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Pseudomonas indica sp. nov., a novel butane-utilizing species

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The taxonomic position of two butane-utilizing bacteria was studied using a polyphasic approach. Biochemical and physiological characteristics indicated these to be members of the genus *Pseudomonas*, showing more similarity to *Pseudomonas mendocina* than to any other species. The major fatty acids found in these two strains also pointed to their similarity to *P. mendocina*. On the other hand, DNA–DNA hybridization studies with seven related *Pseudomonas* species belonging to the *γ-Proteobacteria* and the ΔT_m values of reassociated molecules clearly showed that these two strains do not belong to any of the seven species tested. The 16S rRNA gene was sequenced and compared with the sequences available in the GenBank database. Phylogenetic analysis using the region covering positions 31–1488 (*Escherichia coli* numbering) confirmed these observations and placed these two strains as members of the authentic *Pseudomonas*, but not in any existing species of the genus. On the basis of biochemical characteristics, fatty acid profiles, DNA–DNA reassociation and ΔT_m values, as well as 16S rRNA gene sequence analyses, these two isolates were shown to belong to one species but to have characteristics distinct from those of validly described species of *Pseudomonas* (*sensu stricto*). These strains, therefore, should be recognized as a novel species, for which the name *Pseudomonas indica* sp. nov. is proposed. The type strain is strain IMT37^T (= MTCC 3713^T = DSM 14015^T).

Keywords: *Pseudomonas indica*, butane, phylogeny, 16S rRNA gene sequence, DNA–DNA hybridization

INTRODUCTION

Until recently, the genus *Pseudomonas* Migula 1894 included a large number of well-characterized organisms, as well as poorly characterized organisms. It was realized some time ago that the diversity and heterogeneity within the genus was too broad. As a result, the members of this genus were divided into five groups based on rRNA–DNA hybridization data (Palleroni, 1984), of which species belonging to rRNA similarity groups II–V have been reclassified and transferred to other genera. At present, only the members of rRNA group I are considered to represent authentic *Pseudomonas* species (Palleroni, 1992a, b; see Anzai *et al.*, 2000, for an excellent review).

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The GenBank accession numbers for the 16S rDNA sequences of strains IMT37^T and IMT40 are respectively AF302795 and AF302796.

The members of the genus *Pseudomonas* are well known for their metabolic diversity and ability to utilize and degrade a large variety of natural and xenobiotic compounds. Although some species of *Pseudomonas* are useful specifically for the degradation of aromatic hydrocarbons and their derivatives and have been well studied at the biochemical, genetic and molecular levels, only one species, '*Pseudomonas butanovora*', has been reported to utilize a gaseous alkane (butane) (Takahashi, 1980). This organism has now been shown to have strong homology with *Thauera* species, and has been classified in the *Rhodocyclus* group of the *β-Proteobacteria* (Anzai *et al.*, 2000). It is reported that a large number of bacteria, particularly those belonging to the Gram-positive *Corynebacterium–Nocardia–Mycobacterium–Rhodococcus* complex, can utilize propane and butane as sole sources of carbon and energy (Ashraf *et al.*, 1994). Although 25 of 29 strains of *Pseudomonas aeruginosa* were reported to grow on one of two

Table 1. Biochemical and physiological characteristics of strains IMT37^T and IMT40

P. aeruginosa and *P. mendocina* were used as reference strains. The characteristics of *P. stutzeri* were taken from Palleroni (1984) for comparison. All of the taxa were Gram-negative, aerobic, motile, catalase- and oxidase-positive and could grow at between 25 and 42 °C (but not at 4 or ⩾ 50 °C) and in the pH range 5·7–10·0. All taxa were positive for: utilization of glucose, L-arginine, L-valine, L-serine, L-histidine, DL-alanine, L-phenylalanine, betaine, pimelic acid, sebacic acid, adipic acid, sodium succinate, 2-ketogluconate, propionate, sodium pyruvate and ethanol, tolerance of sodium azide (0·02%), growth on MacConkey agar and hydrolysis of Tween 60 and Tween 80. All taxa were negative for utilization of adonitol, cellobiose, inositol, lactose, L-rhamnose, salicin, sorbitol and D-trehalose. The characteristics that distinguish strains IMT37^T and IMT40 from each other are indicated by asterisks. ND, No data available; d, 11–89% of tested strains were positive; W, weak reaction.

