Pseudomonas panipatensis sp. nov., isolated from an oil-contaminated site

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A Gram-negative, motile, rod-shaped, non-sporulating, aerobic bacterial strain (Esp-1^T) was isolated from oil-contaminated soil of Panipat Oil Refinery, India, and its taxonomic position was determined using a polyphasic approach. Strain Esp-1^T grew in the presence of 2 % NaCl at 30 °C and was characterized chemotaxonomically by having C_{16:0} as the major fatty acid followed by C_{17:0} cyclo and C_{18:1ω7c}. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain Esp-1^T formed a cluster together with *Pseudomonas knackmussii* DSM 6978^T (98.9 % sequence similarity), *Pseudomonas delhiensis* MTCC 7601^T (98.5 %), *Pseudomonas nitroreducens* DSM 14399^T (98.5 %), *Pseudomonas citronellolis* DSM 50332^T (98.7 %), *Pseudomonas multiresinivorans* ATCC 700690^T (98.9 %) and *Pseudomonas jinjuensis* DSM 16612^T (97.8 %). DNA–DNA hybridization values of strain Esp-1^T with *P. knackmussii* DSM 6978^T, *P. delhiensis* MTCC 7601^T, *P. jinjuensis* DSM 16612^T, *P. citronellolis* DSM 50332^T, *P. multiresinivorans* ATCC 700690^T and *P. nitroreducens* DSM 14399^T were 32.9, 30.2, 20.6, 23.4, 23.4 and 20.0 %, respectively. Low levels of DNA–DNA hybridization and phenotypic and chemotaxonomic results are sufficient to delineate strain Esp-1^T from other closely related species of *Pseudomonas*. Phenotypic and chemotaxonomic data confirm that strain Esp-1^T represents a novel species, for which the name *Pseudomonas panipatensis* sp. nov. is proposed. The type strain of *Pseudomonas panipatensis* sp. nov. is Esp-1^T (=MTCC 8990^T=CCM 7469^T).

The genus *Pseudomonas* is an extremely heterogeneous group that has been reclassified several times on the basis of phenotypic features (Sneath *et al.*, 1981), DNA–DNA hybridization (Palleroni, 1984), 16S rRNA gene sequence similarity (Anzai *et al.*, 2000) and chemotaxonomic data (Oyaizu & Komagata, 1983; Vancanneyt *et al.*, 1996). Its members grouped previously into the α , β and γ subclasses of the proteobacteria (Palleroni, 1984), but are now restricted to the γ subclass, with *Pseudomonas aeruginosa* as the type species; members of the α and β subclasses have been transferred to other genera (Kerstens *et al.*, 1996; Anzai *et al.*, 2000).

Members of the genus *Pseudomonas* are ubiquitous in nature due to their metabolic versatility (Palleroni, 1993; Elkin & Geddes, 2003; López-Romalde *et al.*, 2003; Levitski-Heikkilä & Ullian, 2005). A number of pseudomonads are known to utilize a variety of xenobiotics as

sources of carbon and energy (Kiyohara *et al.*, 1992; Johnsen *et al.*, 1996; Stolz *et al.*, 2007) and are thus exploited for the bioremediation of such compounds (O'Mahony *et al.*, 2006; Onaca *et al.*, 2007). Owing to their success in the bioremediation of soils with oily sludge (Lal & Khanna, 1996; Mishra *et al.*, 1999, 2001; Whyte *et al.*, 2001), there have been quite a large number of attempts to isolate *Pseudomonas* from such sites (Bhattacharya *et al.*, 2003; Prakash *et al.*, 2007a).

In an ongoing study, a bacterial strain (Esp-1^T) was isolated from an oil refinery (Panipat, India) that degrades citronellol (an isoprenoid and a component of oily sludge). Taxonomic studies based on 16S rRNA gene sequence analysis, morphological and physiological analysis, DNA–DNA relatedness and fatty acid patterns indicate that strain Esp-1^T represents a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas panipatensis* sp. nov. is proposed.

