Rhodococcus imtechensis sp. nov., a nitrophenol-degrading actinomycete

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A Gram-positive actinobacterium, strain RKJ300^T, capable of utilizing *p*-nitrophenol and 2,4-dinitrophenol, was isolated from a pesticide-contaminated site in India. The morphological and chemotaxonomic properties of the isolate were typical of members of the genus *Rhodococcus*. The DNA G + C content was 72 mol%. Strain RKJ300^T exhibited the highest level of sequence similarity with *Rhodococcus wratislaviensis* NCIMB 13082^T (99.3%), followed by *Rhodococcus opacus* DSM 43205^T (98.8%), *Rhodococcus percolatus* MBS1^T (98.6%) and *Rhodococcus koreensis* DNP505^T (98.1%). The low levels of DNA–DNA relatedness (49–58%) with the above micro-organisms, and the differences in the biochemical and physiological properties, suggest that strain RKJ300^T should be classified within a novel species of the genus *Rhodococcus*, for which the name *Rhodococcus imtechensis* sp. nov. is proposed. The type strain is RKJ300^T (=MTCC 7085^T=JCM 13270^T).

In recent times, the application of polyphasic procedures has led to marked improvements in the classification of the genus *Rhodococcus* (Goodfellow *et al.*, 1998, 1999). At the time of writing, the genus *Rhodococcus* consists of more than 30 recognized species (<http://www.bacterio.cict.fr/qr/rhodococcus.html>). Rhodococci are ubiquitous in the environment, but occur primarily in soils and sludges. As these micro-organisms exhibit broad metabolic diversity, particularly with respect to hydrophobic compounds, they have attracted increased interest with regard to the biochemical and genetic characterization of their metabolic capabilities, and they have also been considered to be excellent candidates for bioremediation treatments (Finnerty, 1992; Warhurst & Fewson, 1994). In this work, a polyphasic approach was used to determine the taxonomic position of bacterial strain RKJ300^T, which is capable of utilizing toxic and recalcitrant compounds such as *p*-nitrophenol and 2,4-dinitrophenol as sole sources of carbon and energy.

Strain RKJ300^T was isolated from a pesticide-contaminated site in Punjab State, India, on tryptone soya agar (TSA; HiMedia) at 30 °C by using the dilution plating technique. Subcultivation was done on TSA at 30 °C for 48 h and the bacterial isolate was maintained as a glycerol stock at –70 °C. The reference strains *Rhodococcus wratislaviensis* MTCC

6421^T, *Rhodococcus opacus* MTCC 6420^T, *Rhodococcus percolatus* MTCC 6422^T and *Rhodococcus koreensis* MTCC 6425^T were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India.

The colony morphology was examined following growth of the strain on TSA at 30 °C for 3 days. The cell morphology was investigated using light microscopy (Zeiss) at ×1000 magnification and scanning electron microscopy (Stereoscan 260; Leica). Cells showing different morphological stages of the growth cycle in liquid (tryptone soya broth) (TSB; HiMedia) and solid (TSA) media were processed for scanning electron microscopy as described previously (Richard & Wilson, 1993). Motility was checked using the method described by Skerman (1967). The Gram reaction was determined by using the HiMedia Gram staining kit according to the manufacturer's instructions. Physiological tests such as growth at different temperatures, pH values and NaCl concentrations were performed as described by Goodfellow (1986). Catalase activity and urea hydrolysis were determined as mentioned by Cowan & Steel (1965). The ability to hydrolyse arbutin, casein, gelatin, elastin, Tween 80 and starch was examined by following the protocol of Smibert & Krieg (1994). Nitrate reduction was determined as described by Lanyi (1987). The following tests were performed as described by Goodfellow (1986): Voges–Proskauer, methyl red, oxidation/fermentation, growth on citrate agar and MacConkey agar and the production of H₂S and indole. Oxidase activity was determined from the oxidation of tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma) (Smibert & Krieg, 1994). Acid

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RKJ300^T is AY525785.