Test	IMT37^T	IMT40	*P. aeruginosa*	*P. mendocina*	*P. stutzeri*
Sugar utilization:					
D-Ribose	−	−	+	−	−
D-Xylose*	−	+	+	−	−
D-Arabinose	−	−	+	−	−
L-Arabinose	+	+	−	−	−
D-Galactose*	−	+	+	−	−
D-Mannose	−	−	+	−	−
Fructose*	−	+	+	+	+
Maltose	+	+	−	−	+
Raffinose	−	−	−	−	ND
Mannitol	+	+	+	−	+
Sucrose*	−	+	+	+	ND
Acid production from:					
Rhamnose	−	−	−	−	ND
Salicin	−	−	−	−	ND
Arabinose	+	+	+	−	ND
Lactose	−	−	−	−	ND
Raffinose	−	−	−	−	ND
Inositol	−	−	−	−	ND
Sucrose	+	+	−	−	ND
Xylose*	−	+	+	−	ND
Fructose	−	−	+	−	ND
Galactose*	−	+	+	−	ND
Mannitol	+	+	+	−	ND
Adonitol	−	−	−	−	ND
Mannose	−	−	+	−	ND
Maltose	+	+	−	−	ND
Trehalose	−	−	−	−	ND
Sorbitol	−	−	−	−	ND
Cellobiose	−	−	−	−	ND
Melibiose*	−	+	+	−	ND
Inulin*	−	+	−	−	ND
Dulcitol	−	−	−	−	ND
Growth in NaCl at:					
2·5%	+	+	+	+	ND
5%	−	−	+	+	ND
7%	−	−	−	+	ND
10%	−	−	−	−	ND
Utilization of:					
L-Arginine	+	+	+	+	−
Tryptamine	−	−	+	+	−
Nicotinic acid	−	−	+	+	−
L-Histidine	+	+	+	+	−
L-Leucine	+	+	−	+	+

Table 1 (*cont.*)

Test	IMT37^T	IMT40	*P. aeruginosa*	*P. mendocina*	*P. stutzeri*
Utilization of:					
Tartrate	−	−	+	−	−
Betaine	+	+	+	+	−
Geraniol	−	−	−	+	−
L-Malic acid	+	+	−	+	+
PEG-6000	−	−	+	+	ND
Pimelic acid	+	+	+	+	−
Sodium gluconate	+	+	−	+	d
p-Hydroxybenzoate	+	+	+	−	d
Glycerol*	−	+	−	+	+
Isobutyric acid*	−	+	−	+	−
2-Ketogluconate	+	+	+	+	−
Ethanol	+	+	+	+	ND
n-Butanol	+	+	+	−	d
Butyric acid	+	+	+	−	+
Hexanes	+	+	−	−	−
Butane	+	+	−	−	ND
Simmons' citrate agar	+	+	+	+	ND
Indole	−	−	−	−	ND
Hydrolysis of:					
Starch	+	+	+	−	+
Casein*	−	+	+	−	ND
Gelatin	−	−	+	−	−
Tyrosine degradation*	+	−	−	−	ND
Urease	−	−	−	−	ND
Methyl red test	−	−	−	−	ND
Voges–Proskauer test	−	−	−	−	ND
Oxidation/fermentation (O/F)	O	O	O	O	ND
Nitrate reduction	−	−	+(w)	−	+
Fluorescence	−	−	+	−	ND
Lysine decarboxylase*	+	−	−	−	ND
Arginine dihydrolase	−	−	−	−	ND
Ornithine decarboxylase	−	−	−	−	ND

aliphatic hydrocarbons, hexadecane or dodecane (Stanier *et al.*, 1966), no species of authentic *Pseudomonas* was reported to use a gaseous alkane (butane) as a sole source of carbon and energy. We have recently described the discovery of a *Pseudomonas* strain, IMT37^T, capable of butane utilization, and have characterized a gene encoding a 54 kDa polypeptide that is involved in the initial step of butane utilization (Padda *et al.*, 2001). However, the taxonomic position of the strain was not clear.

Here, we report detailed characterization of two strains, IMT37^T and IMT40, isolated from oilfields in Gujarat (India). On the basis of a polyphasic study involving biochemical and physiological tests, fatty acid analysis, DNA–DNA hybridization and 16S rRNA gene sequence analysis, we propose that strains IMT37^T and IMT40 belong to a novel species, *Pseudomonas indica* sp. nov.

METHODS

Bacterial strains and growth conditions. For routine maintenance, all strains were grown on Luria–Bertani medium (Sambrook *et al.*, 1989) at 30 °C. Butane-utilizing strains IMT37^T and IMT40 were isolated from soil samples by the enrichment method, using liquefied petroleum gas as the sole source of carbon (Padda *et al.*, 2001).