Soil samples collected from Panipat Oil Refinery were used to isolate bacterial strains. Strain Esp-1^T was isolated by a serial dilution plating method on Luria–Bertani (LB) agar (1.0 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, 0.1 % glucose) at 30 °C. Cell morphology was examined after 72 h incubation at 30 °C by light microscopy (Motic B1

Abbreviation: TEM, transmission electron microscope.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Pseudomonas panipatensis* Esp-1^T (=MTCC 8990^T=CCM 7469^T) is EF424401.

DNA–DNA hybridization data for strain Esp-1^T and related species are available with the online version of this paper.

series). The presence of flagella was determined with a transmission electron microscope (TEM) using cells from exponentially growing cultures. Cells were negatively stained with 0.5 % uranyl acetate on copper grids and, after air drying, the grids were examined with a TEM (Morgagni 269D). Motility of the organism was studied by the hanging drop method as well as in motility agar (Farmer, 1999). Gram staining and spore staining were done using a Himedia kit. Growth at various temperatures, pH values and salt (NaCl) concentrations was investigated in LB broth. Catalase activity was confirmed by adding 3 % (v/v) hydrogen peroxide solution to colonies grown on LB agar. Cytochrome oxidase activity was determined by Kovac's reagent. Citronellol degradation, nitrate reduction, urease activity, and hydrolysis of starch, aesculin, hypoxanthine, xanthine, casein, gelatin, and Tweens 20 and 80 were determined as described by Prakash *et al.* (2007a). Degradation of phenanthrene, anthracene and naphthalene was determined according to the protocol described by Kiyohara *et al.* (1982). Acid production from carbohydrates was determined as described previously by Gordon *et al.* (1974). Antibiotic sensitivity tests were performed on Mueller–Hinton II medium using a readymade Sensi-Disc (Himedia) with various amounts of antibiotics. Other physiological tests were performed as described by Collins *et al.* (1989) and Stanier *et al.* (1966). Type strains were obtained from the DSMZ, Braunschweig, Germany, and used for comparative studies.

Fatty acid methyl ester analysis was carried out at the Institute of Microbial Technology, Chandigarh, India, as follows. Two to four loops of culture were scraped from a Petri dish and subjected to saponification, methylation and extraction using the methods of Miller (1982) and Kuykendall *et al.* (1988). Fatty acid methyl ester mixtures were separated using the Sherlock Microbial Identification system (MIDI) consisting of GC (Agilent 68900) and a flame ionization detector. Identification and comparisons were made using the Aerobe (TBSA50, version 5) database of the Sherlock Microbial Identification system. Genomic DNA extraction was performed as described by Pal *et al.* (2005).

Strain Esp-1^T was a Gram-negative, non-sporulating, rod-shaped (1.0–1.2 × 2.8–3.5 µm) bacterium. It possessed a single polar flagellum (Fig. 1). Colonies were muddy-white,

Fig. 1. TEM of negatively stained cells of strain Esp-1^T showing the presence of a single polar flagellum. Bar, 0.5 µm.

