Pseudomonas turukhanskensis sp. nov., isolated from oil-contaminated soils

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A bacterial strain named IB1.1T was isolated in a screening of hydrocarbon-degrading bacteria from oil-contaminated soils on the territory of the Turukhansk District of Krasnoyarsk Krai, East Siberia, Russia. The 16S rRNA gene sequence had 98.7 % identity with respect to the closest phylogenetic relative, Pseudomonas granadensis F-278,770T, and the next most closely related species with 98.6 % similarity was Pseudomonas punonensis, suggesting that IB1.1T should be classified within the genus Pseudomonas. The analysis of housekeeping genes rpoB, rpoD and gyrB showed similarities lower than 90 % in all cases with respect to the closest relatives, confirming its phylogenetic affiliation. The strain showed a polar flagellum. The respiratory quinone was Q9. The major fatty acids were 16:1ω7c/16:1ω6c (summed feature 3), 18:1ω7c and 16:0. The strain was oxidase- and catalase-positive, but the arginine dihydrolase system was not present. Nitrate reduction, urease and β-galactosidase production, and aesculin hydrolysis were negative. The temperature range for growth was 4–34 °C, and the strain could grow at pH 11. The DNA G+C content was 58.5 mol%. DNA–DNA hybridization results showed values of less than 30 % relatedness with respect to the type strains of the eight most closely related species. Therefore, the dataset of genotypic, phenotypic and chemotaxonomic data support the classification of strain IB1.1T into a novel species of the genus Pseudomonas, for which the name Pseudomonas turukhanskensis sp. nov. is proposed. The type strain is IB1.1T (=VKM B-2935T=CECT 9091T).

The Turukhansk District is a large administrative unit of Krasnoyarsk Krai (East Siberia, Russia). Its territory includes one of the sites of the promising Vankor oil and gas field, the development of which has a negative environmental impact. Because of the very continental climate (8-month-long severe winters up to −60 °C, short hot summers up to +35 °C, annual average air temperature −5.8 °C) and peculiar soil features (deep-seated permafrost zones, low nutrient levels, poor aeration, etc.), natural self-purification of oil-contaminated soils by native microbiota proceeds at a slow rate. Under such conditions, the best biological way for accelerating the process is the introduction of psychrotrophic micro-organisms that have sufficient activity at low temperatures and show considerable ability for degradation of petroleum hydrocarbons. The genus Pseudomonas includes several species reported as xenobiotic-degrading bacteria such as Pseudomonas panipatensis (Gupta et al., 2008), P. oleovorans (Saha et al., 2010), P. benzenivorans and P. saponiphila (Lang et al., 2010), and some of them were isolated from oil-contaminated soil, such as Pseudomonas frederiksgenensis (Andersen et al., 2000), P. taeanensis (Lee et al., 2010), P. sagittaria (Lin et al., 2013) and the recently described P.astesunigri, isolated from beach intertidal sand samples contaminated after the Prestige oil spill (Sanchez et al., 2014). Moreover, the presence of psychrotolerant members of the genus Pseudomonas isolated from soil and able to utilize petroleum hydrocarbons at low temperatures have been reported, such as P. toyotomiensis (Hirota et al., 2011).

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

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, rpoD, rpoB and gyrB sequences of strain IB1.1T are KP306892, LT219438, LT219439 and LT219440, respectively.

Two supplementary figures and one supplementary table are available with the online Supplementary Material.

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In a previous study focusing on the screening of hydrocarbon-degrading bacteria from oil-contaminated soils on the territory of the Turukhansk District of Krasnoyarsk Krai, we isolated a collection of strains and checked their ability to assimilate deane, toluene and methylnaphthalene in liquid Raymond’s medium at 4–6 °C (Korshunova et al., 2012). From all the isolates, strain IB-1.1T was the most effective one. Based on the preliminary study of morphological, physiological and biochemical characteristics, we provisionally assigned it as a representative of the genus Pseudomonas (Korshunova et al., 2012). In the present study we performed a polyphasic taxonomy study, and the phylogenetic analysis of 16S rRNA and housekeeping genes as well as phenotypic and chemotaxonomic features confirmed that strain IB-1.1T belongs to the genus Pseudomonas and represents a novel species for which we propose the name Pseudomonas turukhanskensis sp. nov.