Physiological and biochemical tests. The two strains isolated from the oilfields in Gujarat were tested for a number of phenotypic characteristics, as described by Palleroni (1984). Two type strains (*P. aeruginosa* MTCC 2453^T and *Pseudomonas mendocina* MTCC 1602^T) were used for comparison. All the strains were tested for morphological characteristics, flagellar structure, Gram staining, motility, oxidase, catalase, aerobic or anaerobic growth, hydrolysis of Tween, starch, gelatin and casein and other biochemical properties, as listed in Table 1.

To study the growth of these strains on gaseous alkanes, cells were streaked on plates of minimal medium (Whittenbury *et*

al., 1970) and were placed in a desiccator. The desiccator was evacuated and then filled with an alkane/air mixture (40:60). To check growth on various hydrocarbons (C_5–C_{10}), the cultures streaked on minimal plates were placed in a desiccator along with a glass Petri dish containing a few drops of the hydrocarbon to saturate the desiccator atmosphere with the vapours of the hydrocarbon.

Cellular fatty acid profiles. The gaseous alkane-utilizing strains IMT37^T and IMT40 were grown on tryptic soy agar (Difco) plates at 30 °C for 24 h. Fatty acid methyl esters were obtained from freshly grown cultures by saponification, methylation, extraction and washing according to the instructions of the MIDI system (Microbial ID). The samples were injected into a Hewlett Packard model 5890A gas chromatograph equipped with an HP7673A autosampler and a flame-ionization detector fitted with a 5% phenylmethyl silicone column (0·2 mm × 25 m). The injection-port temperature was 300 °C and the oven temperature was set between 170 and 270 °C, increasing at a rate of 5 °C min^{-1}. The gas chromatograph was connected to an integrator (HP3392A). Fatty acid profiles were compared with the profiles in the MIS library of MIDI. The strains were also kindly analysed by the DSMZ for confirmation of the fatty acid profile.

DNA isolation and general techniques. The genomic DNA used for amplification of the 16S rRNA gene and DNA–DNA hybridization studies was isolated by using the CTAB/phenol/chloroform method, as described by Ausubel *et al*. (1992). pUC19 was isolated using the Qiagen Plasmid Midi kit according to the manufacturer's instructions. Other general molecular biology techniques used in the study were performed according to Sambrook *et al*. (1989). The G+C content (mol %) of strains IMT37^T and IMT40 was determined by the thermal denaturation method and was calculated by the equation given by Mandel & Marmur (1968). Genomic DNA of *Bacillus megaterium*, with a G+C content of 37·6 mol %, was used as a control for G+C content determinations.

DNA–DNA hybridization. DNA–DNA hybridization was performed by following the filter hybridization method as described by Johnson (1994), with some modifications. Equal amounts of chromosomal DNA of strains IMT37^T and IMT40 and seven other *Pseudomonas* type strains were denatured in an alkaline solution (0·4 M NaOH, 10 mM EDTA) at 100 °C for 10 min. Samples were chilled immediately on ice and vacuum-blotted on nylon membrane (Hybond-NX) using the Bio-Dot SF apparatus (Bio-Rad). Blots were washed twice with 0·4 M NaOH. DNA was fixed on the membrane by UV cross-linking for 5 min. Prehybridization and hybridization were done in Pyrex bottles in a hybridization oven (Amersham Life Science) at 42 °C in 25% formamide. Hybridization buffer contained 6× SSC, 5× Denhardt's solution, 0·2 mg calf-thymus DNA ml^{-1} and 0·5% SDS. Probes for hybridization were prepared by radiolabelling genomic DNA from strains IMT37^T and IMT40 separately with [α-^{32}P]dCTP, using the Megaprime labelling kit (Amersham). Hybridization and washings were done according to the protocol given by the manufacturer of Hybond-NX membrane. After the unbound probe was washed off, the membrane was exposed to X-ray film. The percentage similarity between the labelled DNA and test strains was determined by measuring the densities of dark spots formed on X-ray film by using a Shimadzu double-wavelength flying-spot scanning densitometer.