smooth and circular in appearance. Citronellol degradation, nitrate reduction, and catalase and oxidase tests gave positive results. Xanthine, Tween 20, starch and gelatin were not hydrolysed, whereas hypoxanthine and Tween 80 were hydrolysed. Strain Esp-1^T was sensitive to oxytetracycline (30 µg), tetracycline (30 µg), kanamycin (30 µg), gentamicin (10 µg), neomycin (30 µg) and chloramphenicol (30 µg), but resistant to streptomycin (10 µg), novobiocin (30 µg), penicillin G (10 µg) and erythromycin (15 µg). Strain Esp-1^T did not produce a clear zone when cultured on LB agar or minimal salt agar plates containing phenanthrene, anthracene or naphthalene. Phenotypic characteristics of strain Esp-1^T were compared with those of six closely related species of *Pseudomonas* (Table 1). Strain Esp-1^T showed remarkable differences from the closely related strain *Pseudomonas knackmussii* DSM 6978^T with respect to colony colour, citronellol degradation, growth in 3 % NaCl (w/v), nitrate reduction, hydrolysis of Tween 80 and hypoxanthine, production of urease, assimilation of D-glucose, D-fructose, liquid paraffin, heptane, L-histidine, L-serine, α-naphthol, trehalose, sorbitol and acetate, and acid production from maltose and D-fructose. Phenotypic characteristics are important in delineating newly isolated strains from existing species (Stackebrandt *et al.*, 2002). Hence, marked phenotypic differences between strain Esp-1^T and *P. knackmussii* enable them to be differentiated. Other phenotypic properties of strain Esp-1^T are given in the species description.

Analysis of the 16S rRNA gene sequence was carried out using a single colony from an overnight grown culture of the bacterium. This colony was picked up with a sterile tip, boiled in deionized water and centrifuged at 10 000 g for 10 min. The supernatant was diluted 10-fold and used for PCR amplification. PCR amplification was carried out with Robocycler 96 (Stratagene) using a universal primer set corresponding to positions 27F (16S forward primer; 5'-AGAGTTTGATCTGGCTCAG-3') and 1492R (16S reverse primer; 5'-TACGTTACCTTGTTACGACTT-3') of *Escherichia coli* (Prakash *et al.*, 2007b). The PCR product (1.5 kb) was purified using a Qiagen kit according to the manufacturer's instructions and run on a 3100-Avant Genetic Analyzer Sequencer (Applied Biosystems) in the Department of Zoology, University of Delhi, Delhi, India, with a MicroSeq Fullgene 16S rRNA gene sequencing kit. The 16S rRNA gene sequences of closely related strains were obtained using the sequence match program of the Ribosomal Database Project (RDP; <http://rdp.cme.msu.edu/>) and BLAST (<http://www.ncbi.nlm.nih.gov>).

A phylogenetic tree was constructed using 16S rRNA gene sequences of strain Esp-1^T and the 12 most closely related species of the genus *Pseudomonas* with validly published names, along with an outgroup sequence. Multiple alignments of these 14 sequences were performed using CLUSTAL_X (Thompson *et al.*, 1997), common gaps from all the selected sequences were removed and the alignment was checked manually for quality. Terminal nucleotides

Table 1. Differential phenotypic characteristics of strain Esp-1^T and related *Pseudomonas* species

Strains: 1, *P. panipatensis* Esp-1^T; 2, *P. knackmussii* DSM 6978^T; 3, *P. delhiensis* MTCC 7601^T; 4, *P. citronellolis* DSM 50332^T; 5, *P. jinjuensis* DSM 16612^T; 6, *P. nitroreducens* DSM 14399^T; 7, *P. multiresinivorans* ATCC 700690^T. All tests were performed under reproducible laboratory conditions. +, Positive; ++, strong positive; (+), weak positive; –, negative; ND, not done. All strains tested positive for: growth on MacConkey agar and allantoin; DNase, catalase and oxidase; assimilation of L-proline, *o*-cresol, allantoin, pyruvate and citrate; and acid formation from D-glucose. All strains tested negative for: lecithinase; assimilation of inositol, pyrocatechol, aniline and *para*-nitrophenol; and acid formation from L-serine, sucrose, adonitol, sorbose, Triton X-100, α -naphthol, inositol, rhamnose, trehalose, propionate, sorbitol, acetate, pyridine, heptane, cystine, L-histidine, L-arabinose, glycerol and toluene. All data are from this study unless otherwise indicated.

