Pseudomonas delhiensis sp. nov., from a fly ash dumping site of a thermal power plant

Om Prakash, Kirti Kumari and Rup Lal

Molecular Biology Laboratory, Department of Zoology, University of Delhi, Delhi 110 007, India

Correspondence

Rup Lal

duzdel@vsnl.com

A phenanthrene- and citronellol-degrading bacterium, strain RLD-1^T, was isolated from the fly ash dumping site of a thermal power plant in Delhi, India. The 16S rRNA gene sequence indicated that this strain belongs to the genus *Pseudomonas*; high levels of sequence similarity were found with respect to *Pseudomonas citronellolis* DSM 50332^T (98.9%), *Pseudomonas jinjuensis* DSM 16612^T (97.6%) and *Pseudomonas nitroreducens* DSM 14399^T (97.5%). Phylogenetic analysis based on 16S rRNA gene sequences placed the strain within the clade represented by these three strains. Strain RLD-1^T showed low levels of DNA–DNA hybridization with respect to *P. citronellolis* DSM 50332^T (36%), *P. jinjuensis* DSM 16612^T (4%) and *P. nitroreducens* (13.7%). Strain RLD-1^T can also be distinguished from these three strains on the basis of several biochemical and physiological attributes. The novel strain contained high levels of cellular fatty acids 18:1 ω 7c, 16:0 and 16:1 ω 7c, along with 10:0 3-OH and 12:0 3-OH. Thus, strain RLD-1^T represents a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas delhiensis* sp. nov. is proposed. The type strain is RLD-1^T (=MTCC 7601^T =CCM 7361^T).

The genus *Pseudomonas* (Migula, 1894) represents a group of Gram-negative, non-spore-forming, motile, rod-shaped bacteria. It is an extremely heterogeneous group, and has been reclassified several times on the basis of phenotypic features (Sneath *et al.*, 1981), rRNA–DNA hybridization (Palleroni, 1984), 16S rRNA gene sequence similarity (Anzai *et al.*, 2000) and chemotaxonomic data (Oyaizu & Komagata, 1983; Vancanneyt *et al.*, 1996). Previously (Palleroni, 1984), members of the genus *Pseudomonas* were scattered throughout the classes *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*, but they are now restricted to the *Gammaproteobacteria* (with *Pseudomonas aeruginosa* as the type species); the members of the *Alphaproteobacteria* and *Betaproteobacteria* were transferred to other genera (Kerstens *et al.*, 1996; Anzai *et al.*, 2000).

In the present study, a phenanthrene- and citronellol-degrading bacterial strain, RLD-1^T, was isolated from the fly ash dumping site of a thermal power plant in Delhi, India, and found to use phenanthrene as the source of carbon and energy. Phylogenetic and taxonomic studies based on 16S rRNA gene sequences, DNA–DNA hybridization data, fatty acid patterns and physiological and biochemical

characteristics showed that strain RLD-1^T represents a novel species of the genus *Pseudomonas*.

Strain RLD-1^T was isolated by using an enrichment culture method (Samanta *et al.*, 1999) with phenanthrene as the source of carbon and energy (Kiyohara *et al.*, 1982). Colonies producing a clear zone due to phenanthrene degradation were isolated and purified on nutrient agar (NA) plates. Strain RLD-1^T showed luxuriant growth on citronellol, a natural acyclic monoterpenoid, which it degraded in liquid culture (data not shown). Interestingly, the phenanthrene-degrading ability was lost after two to three transfers on NA or Luria–Bertani (LB) agar, but the ability to degrade citronellol persisted.

Genomic DNA from strain RLD-1^T was extracted and purified as described by Pal *et al.* (2005). The 16S rRNA gene sequence was PCR-amplified (Robocycler 96; Stratagene) using a universal primer set corresponding to *Escherichia coli* positions 27F (16S forward primer: 5'-GAGTTTGAT-CCTGGCTCAG-3') and 1492R (16S reverse primer: 5'-TACGGTTACCTTGTTACGACTT-3'). The PCR product was run on a 3100 Avant Genetic Analyzer (Applied Biosystems) in the Department of Zoology, University of Delhi, India, using the MicroSeq Fullgene 16S rRNA gene sequencing kit. 16S rRNA gene sequence similarity values were obtained using the SEQUENCE MATCH program of the Ribosomal Database Project (<http://rdp.cme.msu.edu/html/>) and BLAST (<http://www.ncbi.nlm.nih.gov/blast/>).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RLD-1^T is DQ339153.

DNA–DNA relatedness data are available in a supplementary table in IJSEM Online.