Thermal stability of reassociated DNA. The extent of base-pair mismatching was estimated by comparing the thermal stability of heteroduplexes with the stability of homologous duplexes. Genomic DNA from strains IMT37^T and IMT40 and the other seven *Pseudomonas* type strains was blotted and hybridized in the same way as for the DNA–DNA hybridization experiment. After the unbound probe was washed off, the membrane was cut into small pieces, each piece having heteroduplex or homoduplex DNA. Membrane pieces were put separately into 2 ml microcentrifuge tubes containing 1 ml elution buffer (0·5× SSC) and incubated at 55 °C for 6 min. Membrane pieces were taken out and incubated in fresh elution buffer at a temperature 5 °C higher than the previous one. The elution profile was monitored in this way up to 90 °C at 5 °C intervals, maintaining each temperature for 6 min. Radioactivity (c.p.m.) was measured in a β-scintillation counter (Vallac) for each eluate by taking 0·5 ml eluate and mixing it with 4·5 ml scintillation fluid [0·55% (w/v) PPO; 0·01% (w/v) POPOP; 33·3% (v/v) Triton X-100 in toluene]. An integral elution-profile curve was made by summing the radioactivity at each temperature and dividing that by the total radioactivity.

16S rRNA gene sequence determination. Full-length 16S rRNA genes of strains IMT37^T and IMT40 were amplified by a PCR using 100 ng chromosomal DNA and 25 pmol primers designed from the conserved region in ribosomal genes [8-27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1500r (5′-AGAAAGGAGGTGATCCAGGC-3′); *Escherichia coli* numbering system, Brosius *et al*., 1978]. PCR cycling parameters included an initial denaturation at 95 °C for 4 min followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and amplification at 72 °C for 2 min and final extension at 72 °C for 10 min. Amplified fragments were separated by electrophoresis on an agarose gel, and the expected 1·5 kb full-length 16S rRNA gene fragment was eluted by using the Qiaquick gel extraction kit. These fragments were blunt-ended by T4 DNA polymerase and then cloned in pUC19, digested with *Sma*I and dephosphorylated with calf intestinal alkaline phosphatase. The rDNA sequence was determined by the dideoxy chain-termination method using the Big-Dye terminator kit (Applied Biosystems). Sequencing reaction products were analysed by capillary electrophoresis on an ABI 310 Genetic Analyzer.

Analyses of sequence data. The 16S rRNA gene sequences of strains IMT37^T and IMT40 were used as a query to search for homologous sequences in the GenBank database. The 16S rDNA sequences of 39 species of *Pseudomonas* showing high similarity were retrieved from the GenBank database and aligned with those of strains IMT37^T and IMT40 using CLUSTAL W version 1.81 at the European Bioinformatics Institute website (http://www.ebi.ac.uk/clustalw). In constructing the phylogenetic tree, gaps present in the sequences retrieved from GenBank were removed manually. Aligned sequences were analysed by various phylogenetic methods available in the PHYLIP package (Felsenstein, 1993) at http://bioweb.pasteur.fr to assess the phylogenetic position of these strains. Pairwise evolutionary distances were calculated using DNADIST (Kimura's two-parameter method; Kimura, 1980). A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) and the unweighted pair group arithmetic average-linkage algorithm. A maximum-likelihood analysis was also done with fastDNAml (Felsenstein, 1989; Olsen *et al*., 1994). Distance-matrix data obtained from DNADIST were also used to construct a phylogenetic tree by using KITSCH. To

determine the confidence values for individual branches, 100 bootstrap replications were done for each generated tree, using SEQBOOT and CONSENSE from the PHYLIP package.

RESULTS AND DISCUSSION

Physiological and biochemical characteristics

Both strains IMT37^T and IMT40 were isolated on the basis of their ability to utilize gaseous alkanes as the sole source of carbon and energy. They were also shown to utilize higher straight-chain alkanes (tested up to C_{10}), *p*-hydroxybenzoate and protocatechuate. Both strains could grow at between 25 and 42 °C and within a pH range of 5·7–10·0. They could tolerate NaCl concentrations up to 2·5%. Microscopic, biochemical and physiological observations revealed strains IMT37^T and IMT40 to be Gram-negative rods, motile, non-fluorescent, aerobic and catalase- and oxidase-positive. Transmission electron micrographs show that cells of both strains have a single polar flagellum (Fig. 1). Both could hydrolyse Tween 60, Tween 80 and starch but not gelatin. Both strains were able to utilize many sugars as carbon sources (listed in Table 1). Under oxidative conditions, both strains produced acid from arabinose, sucrose, glucose, mannitol and maltose but not from rhamnose, salicin, lactose, raffinose, inositol, fructose, adonitol, mannose, trehalose, sorbitol, cellobiose or dulcitol. Under anaerobic conditions, none of the strains could produce acid from glucose. The two isolates were negative for the methyl red and Voges–Proskauer tests, nitrate reduction, arginine dihydrolase, ornithine decarboxylase and urease. The results of all these tests are summarized in Table 1. Thus, based on these properties, both strains could be placed in the genus *Pseudomonas* (Palleroni, 1984). The presence of a single polar flagellum in both these isolates is also a characteristic typical of *Pseudomonas* spp.