Characteristic	1	2	3	4	5	6	7
Cell size (μm)	1.2 $\times$ 3.5	ND	0.5 $\times$ 1.5*	1.1 $\times$ 3.1†	0.4 $\times$ 2.1‡	1.0 $\times$ 3.0†	ND
Growth at/in:							
4 °C	–	–	+	+	–	+	ND
41 °C	++	++	+	+	–	–	–
3 % NaCl	–	+	++	+	–	+	ND
Nitrate reduction	+	–	–	+	+	+	ND
Citronellol degradation	+	–	+	+	–	–	+
Hydrolysis of:							
Aesculin	–	–	–	+	+	–	–
Tween 80	+	–	+	–	–	+	+
Hypoxanthine	++	+	++	+	++	+	+\$
Production of:							
Urease	++	+	+	++	–	+	–\$
Gelatinase	–	–	–	–	+	–	–
Assimilation of:							
Adonitol	–	–	+	–	–	–	–
D-Glucose	++	+	+	+	+	+	+
D-Mannitol	–	–	++	–	–	–	–\$
D-Ribose	–	–	+	–	–	–	–
D-Xylose	–	–	+	–	–	–	–
D-Fructose	–	+	++	+	++	++	+\$
D-Galactose	–	–	–	+	–	–	–
L-Arabinose	–	–	++	–	–	–	–
Glycerol	–	+	+	+	++	+	+
Toluene	–	–	+	(+)	–	–	–
Liquid paraffin	+	(+)	+	–	+	++	+
Pyridine	–	–	(+)	–	–	–	–
Heptane	(+)	–	+	+	+	+	(+)
Cystine	–	–	(+)	–	–	(+)	–
L-Histidine	+	–	+	+	++	+	+
L-Serine	–	+	+	+	(+)	+	(+)
Sorbose	–	–	+	–	–	–	–
Triton X-100	(+)	(+)	(+)	+	(+)	+	+
α -Naphthol	–	+	–	+	–	(+)	–
Trehalose	(+)	–	+	–	–	–	–
Propionate	–	–	+	–	–	–	–
Sorbitol	+	–	(+)	–	–	–	–
Acetate	++	–	+	–	–	–	–
Acid production from:							
L-Arabinose	–	–	++	–	–	–	–
Maltose	+	–	–	–	–	–	–
D-Xylose	–	–	++	–	–	–	–
D-Fructose	–	+	++	+	++	+	+
D-Ribose	–	–	+	–	–	–	–
Galactose	–	–	+	+	–	–	–
Mannitol	–	–	++	–	–	–	–

*Prakash *et al.* (2007a).

†Palleroni (2005).

‡Kwon *et al.* (2003).

\$Mohn *et al.* (1999) and Lang *et al.* (2007).

not common to all 14 sequences were removed. Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Nei & Kumar, 2000) methods using the MEGA3 package (Kumar *et al.*, 2004). The evolutionary distance matrices for the neighbour-joining method were calculated using the Kimura two-parameter method (Kimura, 1980). Similarity values for the 16S rRNA gene sequences were calculated using the FASTA program (Pearson & Lipman, 1988).

The 16S rRNA gene sequence of strain Esp-1^T showed highest similarity to *P. knackmussii* DSM 6978^T (98.9%), followed by *Pseudomonas multiresinivorans* ATCC 700690^T (98.9%), *Pseudomonas delhiensis* MTCC 7601^T (98.5%), *Pseudomonas citronellolis* DSM 50332^T (98.7%), *Pseudomonas nitroreducens* DSM 14399^T (98.5%) and *Pseudomonas jinjuensis* DSM 16612^T (97.8%). The neighbour-joining tree indicated that strain Esp-1^T formed a cluster with these six strains (Fig. 2). A similar phylogenetic relationship was also obtained by the maximum-parsimony method (data not shown). 16S rRNA gene sequence similarities and phylogenetic analysis (with high bootstrap values) indicate that strain Esp-1^T is a member of the genus *Pseudomonas* that belongs to rDNA similarity group I (Palleroni, 1984) and the *P. aeruginosa* group of Anzai *et al.* (2000).

