BLAST analysis was performed via the EZTaxon server, a type strain sequence database (Chun et al., 2007; Kim et al., 2012), and closely related sequences were retrieved manually. Sequences were aligned using the CLUSTAL W program (Thompson et al., 1994) and the results obtained were edited manually. Phylogenetic trees were reconstructed using the neighbour-joining (NJ) (Saitou & Nei, 1987) and maximum-likelihood (ML) (Tateno et al., 1994) methods available in the MEGA6 software (Tamura et al., 2013). Evolutionary distances of the NJ and ML trees were calculated using Kimura’s two-parameter (Kimura, 1980) and Tamura–Nei (Tamura & Nei, 1993) models, respectively. The heuristic search option NNI (Nearest-Neighbour-Interchange) was used for ML phylogenetic analysis with very strong branch swap filter. A discrete gamma distribution was used to model evolutionary rate differences among sites (five categories; + G, parameter = 0.3358). All positions containing gaps and missing data were eliminated, and bootstrap analyses were performed using 1000 replications for both the NJ and the ML analyses.

**Fig. 1.** NJ (Saitou & Nei, 1987) phylogenetic tree showing the relationship between strain DW151BT and its closest relatives, based on 16S rRNA gene sequences. Evolutionary distances were calculated using Kimura’s two-parameter model (Kimura, 1980). A discrete gamma distribution was used to model evolutionary rate differences among sites (five categories; + G, parameter = 0.3358). All positions containing gaps and missing data were eliminated for both the NJ and the ML analyses. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points; only values above 50% are given. Filled circles indicate that the corresponding nodes were retrieved in the ML phylogenetic tree. Bar, 0.01 substitutions per site.
R. rhodochrous and R. artemisiae with significant bootstrap support values (Fig. 1). The results of DNA–DNA hybridization revealed that strain DW151B\textsuperscript{T} shared 52.0 % (± 4.7), 48.0 % (± 1.3) and 44.9 % (± 1.7) DNA–DNA relatedness with *R. pyridinivorans* DSM 44555\textsuperscript{T}, *R. rhodochrous* DSM 43241\textsuperscript{T} and *R. gordoniae* DSM 44689\textsuperscript{T}, respectively, at the whole genome level. The values obtained are significantly lower than the 70 % threshold value suggested for the delineation of genomic species (Wayne et al., 1987; Stackebrandt & Goebel, 1994). However, DNA–DNA hybridization was not performed between strain DW151B\textsuperscript{T} and *R. artemisiae* YIM 65754\textsuperscript{T} as it displayed low 16S rRNA gene sequence similarity and formed an outgroup to the cluster containing strain DW151B\textsuperscript{T} and other close relatives in the phylogenetic tree. Therefore, it is assumed that they exhibit low similarity at the whole genome level. These results are in agreement with the conclusion that representatives of *Rhodococcus* species sharing 16S rRNA gene sequence similarity of more than 98 % can also share low whole genome relatedness (Yassin, 2005; Zhao et al., 2012). Thus, differences observed in phenotypic properties and low DNA–DNA relatedness between strain DW151B\textsuperscript{T} and its nearest phylogenetic relatives clearly suggest that it should be assigned to a novel species of the genus *Rhodococcus*, for which the name *Rhodococcus lactis* sp. nov. is proposed.

**Description of *Rhodococcus lactis* sp. nov.**

*Rhodococcus lactis* (lac’tis. L. gen. n. lactis from milk, pertaining to the isolation source ‘dairy waste`).

Cells are Gram-stain-positive, non-motile, non-spore-forming and strictly aerobic. Displays a rod–coccus cycle. Optimum growth occurs at pH 7.0 and 30 °C; tolerates up to 6 % (w/v) NaCl. Positive for catalase but negative for oxidase. Utilizes citrate, Tween 20, 40 and 60, dextrin, D-fructose, D-glucose, D-psicose, D-ribose, D-xyllose, acetic acid, \( \gamma \)-hydroxybutyric acid, D-sorbitol, D-galactose, D-ribose, L-lactate, D-mannitol, trehalose, cycloextrin, methyl \( \beta-D \)-glucopyranosside and pullulan. Weakly positive for asacchar hydrolysis. Positive for leucine arylamidase, alanine arylamidase and L-proline arylamidase but negative for arginine dihydrolase-1, L-aspartate arylamidase, tyrosine arylamidase, Ala–Phe–Pro arylamidase, amygdalin, phosphatydinositol, phospholipase C, \( \beta \)-galactosidase, \( \alpha \)-glucosidase, \( \alpha \)-mannosidase, phosphatase, \( \beta \)-glucuronidase, \( \alpha \)-galactosidase, L-tyrosylidipyl arylamidase, \( \beta \)-glucuronidase and \( \beta \)-galacto-pyranosidase. Susceptible to optochin, polymixin B, bacitracin and novobiocin. Acid production from different sugars such as L-arabinose, inositol, maltose, melibiose, salicin, sucrose, inulin, lactose, raffinose and mannose is not observed. Does not hydrolyse starch, gelatin or urea.

The type strain is DW151B\textsuperscript{T} (\( = \)MTCC 12279\textsuperscript{T} = DSM 45625\textsuperscript{T}). The genomic DNA G+C content of the type strain is 69.9 mol%.

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**References**