The cells were stained according to the Gram procedure described by Doetsch (1981). Motility was checked by phase-contrast microscopy after growing cells on nutrient agar (NA; BD) medium at 22 °C for 48 h. The cell size and flagellation type was determined by a scanning probe microscope Solver PRO-M (NT-MDT) after 7 h of incubation on NA at 26 °C as was previously described (Bolskova et al., 2001, 2004). Strain IB1.1T was Gram-stain-negative, rod-shaped (1–1.1×2.5–3.3 mm) and motile by a polar flagellum (Fig. S1, available in the online Supplementary Material). Cells grew as round, translucent, beige-coloured colonies on NA.

For 16S rRNA gene sequencing and comparison analysis, DNA extraction, amplification and sequencing were performed as reported by Rivas et al. (2007). The amplification and partial sequencing of the gyrB, rpoB and rpoD housekeeping genes was performed as described by Mulet et al. (2010), using the primers PseEG50F/PseEG790R for the rpoD gene (Mulet et al., 2009) and LAPS5F/LAPS27R for the rpoB gene (Tayeb et al., 2005). For gyrB, the following primers were used (sequencing primers are underlined): (UP-1, 5’-GAAGTCATCATGACCGTCTGCGAYGCNGG-NNGAARTTYGA-3’; and UP-2r, 5’-AGCAAGGTACGGATGTGCCAGCRTCNGCRTCNGT-3’) (Yamamoto & Hayama, 1998).

The sequences obtained (1412 nt for 16S, 724 nt for rpoD, 1129 nt for rpoB and 1149 nt for gyrB) were compared with those from GenBank using the BLASTN (Altschul et al., 1990) and EzTaxon (Kim et al., 2012) programs, and identities were calculated after pairwise comparison. For phylogenetic analysis, sequences were aligned using the CLUSTAL X software (Thompson et al., 1997). The distances were calculated according to Kimura’s two-parameter model (Kimura, 1980). Phylogenetic trees of 16S rRNA gene sequences were inferred using neighbour-joining analysis (NJ; Saitou & Nei, 1987) and maximum-likelihood (ML; Rogers & Swofford, 1998). MEGA5 software (Tamura et al., 2011) was used for all analyses.

The comparison of the 16S rRNA gene sequence of strain IB1.1T against those of the type strains of bacterial species recorded in the EzTaxon-e database showed the affiliation of the novel strain to genus Pseudomonas. The most closely related species was Pseudomonas grandensis F-278,770T (Pascual et al., 2015) with 98.7 % pairwise similarity (17 nucleotides difference), and the next most closely related species with 98.6 % similarity (19 different nucleotides) was Pseudomonas punonensis (Ramos et al., 2013). The remaining related species of the genus Pseudomonas showed less than 98.5 % identity.

The phylogenetic analysis of 16S rRNA gene sequences was carried out including all the species closely related to the novel species showing more than 97 % sequence similarity as well as representative strains from the Pseudomonas groups according to Mulet et al. (2010), including the type strain of the type species of the genus, Pseudomonas aeruginosa LMG 1242T. According to the NJ phylogenetic tree (Fig. 1), IB1.1T clustered in a separate branch associated with a wide group that includes the closest relative P. grandensis F-278,770T and other related species such as Pseudomonas reinekei MT-1T, P. vancouverensis DhA-51T, P. moorei RW10T and P. helmancticensis OHAI1T. The same tree topology was obtained after ML phylogenetic analysis (data not shown).

Additionally to the 16S rRNA gene, three housekeeping genes widely used in the phylogenetic analysis of species of the genus Pseudomonas were analysed in this work (Tayeb et al., 2005; Mulet et al., 2009, 2010, 2012; Ramos et al., 2013; Ramirez-Bahena et al., 2015). The NJ phylogenetic tree of the concatenated rpoD, rpoB and gyrB gene sequences was congruent with the phylogeny based on the 16S rRNA gene analysis, supporting the affiliation of IB1.1T to genus Pseudomonas as a representative of a separated species related to a group formed by P. grandensis, P. reinekei, P. vancouverensis, P. moorei and P. helmancticensis, almost equidistant to the group formed by P. punonensis, P. straininea and P. argentinensis (Fig. 2). Again, the results were congruent with those of the ML phylogenetic analysis (data not shown). The identities of the housekeeping gene sequences with respect to P. grandensis, P. punonensis, P. argentinensis, P. reinekei, P. vancouverensis, P. straininea, P. moorei and P. helmancticensis were about 77–78 % for rpoD, 90 % for rpoB and 84–87 % for the gyrB gene. These values evidence the great phylogenetic distance of strain IB1.1T from the described species of the genus Pseudomonas, and the results of the rpoD, rpoB and gyrB gene analysis also indicated that IB1.1T represents an undescribed species of this genus.