DNA–DNA hybridization of strain Esp-1^T was performed using the membrane filter method (Bala *et al.*, 2004; Tourouva & Antonov, 1987) with the six most related strains based on 16S rRNA gene sequencing. DNA–DNA relatedness values of strain Esp-1^T with *P. knackmussii* DSM 6978^T, *P. delhiensis* MTCC 7601^T, *P. jinjuensis* DSM 16612^T, *P. citronellolis* DSM 50332^T, *P. multiresinivorans* ATCC 700690^T and *P. nitroreducens* DSM 14399^T were 32.9, 30.2, 20.6, 23.4, 23.4 and 20%, respectively. Percentage relatedness calculated on the basis of the data obtained (mean of four replicates) by DNA–DNA hybridization (summarized in Supplementary Table S1, available in IJSEM Online) showed less than 70% relatedness, which is within the threshold value for bacterial species delineation (Wayne *et al.*, 1987). These data further

indicate that strain Esp-1^T represents a novel species of the genus *Pseudomonas*.

Strain Esp-1^T contained C_{10:0} 3-OH (0.99%), C_{12:0} (2.13%), C_{12:0} 2-OH (5.30%), C_{12:0} 3-OH (4.49%), C_{14:0} (2.62%), C_{16:0} (32.27%), C_{17:0} cyclo (12.41%), C_{18:1}ω7c (15.29%), C_{18:0} (3.37%), C_{19:0} cyclo ω8c (8.04%) and summed feature 3 (C_{16:1}ω7c and/or C_{15:0} 2-OH iso; 11.12%). Fatty acid analysis revealed that C_{16:0} and C_{18:1}ω7c were the dominant fatty acids in the cell wall of strain Esp-1^T. The fatty acid profile of strain Esp-1^T was compared with those of the type strains of some *Pseudomonas* species. The presence of high levels of C_{18:1}ω7c and C_{16:0}, along with C_{10:0} 3-OH, C_{12:0} 2-OH and C_{12:0} 3-OH (Table 2) in the fatty acid profiles corresponds with the profiles of other members of the genus *Pseudomonas*.

The position of strain Esp-1^T in the genus *Pseudomonas* is indicated by its distinctive phenotypic features, 16S rRNA gene sequence and chemotaxonomic features. Sufficient divergence from other species is emphasized by its phenotypic features, such as degradation of citronellol, urease production, hydrolysis of Tween 80, growth at different NaCl concentrations, nitrate reduction, and acid production from and assimilation of different substrates (Table 1), as well as low DNA–DNA hybridization values. Thus, it is concluded that strain Esp-1^T represents a novel species of *Pseudomonas* for which the name *Pseudomonas panipatensis* sp. nov. is proposed.

Description of *Pseudomonas panipatensis* sp. nov.

Pseudomonas panipatensis (pa.ni.pa.ten'sis. N.L. fem adj. *panipatensis* pertaining to Panipat, the place of isolation of the bacterium).

Cells are Gram-negative, aerobic, non-sporulating, motile rods (1.0–1.2 × 2.8–3.5 μm). Possesses a single polar flagellum. Colonies are smooth, circular and muddy-white, growing up to 1.5 mm diameter on LB agar after 72 h incubation at 30 °C. Grows well at 28–37 °C and pH 5–9, but growth does not occur in the presence of 3% NaCl.

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain Esp-1^T with other related taxa. The tree was rooted by using *Rhodanobacter lindanoclasticus* RP5557^T as outgroup. Numbers at branches indicate bootstrap values of 100 resamplings.