Although strains IMT37^T and IMT40 have many biochemical and physiological properties in common, they also differ in a few biochemical and physiological characteristics. Strain IMT40 could utilize D-xylose, galactose, fructose, sucrose, glycerol and isobutyric acid as carbon sources, whereas strain IMT37^T was unable to utilize any of these substrates. Strain IMT40 could hydrolyse casein but strain IMT37^T could not, and the latter was able to degrade tyrosine and showed a positive lysine decarboxylase reaction. The two isolates showed differences in their ability to produce acids under aerobic conditions: strain IMT40 produced acid from xylose, galactose, melibiose and inulin, unlike strain IMT37^T. Therefore, although strains IMT37^T and IMT40 belong to the genus *Pseudomonas* and were shown to be closely related in terms of most phenotypic properties, they differ in a few characteristics.

The G+C content of the DNA of strains IMT37^T and IMT40 was found to be 72 mol%. The value observed for these two isolates is the highest reported so far for *Pseudomonas* (58–70 mol%; Palleroni, 1984).

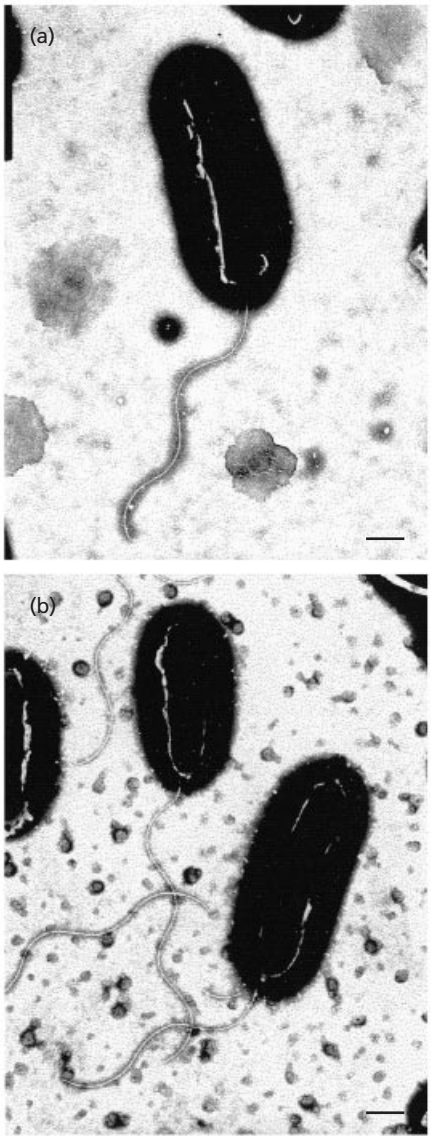

Fig. 1. Transmission electron micrographs of cells of strains IMT40 (a) and IMT37^T (b), showing the single polar flagella. Bars, 500 nm.

The original genus *Pseudomonas* Migula 1894 contained a wide variety of Gram-negative aerobic bacteria, which were divided into five subgroups based on rRNA–DNA hybridization. Various members of the genus *Pseudomonas* were distributed among the α-, *β*- and *γ*-subclasses of the *Proteobacteria* (Palleroni, 1992a). In recent years, modern classification approaches have led to the transfer of most of the species from rRNA groups II–V to other genera, leaving rRNA group I as *Pseudomonas sensu stricto* (Palleroni, 1992b), with *P. aeruginosa* as the type species of the genus. The phenotypic characteristics of strains IMT37^T and IMT40 are very similar, though not exactly identical. On the basis of these characteristics, these two strains could not be placed in any

Table 2. DNA relatedness and ΔT_m between strains IMT37^T and IMT40 and other type strains of *Pseudomonas*

Values were obtained by DNA–DNA hybridization and thermal stability determination.