Scanning electron micrographs of strain RKJ300^T and a table showing fatty acid compositions are available as supplementary material in IJSEM Online.

production from various carbohydrates was determined after 3 days incubation at 30 °C, using bromocresol purple as an acid/base indicator (Gordon *et al.*, 1974). Tests for the utilization of various substrates as sole carbon (1 %, w/v) (Shirling & Gottlieb, 1966) and sole nitrogen (0.1 %, w/v) (Williams *et al.*, 1983) sources were carried out and the utilization results were observed over a period of 1 week. The utilization of substrates as sole carbon and energy sources was also determined by using the Biolog GP2 MicroPlate system. The plate was used according to the manufacturer's instructions, with the modification that TSA medium was used instead of Biolog Universal Growth agar medium. The inoculated plates were incubated for 24 h and the results were read with a MicroPlate Reader, using Microlog 4.2 computer software to perform automated reading. The strains were examined for their ability to degrade *p*-nitrophenol and 2,4-dinitrophenol by using the method described by Prakash *et al.* (1996) following incubation at 30 °C for 3 days. The sensitivity of the strain to various antibiotics was tested using antibiotic-susceptibility discs supplied by HiMedia.

Freeze-dried cells for chemotaxonomic analyses (excepting fatty acid studies) were prepared following growth of the strain in TSB for 4 days at 30 °C. The diagnostic cell-wall amino acids and sugars were determined using TLC as described by Staneck & Roberts (1974). Polar lipids were extracted, examined by two-dimensional TLC and identified using the method described by Minnikin *et al.* (1984). Menaquinones were extracted (Minnikin *et al.*, 1984) and separated by reversed-phase HPLC (Kroppenstedt, 1982). Mycolic acids were extracted and analysed according to the protocol of Minnikin *et al.* (1975). For cellular fatty acid analysis, the strains were grown on TSA at 30 °C for 36 h, and fatty acid methyl esters were analysed by using the Sherlock Microbial Identification System (MIDI) as described previously (Pandey *et al.*, 2002). Genomic DNA was isolated using a Qiagen genomic DNA isolation kit. DNA–DNA relatedness was studied by using the membrane filter method (Tourova & Antonov, 1987) as described by Shivaji *et al.* (1992); this was performed three times starting from the isolation of genomic DNA. The G + C content of the genomic DNA was determined spectrophotometrically (Lambda 35; Perkin Elmer) using the thermal denaturation method (Mandel & Marmur, 1968).

PCR amplification of the 16S rRNA gene was performed with universal primers 27f (5'-AGAGTTTGATCMTGGC-TCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGAC-TT-3') (*Escherichia coli* 16S rRNA numbering system; Brosius *et al.*, 1978). The reaction mixture contained 70 ng chromosomal DNA, 1 U Deep Vent DNA polymerase, 1 × ThermoPol reaction buffer, 200 µM each dNTP (New England Biolabs) and 20 pmol each primer (BioBasic). The PCR cycling parameters consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 75 °C for 2 min and a final extension

for 10 min at 75 °C. An amplicon of approximately 1.5 kb was separated by gel electrophoresis, eluted using the Qiaquick gel extraction kit (Qiagen) and cloned into electrocompetent *E. coli* DH5α cells using the *Sma*I-digested cloning vector pBluescript II KS(+). Transformants were selected on the basis of the blue/white screening procedure (Sambrook & Russell, 2001). Recombinant plasmids were isolated using the QIAprep spin miniprep kit (Qiagen) and the insert was completely sequenced using five forward primers and four reverse primers, namely KS (5'-TCGAGGTCGACGGTATC-3'), 27f, 357f, 704f, 1114f, 685r, 1110r, 1492r and SK (5'-CGCTCTAGAACTAGTG-GATC-3') (KS and SK, vector-specific primers) (Johnson, 1994), using the dideoxy chain-termination method as described previously (Pandey *et al.*, 2002). The 16S rRNA gene sequences of closely related taxa with validly published names were retrieved from the GenBank database using BLASTN (Altschul *et al.*, 1997) and aligned using the CLUSTAL X program (Thompson *et al.*, 1997); the alignment was edited manually. For the neighbour-joining analysis (Saitou & Nei, 1987), the distances between sequences were calculated on the basis of the method of Jukes & Cantor (1969). A bootstrap analysis of 1000 replications was performed to assess the confidence limits of the branching (Felsenstein, 1985).