DNA–DNA hybridization was carried out by the method of Ezaki et al. (1989), following the recommendations of Willems et al. (2001). Strain IB1.1T was hybridized with the type strains of the eight species of the genus Pseudomonas showing more than 98 % identity in 16S rRNA gene sequences, P. grandensis F-278,770T, P. punonensis LMT03T, P. argentinensis CH01T, P. reinekei MT-1T, P.
Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1242 nt) of *Pseudomonas turukhanskensis* sp. nov. IB1.1<sup>T</sup> and closely related species of the genus *Pseudomonas*. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branching points. Bar, one nucleotide substitution per 100 nt.

*Pseudomonas turukhanskensis* sp. nov.

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For base composition analysis, DNA was prepared according to the method of Chun & Goodfellow (1995). The G+C content of DNA (mol%) was determined using the thermal denaturation method (Mandel & Mamur, 1968). The DNA G+C content of strain IB1.1T was 58.5 mol%. This value is within the range obtained for species of the genus *Pseudomonas* (Palleroni, 2005).

The cellular fatty acids were analysed by using the Microbial Identification System (MIDI; Microbial ID) Sherlock 6.1 and the library RTSBA6 according to the technical instructions provided by this system (Sasser, 1990). Strain IB1.1T and the type strains of the same closely related species considered for DNA–DNA hybridization experiments were grown on TSA plates (Becton Dickinson, BBL) for 24 h at 28°C and harvested in the late exponential growth phase. The major fatty acids of strain IB1.1T 16:0 (22%), 16:1ω7c/16:0ω6c in summed feature 3 (34.3%) and C18:1ω7c/C18:1ω6c in summed feature 8 (26.4%). As expected, all the relatives clustering in the same phylogenetic group as IB1.1T shared similar fatty acid profiles (Table 1). IB1.1T had the three fatty acids typically present in members of the genus *Pseudomonas* according to Palleroni (2005), which are 10:0 3-OH, 12:0 and 12:0 3-OH.

Strain IB1.1T was cultivated for 24 h on TSA plates (Becton Dickinson, BBL) at 28°C to obtain the cell mass required for quinone analysis that was carried out from freeze-dried cells using the methods described by Collins & Jones (1981), Tamaoka et al. (1983) and Collins (1985). The novel isolate, IB1.1T, contained Q9 as respiratory quinone (100%). The presence of Q9 as the major ubiquinone is in agreement with the results obtained for the species of the genus *Pseudomonas* (Palleroni, 2005).

For fluorescent pigment analysis, cells were grown in King B broth and testing for pigment production (King et al., 1954). Strain IB1.1T produced a fluorescent pigment in this medium.