Test DNA	Reference DNA (probe)			
	IMT37^T		IMT40	
	Relatedness (%)	ΔT_m (°C)	Relatedness (%)	ΔT_m (°C)
Strain IMT37^T	100	–	110·82	2
Strain IMT40	88·70	0·5	100	–
P. aeruginosa MTCC 2453^T	70·71	19	68·61	17·5
P. alcaligenes MTCC 493^T	64·79	16·5	70·81	15
P. fluorescens MTCC 617^T	50·68	16·5	52·45	14
P. fragi MTCC 510^T	50·41	18	44·86	13
P. mendocina MTCC 1602^T	66·48	18	71·18	11·5
P. oleovorans MTCC 617^T	71·47	14·5	61·06	17·5
P. putida MTCC 102^T	70·28	13·5	49·35	12

validly described species of *Pseudomonas*, and thus they warrant the creation of a novel species to accommodate them. Among the existing species, only *P. mendocina* and *Pseudomonas stutzeri* are phenotypically close to these two isolates. The fatty acid and lipid profiles of strains IMT37^T and IMT40 are also indicative of their closeness to *P. mendocina* and *P. stutzeri*.

Fatty acid analysis

The fatty acid profiles of strains IMT37^T and IMT40 were very similar. The predominant fatty acids were 18:1ω7*c* (36%), 16:0 (18·4%), 12:0 (7·6%), 10:0 3OH (3·5%) and 16:1ω7*c*/15 iso2OH (22–24%). Other fatty acids present in smaller amounts were 12:0 3OH (2·5%), 17:1ω8*c* (1·2–1·47%) and 15:0 (0·99–1·36%). The fatty acid and lipid profiles of the two strains closely matched those of *Pseudomonas*, particularly those of *P. mendocina*, followed by those of *P. stutzeri*.

Genomic relatedness

Labelled genomic DNA of strains IMT40 and IMT37^T was reciprocally hybridized with that of seven type strains of *Pseudomonas*. All the type strains chosen for the analysis belonged to rRNA group I (Palleroni, 1984) of the genus *Pseudomonas* in the *γ-Proteobacteria* (Anzai *et al.*, 2000). The results of DNA–DNA hybridization studies are shown in Table 2. DNA relatedness between strains IMT37^T and IMT40 was between 88 and 100%. Strain IMT40 had relatively high similarity, in the range 61–71%, to four type strains belonging to the *P. aeruginosa* group (Anzai *et al.*, 2000): *P. aeruginosa*, *P. mendocina*, *Pseudomonas oleovorans* and *Pseudomonas alcaligenes*. The extent of similarity was lower (49–52%) to the type strains of *Pseudomonas fragi*, *Pseudomonas putida* and *Pseudomonas fluorescens*. The hybridization profile was found to be similar when DNA of strain IMT37^T was used as a probe, except that *P. putida* showed relatively high similarity (70%).

Genomic relatedness between strains IMT37^T or IMT40 and each of seven type strains of the genus *Pseudomonas* was determined by measuring the thermal stability of reassociated homoduplex and heteroduplex DNA molecules. Heterogeneity between strain IMT37^T and IMT40 DNA was minimal, as evident from the low ΔT_m (0·5–2·0 °C). Although a few species of *Pseudomonas* showed 61–71% DNA relatedness with strains IMT37^T or IMT40 on the basis of hybridization studies, all seven species showed a high degree of base-pair mismatch, which was reflected in low T_m values of heteroduplex molecules and consequently in high ΔT_m values, ranging from 11·5 to 19 °C (Table 2). Considering that a ΔT_m of 1 °C represents mispairing of 1–2·2% (Johnson, 1994), DNA of strain IMT37^T and that of strain IMT40 was found to have close similarity to each other but not to that of other species tested.

DNA–DNA hybridization of more than 70% and a ΔT_m of less than 5 °C are two key features of members belonging to the same species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Since our two isolates showed 88% or more DNA relatedness to each other and a ΔT_m of 2 °C, strains IMT37^T and IMT40 fulfil these criteria. The results of DNA–DNA hybridization of these two isolates with related strains of *Pseudomonas* from rRNA group I and a ΔT_m value of more than 5 °C indicate that strains IMT37^T and IMT40 are significantly different from other species of *Pseudomonas*, even if they did show some degree of relationship in terms of phenotypic characteristics and fatty acid profiles. Our conclusion is that strains

IMT37^T and IMT40 should be considered as members of a novel species.