Scanning electron micrographs revealed that the cells of strain RKJ300^T formed filaments or showed preliminary branching at an early phase of growth (12 h) and fragmented into short rods during the exponential phase (32 h); most cells appeared as cocci in stationary phase (64 h) (see Supplementary Fig. S1 available in IJSEM Online). Growth of the strains on minimal agar medium supplemented with *p*-nitrophenol (0.5 mM) or 2,4-dinitrophenol (0.5 mM) as the sole source of carbon and energy is indicated in Table 1. Cells of strain RKJ300^T were resistant to cephaloridin (30 µg per disc) and ampicillin (10 µg per disc). They were sensitive to the following antibiotics (µg per disc): rifampicin (5), tetracycline (30), chloramphenicol (10), chlorotetracycline (30), gentamicin (10), streptomycin (10), neomycin (30), tobramycin (10), norfloxacin (10), vancomycin (30) and oleandomycin (15). Most of the chemotaxonomic properties of the strain (presented in the species description) were typical of members of the genus *Rhodococcus*. No significant qualitative differences in the fatty acid profiles were found when strain RKJ300^T was compared with phylogenetically close taxa (see Supplementary Table S1 available in IJSEM Online). The DNA G + C content of strain RKJ300^T was estimated to be 72 mol% (mean of three replications; Table 1), a value within the range (63–72 mol%) for the genus *Rhodococcus* (Takeuchi & Hatano, 1998).

An almost-complete sequence (1510 bp) for the 16S rRNA gene of strain RKJ300^T was determined and was compared with those of other closely related taxa retrieved from the GenBank database. Sequence analysis revealed that strain RKJ300^T clustered with the members of the genus

Table 1. Distinguishing characteristics of strain RKJ300^T and the type strains of its closest phylogenetic relatives

Strains: 1, strain RKJ300^T; 2, *R. wratislaviensis* MTCC 6421^T; 3, *R. opacus* MTCC 6420^T; 4, *R. percolatus* MTCC 6422^T; 5, *R. koreensis* MTCC 6425^T. All of the strains were found to produce acid from glucose, fructose^{a*}, D-galactose, inositol^a, lactose^a, maltose^a, D-mannitol^a, D-raffinose, sorbitol^a and sucrose and not to produce acid from adonitol, cellobiose or dulcitol. All strains were positive for the utilization of fructose^{ab}, α -D-glucose^{ab}, pyruvic acid and Tween 40 as sole sources of carbon and energy (Biolog). All strains were negative for the utilization of *N*-acetyl-L-glutamic acid, adenosine 5'-monophosphate, 2'-deoxyadenosine, β -dextrin, D-fructose 6-phosphate, α -D-glucose 1-phosphate, L-glutamic acid, glycogen, inosine, lactamide, mannan, methyl α -D-galactoside, thymidine, thymidine 5'-monophosphate and uridine as sole sources of carbon and energy (Biolog). Data were obtained from this study unless stated otherwise. Characteristics are scored as follows: +, positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5
Hydrolysis of Tween 80 ^{c*}	+	–	–	+	+
Utilization of substrate as sole source of carbon and energy					
L-Arabinose ^c	–	–	–	–	w
L-Arabitol ^a	–	+	+	+	+
D-Cellobiose ^{ac}	–	+	+†	–	+†
D-Maltose ^{ac}	+	+	+	–†	+
D-Melezitose	–	–	+	+	+
Lactose ^{ac}	+	+	+	–†	+
D-Raffinose ^d	–	–	+	+	+
L-Rhamnose ^{acd}	+	+	–	–	+
D-Ribose	–	+	w†	–†	+
2,4-Dinitrophenol	+	–	–	+	+
<i>p</i> -Nitrophenol	+	–	–	–	+
Utilization of substrate as sole source of nitrogen					
L-Proline ^{acd}	+	+	–	+	+
L-Serine ^a	+	–	+	+	+
Utilization of substrate as carbon source (using Biolog GP2 plate)					
Acetic acid	+	+	–	+	w
Amygdalin	–	–	–	+	+
2,3-Butanediol	–	–	+	+	+
α -Cyclodextrin	+	–	–	–	–
L-Fucose	+	w	–	–	+
D-Galacturonic acid	+	–	–	–	w
Gentiobiose	–	w	–	+	+
D-Lactic acid methyl ester	+	–	–	–	–
Lactulose	+	–	–	–	–
L-Malic acid	–	–	–	+	w
D-Melibiose	–	w	w	+	+
Methyl β -D-galactoside	–	–	–	+	–
Methyl α -D-mannoside	+	–	–	–	–
Propionic acid	+	w	–	+	+
D-Psicose	+	+	w	w	–
Putrescine ^{ad}	–	+	+	w	–
L-Pyroglutamic acid	+	–	–	–	–
Salicin	–	+	–	+	–
Stachyose	–	+	–	–	–
D-Trehalose ^b	+	+	–	w	+
Uridine 5'-monophosphate	+	w	–	–	w
D-Xylose ^b	–	+	+	+	+
DNA G+C content (mol%)‡	72	66–68 ^e	67·6 ^f	67·4 ^g	66 ^h

*Tests (based on a different method) were also performed in another study as shown and gave congruent results: *a*, Klatte *et al.* (1994) with *R. opacus*; *b*, Yoon *et al.* (2000) with *R. koreensis*; *c*, Briglia *et al.* (1996) with *R. percolatus*; *d*, Goodfellow *et al.* (2002) with *R. wratislaviensis*.