To construct the phylogenetic tree, the 16S rRNA gene sequence of strain RLD-1^T was compared with 16S rRNA gene sequences of 20 of the most closely related *Pseudomonas* species with validly published names. These 20 sequences were aligned using CLUSTALX (Thompson *et al.*, 1997); common gaps from all of the selected sequences were removed and the alignment was checked manually for quality. Terminal nucleotides not common to all 20 sequences were removed. A phylogenetic analysis was carried out using the PHYLIP software package (version 3.5c; Felsenstein, 1993). An evolutionary distance matrix was calculated by using the distance model of Jukes & Cantor (1969). An evolutionary tree (Fig. 1) was constructed using the neighbour-joining algorithm (Saitou & Nei, 1987), and the resulting tree topology was evaluated by means of bootstrap analysis based on 1000 resamplings, using the SEQBOOT and CONSENSE programs in the PHYLIP package. A parsimony analysis (using DNAPARS) was also performed for the aligned sequence data, with bootstrapping based on 1000 resamplings.

A preliminary comparison of 1500 nt of the 16S rRNA gene sequence, conducted using the GenBank database, indicated that strain RLD-1^T has high levels of similarity with respect to *P. citronellolis* DSM 50332^T (98.9%), *P. jinjuensis* DSM 16612^T (97.6%) and *P. nitroreducens* DSM 14399^T (97.5%). Construction of a 16S rRNA gene sequence-based phylogenetic tree placed strain RLD-1^T in a monophyletic clade represented by *P. citronellolis* DSM 50332^T, *P. jinjuensis* DSM 16612^T and *P. nitroreducens* DSM 14399^T. A similar phylogenetic relationship was also obtained by using a maximum-parsimony method (data not shown). The levels of 16S rRNA gene sequence similarity and the results of the phylogenetic analysis indicate that strain RLD-1^T is a member of the genus *Pseudomonas* and belongs to rDNA similarity group I (Palleroni, 1984) and the *Pseudomonas aeruginosa* group of Anzai *et al.* (2000).

16S rRNA gene sequences are not sufficient to allow discrimination between closely related species. The current gold-standard method for species delineation is DNA–DNA hybridization (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Thus, to clarify the taxonomic status of strain RLD-1^T, DNA–DNA hybridization using the membrane filter method (Tourova & Antonov, 1987) was performed with *P. citronellolis* DSM 50332^T, *P. jinjuensis* DSM 16612^T and *P. nitroreducens* DSM 14399^T obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). After extraction and purification, 10 µg DNA from each strain was transferred onto a nylon membrane (Hybond-N; Amersham) in a dot-blot apparatus (Bio-Rad). The membrane was air-dried and UV cross-linked. DNA probes from each strain were labelled with [α -³²P]dATP (Board of Radiation and Isotope Technology, Hyderabad, India), using a nick-translation kit (Bangalore Genei). Hybridization was performed overnight at 65 °C. After hybridization, the filters were washed twice, first with 0.5 × SSC (3 M sodium chloride, 0.3 M sodium citrate; pH 7) and 0.1 % SDS for 10 min at room temperature and then with 0.1 × SSC and 0.5 % SDS for 20 min at 65 °C to remove the unbound probe. The amount of bound probe DNA was estimated by using a scintillation counter (Beckman Instruments). DNA of strain RLD-1^T was bound to the filters and was hybridized with the DNA probe prepared from each of the reference strains. The DNA–DNA hybridization data showed that, at the genomic level, strain RLD-1^T has DNA–DNA relatedness values of 36, 4 and 13.7 %, respectively, with *P. citronellolis* DSM 50332^T, *P. jinjuensis* DSM 16612^T and *P. nitroreducens* DSM 14399^T. The percentage relatedness values, calculated on the basis of the data obtained (means of four replicates) from DNA–DNA hybridization are summarized in Supplementary Table S1 (available in IJSEM Online). These data both substantiated the results obtained from the 16S rRNA gene sequence analysis and showed that strain RLD-1^T represents a novel species of the genus *Pseudomonas*.

Fig. 1. Neighbour-joining phylogenetic tree, based on almost-complete 16S rRNA gene sequence, showing relationships of strain RLD-1^T with related members of the genus *Pseudomonas*. The tree was rooted by using *Rhodanobacter lindanoclasticus* RP5557^T as the outgroup. Numbers at nodes represent bootstrap values (based on 1000 resamplings). GenBank accession numbers are shown in parentheses. Bar, 0.05 nucleotide substitutions per nucleotide position.