Phylogenetic analyses based on 16S rDNA sequences

The 16S rRNA gene sequences of strains IMT37^T and IMT40 were determined and compared with sequences in the GenBank database. A BLAST search was done using the 16S rDNA sequences of strains IMT37^T and IMT40 as queries. Maximum similarity of the sequences of strains IMT37^T and IMT40 was found to different species of the genus *Pseudomonas*. Representative 16S rRNA gene sequences of the 39 most closely related species of *Pseudomonas* were chosen for phylogenetic comparison. All of the sequences retrieved from GenBank were more than 1400 bp in length. The regions covering positions 31–1488 (*E. coli* numbering) of the rDNA sequences of these species were aligned with the 16S rRNA gene sequences of strains IMT37^T and IMT40, using CLUSTAL W version 1.81. The sequences of strains IMT40 and IMT37^T were 99% identical but exhibited more than 4% dissimilarity with respect to other *Pseudomonas* 16S rRNA gene sequences in the database. Of the 39 *Pseudomonas* 16S rRNA sequences retrieved from GenBank, 23 were from different strains of *P. aeruginosa*; these sequences were 99% identical to each other. There were also two sequences for the type strain of *Pseudomonas pseudoalcaligenes*. To reduce analysis times in various phylogenetic algorithms, only two *P. aeruginosa* strains and one sequence from the *P. pseudoalcaligenes* type strain were finally used in further analyses. Thus, the final phylogenetic analysis was done with 18 sequences of 16S rRNA genes, including those of strains IMT37^T and IMT40. A phylogenetic tree based on the neighbour-joining method is shown in Fig. 2. The phylogenetic tree showed that strains IMT37^T and IMT40 formed a cluster at a level of 99% similarity, with 100% bootstrap support. The branch closest to the cluster containing strains IMT37^T and IMT40 contains three *Pseudomonas* species (*P. aeruginosa*, *Pseudomonas resinovorans* and *P. stutzeri*) belonging to the authentic species of *Pseudomonas* (Anzai *et. al.*, 2000). Similar phylogenetic trees were obtained with the neighbour-joining unweighted pair group arithmetic average-linkage algorithm, maximum-likelihood and KITSCH (not shown). The nearest relative of strains IMT37^T and IMT40, *P. stutzeri*, was found to have 4% dissimilarity to strains IMT37^T or IMT40, which corresponds to 57 nucleotide differences in the 16S rRNA sequence. With *P. resinovorans*, the similarity is 94%, amounting to 86 nucleotide differences. The sequences of strains IMT37^T and IMT40 showed between 95 and 96% similarity to the *P. aeruginosa* sequences, corresponding to a difference of between 72 and 57 nucleotides. Thus, our results clearly indicate that strains IMT37^T and IMT40 are members of the genus *Pseudomonas* but that they differ considerably from other described *Pseudomonas* species.

The 16S rDNA sequences of these two isolates are 99% similar but they are quite different from existing

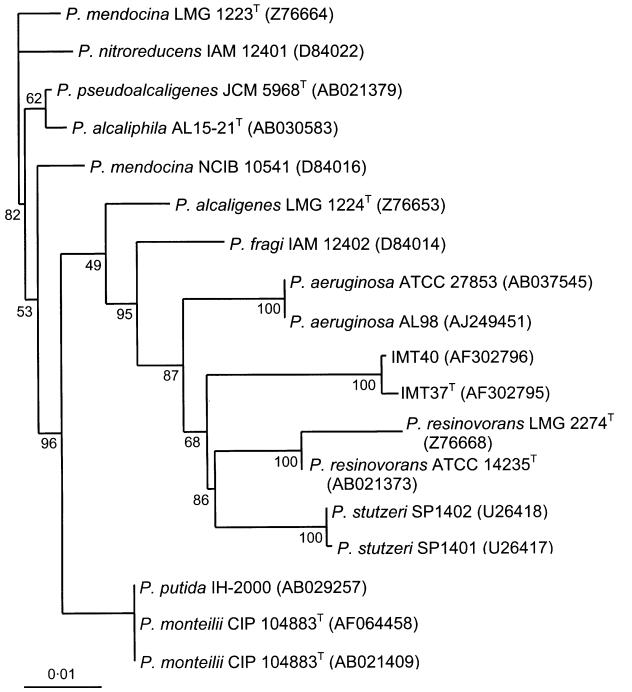

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences, drawn using the neighbour-joining method, showing the relationships between strains IMT37^T, IMT40 and other *Pseudomonas* species. The evolutionary distances were calculated with DNADIST, using Kimura's two-parameter model. Bootstrap values are also shown at the branch nodes. Bar, 0·01 substitutions per site.