Table 2. Cellular fatty acid compositions (%) of strain Esp-1^T and the type strains of related *Pseudomonas* species

Strains: 1, *P. panipatensis* Esp-1^T; 2, *P. knackmussii* DSM 6978^T (Stolz *et al.*, 2007); 3, *P. delhiensis* MTCC 7601^T (Prakash *et al.*, 2007a); 4, *P. citronellolis* DSM 50332^T (Prakash *et al.*, 2007a); 5, *P. nitroreducens* DSM 14399^T (Prakash *et al.*, 2007a); 6, *P. jinjuensis* DSM 16612^T (Prakash *et al.*, 2007a); 7, *P. multiresinivorans* ATCC 700690^T (Lang *et al.*, 2007). All strains were grown on trypticase soy agar at 28 °C for 48 h prior to fatty acid analysis. Empty cells indicate that fatty acids were not detected.

Fatty acids	1	2	3	4	5	6	7
Saturated fatty acids							
C _{10:0}				0.7		0.3	
C _{12:0}	2.1	3.7	4.6	3.3	1.5	6.3	1.8
C _{14:0}	2.6	0.8	1.1	0.8	0.4	0.5	0.8
C _{15:0}		0.3	0.2	0.3		1.0	
C _{16:0}	32.3	19.4	23.8	21.0	21.2	19.6	24.3
C _{17:0}						0.8	
C _{18:0}	3.4	0.3		0.3	0.7	0.4	0.5
Unsaturated fatty acids							
C _{17:1} ω8c						1.2	
C _{18:1} ω7c	15.3	36.6	32.6	35.7	41.8	29.8	36.7
Hydroxy fatty acids							
C _{8:0} 3-OH				0.4			
C _{10:0} 3-OH	1.0	3.5	3.6	6.2	3.8	3.7	3.6
C _{12:0} 2-OH	5.3	3.5	4.8	3.6	5.0	1.5	5.7
C _{12:0} 3-OH	4.5	4.0	4.7	3.5	3.9	3.2	1.1
Summed feature 3*	11.1	24.8	19.0	17.8	16.5	24.5	20.8
Cyclopropane acids							
C _{17:0} cyclo	12.4	2.7	3.7	2.7	1.6	4.7	1.3
C _{19:0} cyclo ω8c	8.0	0.4	2.1	3.2	0.9	2.6	0.5

*Summed features represent groups of two or three fatty acids that can not be separated by GLC with the MIDI system. Summed feature 3 contains one or more of the fatty acids C_{16:1}ω7c and C_{15:0} iso 2-OH.

Positive for oxidase, catalase, DNase, nitrate reductase and urease, but negative for indole, methyl red/Voges-Proskauer, gelatinase and amylase. D-Glucose, D-fructose, D-galactose and D-arabinose are utilized as carbon sources, but not egg yolk, D-xylose, lactose or raffinose. Growth is observed on MacConkey agar. Acid is produced from D-glucose and maltose, but not from adonitol, mannitol, ribose, D-fructose, D-galactose, D-arabinose, sucrose, xylose, raffinose or lactose. The fatty acid profile contains C_{10:0} 3-OH (0.99 %), C_{12:0} (2.13 %), C_{12:0} 2-OH (5.30 %), C_{12:0} 3-OH (4.49 %), C_{14:0} (2.62 %), C_{16:0} (32.27 %), C_{17:0} cyclo (12.41 %), C_{18:1}ω7c (15.29 %), C_{18:0} (3.37 %), C_{19:0} cyclo ω8c (8.04 %) and summed feature 3 (C_{16:1}ω7c and/or C_{15:0} 2-OH iso; 11.12 %). Table 1 summarizes other phenotypic properties.

The type strain is Esp-1^T (=MTCC 8990^T=CCM 7469^T), isolated from oil-contaminated soil of Panipat Oil Refinery, India.

Acknowledgements

The study was supported by National Bureau of Agriculturally Important Microorganisms (NBAIM), Indian Council of Agricultural Research (ICAR), Government of India. We would like to thank J. P.

Euzèby for etymological advice and Hans-Jürgen Busse for providing *Pseudomonas knackmussii* DSM 6978^T.

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