An analysis of the cellular fatty acids of strain RLD-1^T was carried out by Microbial ID (Newark, DE, USA) using the following procedure. Cultures of RLD-1^T were grown on TSBA (tryptic soya broth agar) and fatty acid methyl esters from 40 mg cells scraped from Petri dishes were analysed by saponification, methylation and extraction using the method of Sasser (1990). The fatty acid methyl esters were separated using the Sherlock Microbial Identification System (Microbial ID), consisting of an Agilent model 5980 gas chromatograph fitted with a phenyl methyl silicon column (25 m × 0.2 mm) and a flame ionization detector. Identification and comparison were carried out using the Aerobe (TSBA 50, version 5) databases of the Sherlock Microbial Identification system. Strain RLD-1^T contained 10:0 3-OH (3.62 %), 12:0 (4.61 %), 12:0 2-OH (4.83 %), 12:0 3-OH (4.67 %), 14:0 (1.10 %), 15:0 (trace amount), 16:0 (23.81 %), 17:0 cyclo (3.70 %), 18:1 ω 7c (32.56 %), 19:0 cyclo ω 8c (2.07 %) and summed feature 3 (16:1 ω 7c and/or iso-15:0 2-OH; 19.03 %). A comparative analysis of the fatty acid profile of strain RLD-1^T with those in the TSBA 505 library of MIDI produced the closest match with *P. aeruginosa* (similarity index value, 0.469). The presence of high levels of 18:1 ω 7c, 16:0 and 16:1 ω 7c, along with 10:0 3-OH and 12:0 3-OH, also showed good agreement with the fatty acid patterns of members of the genus *Pseudomonas*, indicating that strain RLD-1^T is a member of the genus *Pseudomonas*.

The morphological features of colonies of strain RLD-1^T (i.e. shape, size, colour, contours and pigment production) were studied on NA and on LB agar plates after 72 h incubation at 37 °C. Colonies were muddy white, each having a greyish spot in the centre. Gram staining and spore staining were done using Himedia kits. The size of the organism (obtained using micrometry) was approximately 0.5 × 1.5 µm. The motility of the micro-organism was studied by using the hanging drop method and by using motility agar (Farmer, 1999). For observation of the cell morphology by transmission electron microscopy, cells were grown on LB media and suspended in physiological saline solution. A small drop of suspension was placed on a carbon-coated copper grid and cells were negatively stained with 0.5 % uranyl acetate for observation under a transmission electron microscope (model 269D; Morgagni). Strain RLD-1^T was found to be a Gram-negative, non-spore-forming, rod-shaped bacterium, each cell bearing a single polar flagellum (Fig. 2). Antibiotic-sensitivity tests were performed on Mueller–Hinton II medium using ready-made Sensi-discs (Himedia) containing various amounts of antibiotic. Strain RLD-1^T was found to be sensitive to oxytetracycline (30 µg), tetracycline (30 µg), chlortetracycline (30 µg), kanamycin (30 µg), streptomycin (10 µg), gentamicin (10 µg) and neomycin (30 µg), but was found to be resistant to nalidixic acid (30 µg), rifamycin (5 µg), novobiocin (30 µg), vancomycin (30 µg), penicillin G (10 µg), ampicillin (10 µg), amoxicillin (10 µg), erythromycin (15 µg) and chloramphenicol (30 µg). A catalase test and tests of growth at different temperatures were carried

Fig. 2. Transmission electron micrograph of negatively stained cells of strain RLD-1^T, showing the single polar flagellum. Bar, 0.5 µm.

out as described by McCarthy & Cross (1984). To study growth at different temperatures, a 1.0 % (v/v) inoculum of strain RLD-1^T (OD₆₀₀ = 0.5) was added to nutrient broth incubated at different temperatures on a Lab-Line Orbit Environ shaker at 200 r.p.m. Growth was monitored by measuring the OD₆₀₀. Optimum growth was observed at 37 °C. Hydrolysis of aesculin and Tween 80 by RLD-1^T and the ability of the strain to grow in the presence of NaCl were tested as described by Arden Jones *et al.* (1979). In contrast to *P. citronellolis* DSM 50332^T and *P. jinjuensis* DSM 16612^T, strain RLD-1^T did not degrade aesculin but did hydrolyse Tween 80 and hypoxanthine (Table 1). Urease activity was detected as described by Christensen (1946). Acid production from carbohydrates and the degradation of xanthine and hypoxanthine were tested as described by Gordon *et al.* (1974). Other physiological tests and methods were as described by Collins *et al.* (1989). In contrast to *P. citronellolis* DSM 50332^T, *P. jinjuensis* DSM 16612^T and *P. nitroreducens* DSM 14399^T, strain RLD-1^T was able to utilize ribose and galactose (Table 1). The differences between the chemical and physiological data for strain RLD-1^T and those for the related strains *P. citronellolis* DSM 50332^T, *P. jinjuensis* DSM 16612^T and *P. nitroreducens* DSM 14399^T support the results of 16S rRNA gene sequence analysis, DNA–DNA hybridizations and fatty acid analysis and indicate that strain RLD-1^T is distinct from these strains. The tree topology also strongly suggested that strain RLD-1^T is a member of the genus *Pseudomonas*. Thus, strain RLD-1^T represents a novel species of *Pseudomonas*, for which the name *Pseudomonas delhiensis* sp. nov. is proposed.