species. Yet, clustering of these two isolates with species belonging to rRNA group I of *Pseudomonas* in the phylogenetic tree (Fig. 2) strongly suggests their inclusion in this group, which contains the authentic species of *Pseudomonas*. 16S rRNA sequence signatures for both strains IMT37^T and IMT40 match that of the *γ-Proteobacteria* described by Woese (1987). Only five of 69 signature nucleotides were different from the consensus in IMT37^T (199R:c–U, 929G–C, 947G–C, 948C:u–A, 976G–A) while, in the case of IMT40, only two nucleotides (44A–G, 199R:c–U) were different. None of the strains considered in the phylogenetic studies showed more than 96% similarity to strain IMT37^T or IMT40, which is a good indication in itself that these two isolates should be considered as a separate species. The percentage similarity of the 16S rDNA sequence and the DNA relatedness values of strains IMT37^T and IMT40 in relation to other related type strains of *Pseudomonas* are in full agreement with the observation of Stackebrandt & Goebel (1994) that, if sequence similarity values are below about 97·5%, it is unlikely that two organisms have more than 60–70% DNA similarity and hence are not related at the species level. Interestingly, when the 16S rDNA sequences of strains IMT37^T and IMT40 were aligned with 16S rDNA sequences of other known higher-alkane-utilizing bacteria such as *Acinetobacter calcoaceticus*, *Nocardioides* sp. CF8, *P. putida*, *P. fluorescens*,

Brucella sp. A4/I, *Alcaligenes faecalis*, *Rhodococcus erythropolis* and *Rhodococcus* sp. 5/14 (GenBank accession numbers AJ009589, AF210769, AJ249825, U71003, U71005, U71008, AJ009591 and AF181690), they did not show any significant similarity, as expected. The sequence of a 2·6 kb fragment of strain IMT37^T DNA that is involved in butane utilization was found to be novel in these two organisms (Padda *et al*., 2001). The G+C content of the gene was 66·8 mol%. The sequence of another 7·3 kb fragment that was determined also contains 67·2 mol% G+C. This fragment contains a *p*-hydroxybenzoate hydroxylase operon involved in the utilization of *p*-hydroxybenzoate. The high G+C content determined from the T_m of genomic DNA is fully reflected in these characterized regions containing genes for hydrocarbon utilization (unpublished data).

Thus, the gaseous-alkane-utilizing isolates IMT37^T and IMT40 can be distinguished from all validly described species of *Pseudomonas* on the basis of morphological, biochemical and physiological characteristics, G+C content and the results of 16S rRNA gene sequence analyses, DNA–DNA hybridization and ΔT_m-determination studies. We conclude, therefore, that isolate IMT37^T should be considered as the type strain of a separate species, designated *Pseudomonas indica* sp. nov. Since strain IMT40 shows close similarity to strain IMT37^T, this isolate should be considered as belonging to *P. indica*. Both strains have been deposited in the Microbial Type Culture Collection and Gene Bank (MTCC, India) under accession numbers MTCC 3713^T (IMT37^T) and MTCC 3714 (IMT40). The type strain, IMT37^T, has also been deposited in the DSMZ as DSM 14015^T.

Description of *Pseudomonas indica* sp. nov.

Pseudomonas indica (in′di.ca. L. fem. adj. *indica* pertaining to India, where the organism was isolated).

Gram-negative, motile with a single polar flagellum, non-fluorescent and catalase- and oxidase-positive. Cells are straight rods, 2–5 μm long and 1 μm wide, and can grow at between 25 and 42 °C over a pH range of 5·7–10·0 on tryptic soy agar and in NaCl concentrations up to 2·5%. Freshly grown colonies are translucent and round with convex surfaces and smooth edges. Gives a positive lysine decarboxylase reaction and is able to degrade tyrosine. Acid is produced from arabinose, sucrose, mannitol, maltose and glucose under oxidative conditions. It cannot hydrolyse casein. It can utilize a number of organic compounds such as betaine, pimelic acid, sodium gluconate, sodium pyruvate, 2-ketogluconate, sebacic acid, adipic acid and L-malic acid. Nitrogenous compounds such as L-arginine, L-serine, L-valine, L-histidine and L-phenylalanine are utilized. One of the most distinguishing properties of this strain is its ability to utilize butane and higher alkanes as well as other hydrocarbons such as *p*-hydroxybenzoate and protocatechuate.

Isolated from oilfields in Gujarat, India. Strain IMT37^T (= MTCC 3713^T = DSM 14015^T) is the type strain. Strain IMT40 (= MTCC 3714) is a reference strain.

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