†Different results were reported by Yoon *et al.* (2000).

‡Data taken from: *e*, Goodfellow *et al.* (2002); *f*, Klatte *et al.* (1994); *g*, Briglia *et al.* (1996); *h*, Yoon *et al.* (2000).

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationship between strain RKJ300^T and related type strains of *Rhodococcus* species. *Corynebacterium diphtheriae* NCTC 11397^T was used as the outgroup. Numbers at nodes indicate levels of bootstrap support $\geq 50\%$, based on a neighbour-joining analysis of 1000 resampled datasets. GenBank accession numbers are given in parentheses. Bar, 1 substitution per 100 nucleotide positions.

Rhodococcus (Fig. 1), showing the highest level of sequence similarity with *R. wratislaviensis* NCIMB 13082^T (99.3%), followed by *R. opacus* DSM 43205^T (98.8%), *R. percolatus* MBS1^T (98.6%) and *R. koreensis* DNP505^T (98.1%).

The genomic relatedness of strain RKJ300^T, as revealed by DNA–DNA hybridization, was $58 \pm 1\%$ with *R. koreensis* MTCC 6425^T, $53 \pm 2\%$ with *R. opacus* MTCC 6420^T, $51 \pm 2\%$ with *R. percolatus* MTCC 6422^T and $49 \pm 2\%$ with *R. wratislaviensis* MTCC 6421^T. These data further supported the conclusion drawn by Yassin (2005) that representatives of *Rhodococcus* species with 16S rRNA gene sequence similarity greater than 98% share whole genomic relatedness values well below the 70% cut-off point recommended for the delineation of bacterial species (Wayne *et al.*, 1987). In view of this, we did not investigate the DNA–DNA relatedness of strain RKJ300^T with respect to species of *Rhodococcus* that showed 16S rRNA gene sequence similarities below 98%. The characteristics that differentiate strain RKJ300^T from closely related species are summarized in Table 1. On the basis of the data presented in this study, we conclude that strain RKJ300^T should be assigned to a novel species, for which the name *Rhodococcus imtechensis* sp. nov. is proposed.

Description of *Rhodococcus imtechensis* sp. nov.

Rhodococcus imtechensis [im.tech.en'sis. N.L. masc. adj. *imtechensis* pertaining to the Institute of Microbial Technology (IMTECH), where the type strain was characterized].

Gram-positive, aerobic, non-motile, catalase-positive, oxidase-negative actinobacterium that shows a hypha-rod-coccus life cycle. On TSA plates, colonies are circular, glistening, opaque, convex, creamish pink in colour and

have smooth margins. Growth occurs at 15–37 °C (optimum 30 °C), at pH 5.0–10.0 (optimum pH 7.0) and at 2.5–5.0% (w/v) NaCl. No growth occurs on citrate agar or MacConkey agar. Tween 80 and urea are hydrolysed, but casein, gelatin, starch, elastin and arbutin are not. Methyl red, Voges–Proskauer and oxidation/fermentation tests give negative results. *p*-Nitrophenol and 2,4-dinitrophenol are degraded. Nitrate and nitrite are not reduced. H₂S and indole are not produced. Utilization of carbon and nitrogen sources and acid production from carbohydrates under aerobic conditions are shown in Table 1. The diagnostic cell-wall amino acid is *meso*-diaminopimelic acid, and arabinose and galactose are the major cell-wall sugars, indicating cell-wall chemotype IV. The major isoprenoid quinone is MK-8(H₂); MK-9(H₂) is present in minor amounts. The cellular polar lipid content is of type II, comprising phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside, phosphatidylglycerol and traces of diphosphatidylglycerol. The predominant fatty acids are C_{16:0} (34.14%), C_{16:1ω7c} (21.18%), C_{17:1ω8c} (15.54%), C_{18:1ω9c} (8.72%), 10-methyl C_{18:0} (1.68%) and 10-methyl C_{17:0} (1.55%). Mycolic acids are present. The DNA G + C content is 72 mol%.

The type strain, RKJ300^T (=MTCC 7085^T =JCM 13270^T), was isolated from a pesticide-contaminated soil in Punjab State, India.

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