Description of *Pseudomonas delhiensis* sp. nov.

Pseudomonas delhiensis (del.hi.en'sis. N.L. fem. adj. *delhiensis* pertaining to Delhi, the place of isolation of the type strain).

Cells are Gram-negative, aerobic, non-spore-forming, motile rods (0.5 × 1.5 µm) bearing a single polar flagellum. Colonies are smooth and circular, muddy white with a greyish central spot (1.5 mm in diameter on NA and

Table 1. Differential phenotypic characteristics of strain RLD-1^T with respect to related members of the genus *Pseudomonas*

Strains: 1, strain RLD-1^T; 2, *P. citronellolis* DSM 50332^T; 3, *P. jinjuensis* DSM 16612^T; 4, *P. nitroreducens* DSM 14399^T. +, Positive; ++, strongly positive; –, negative. Data are from this study unless indicated.

Characteristic	1	2	3	4
Cell size (µm)	0.5 × 1.5	1.1 × 3.1	0.4 × 2.1	1.0 × 3.0
Fluorescence on King's B medium	–	+	–	–
Growth at/with:				
4 °C	+	+	–	+
41 °C	+	+	–	–
5 % NaCl	++	+	–	+
Degradation of citronellol	+	+	–	–
Hydrolysis of:				
Aesculin	–	+	+	–
Tween 80	+	–	–	+
Hypoxanthine	++	+	++	+
Production of:				
Urease	+	++	–	+
Gelatinase	–	–	+	–
Acid production from:				
Adonitol	+	–	+	–
Dulcitol	–	–	+	–
Glucose	+	–	+	–
Mannitol	–	–	+	–
Ribose	+	–	–	–
Xylose	+	–	+	–
Fructose	++	+	++	++
Galactose	+	–	–	–
Arabinose	+	–	+	–
Glycerol	+	+	++	+

*Data from Kwon *et al.* (2003).

2.0 mm on LB agar after 72 h incubation at 37 °C). Positive for oxidase, catalase, nitrate reductase and urease and negative for gelatinase and amylase. Acid is produced from glycerol, lactose, mannitol, glucose, dextrin, sucrose, L-arabinose, D-fructose, D-galactose, xylose, D-trehalose, cellobiose and sorbitol. Sensitive to the following antibiotics (µg): oxytetracycline (30), tetracycline (30), chlortetracycline (30), kanamycin (30), streptomycin (10), gentamicin (10), neomycin (30). Resistant to the following antibiotics (µg): nalidixic acid (30), rifamycin (5), novobiocin (30), vancomycin (30), penicillin G (10), ampicillin (10), amoxicillin (10), erythromycin (15) and chloramphenicol (30). The fatty acid profile contains 10:0 3-OH (3.62 %), 12:0 (4.61 %), 12:0 2-OH (4.83 %), 12:0 3-OH (4.67 %), 16:0 (23.81 %), 17:0 cyclo (3.70 %), 18:1 ω 7c (32.56 %), 19:0 cyclo ω 8c (2.07 %) and summed feature 3 (16:1 ω 7c/iso-15:0 2-OH; 19.03 %). The optimum temperature for growth is 37 °C.

The type strain, RLD-1^T (=MTCC 7601^T=CCM 7361^T), was isolated from the fly ash dumping site of a thermal

power plant in Delhi, India, and has the capacity to degrade phenanthrene and citronellol.

Acknowledgements

Part of this work was supported by grants from the Department of Biotechnology (DBT), Government of India. We gratefully acknowledge SAIF-DST (Sophisticated Analytical Instrumentation Facility-Department of Science and Technology, Department of Anatomy – AIIMS) for providing the transmission electron microscopy facility. O.P. and K.K. acknowledge CSIR-UGC (Government of India) for research fellowships. We would like to thank J. P. Euzéby for etymological advice and J. S. Bhatti for critical reading of the manuscript.

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