argentinensis CH01\textsuperscript{T} and P. helmantiensis OHA11\textsuperscript{T}), from the DSMZ culture collection (P. reinekei DSM 18361\textsuperscript{T}, P. vancouverensis DSM 17555\textsuperscript{T} and P. moorei DSM 12647\textsuperscript{T}), from the LMG culture collection (P. granadensis LMG 27940\textsuperscript{T}) and from the CIP culture collection (P. straminia CIP 106745\textsuperscript{T}). Additionally, API 20NE (BioMérieux) and Biolog GN2 Microplates were used following the manufacturers’ instructions. The results of API 20NE were recorded after 48 h of incubation at 28 °C. Phenotypic characteristics of the novel species are reported below in the species description, and the differences with respect to the closest species of the genus Pseudomonas and the type species of the genus, P. aeruginosa, are recorded in Table 2. The phenotypic characteristics of strain IB1.1\textsuperscript{T} support its classification within the genus Pseudomonas since it is a motile, Gram-negative-staining rod, strictly aerobic, catalase- and oxidase-positive and produces a fluorescent pigment typical of this genus (Hildebrand et al., 1994). Strain IB1.1\textsuperscript{T} can be differentiated from other species of the genus Pseudomonas in the 16S rRNA and housekeeping gene sequences and DNA–DNA hybridization values, as well as in the phenotypic and chemotaxonomic characteristics. Therefore, the novelty of strain IB1.1\textsuperscript{T} was demonstrated genetically by means of phylogenetic analyses of 16S rRNA and housekeeping genes as well as DNA–DNA hybridization values, and also from the point of phenotypic and chemotaxonomic approaches, since 32 phenotypic differences with respect to the closely related species of the genus Pseudomonas were detected (Table 2) and some differences in the fatty acids profiles were also shown, particularly in main fatty acids relevant in species of the genus Pseudomonas such as 12:0, 12:0 2-OH, 16:0, 16:1ω7c/16:1ω6c and 18:1ω7c/18:1ω6c (Table 1). Taking together all the phylogenetic, chemotaxonomic and phenotypic data, strain IB1.1\textsuperscript{T} should be classified into a novel species within the genus Pseudomonas, for which the name Pseudomonas turukhanskensis sp. nov. is proposed.

**Description of Pseudomonas turukhanskensis sp. nov.**

Pseudomonas turukhanskensis [tur.ru.khansk.en’sis. N.L. fem. adj. turukhanskensis pertaining to Turukhansk District,

<table>
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<th>Strain 2</th>
<th>Strain 3</th>
<th>Strain 4</th>
<th>Strain 5</th>
<th>Strain 6</th>
<th>Strain 7</th>
<th>Strain 8</th>
<th>Strain 9</th>
<th>Strain IB1.1\textsuperscript{T}</th>
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*Summed feature 3: 16:1ω7c/16:1ω6c.
†Summed feature 8: 18:1ω7c/18:1ω6c.
Table 2. Differential phenotypic characteristics of *P. turukhanskensis* sp. nov. IB1.1<sup>1</sup>, its phylogenetically most closely related species and the type species of the genus, *P. aeruginosa*

Strains: 1, *P. turukhanskensis* sp. nov. IB1.1<sup>1</sup>; 2, *P. granadensis* LMG 27940<sup>1</sup>; 3, *P. punonensis* LMT03<sup>1</sup>; 4, *P. argentiniensis* CH01<sup>1</sup>; 5, *P. reinkei* DSM 18361<sup>1</sup>; 6, *P. vancouverensis* DSM 17555<sup>1</sup>; 7, *P. straminea* CIP 106745<sup>1</sup>; 8, *P. moorei* DSM 12647<sup>1</sup>; 9, *P. helmanticensis* OHA11<sup>1</sup>; 10, *P. aeruginosa* ATCC 10145<sup>1</sup>. All data were obtained in this study unless specifically indicated. +, Positive; −, negative; w, weakly positive reaction.

<table>
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<td>+</td>
<td>−</td>
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*Data for *P. aeruginosa* ATCC 10145<sup>1</sup> are from Palleroni (2005), Clark et al. (2006) and Xiao et al. (2009).*

the name of the administrative unit of Krasnoyarsk Krai (East Siberia, Russia) where the type strain was isolated.

Gram-stain-negative, strictly aerobic, non-spore forming, rod-shaped cells of 2.5–3.3 mm in length and 1–1.1 mm in diameter, motile by a polar flagellum. Colony morphology on nutrient agar is circular, convex, beige, translucent and usually 2.0 mm in diameter within 3 days of growth at 28 °C. Growth temperature range is 0–34 °C, and pH range for growth is pH 6 to 8. No growth is observed with 5% NaCl concentration in nutrient broth. A diffusible fluorescent pigment is produced on King B medium. Strictly aerobic with oxidative metabolism and no fermentation of sugars in peptone media. The respiratory ubiquinone is Q9. Major fatty acids are 16:0, C<sub>18:1ω7c/C<sub>18:1ω6c</sub> in summed feature 8 and 16:1ω7c/16:1ω6c in summed feature 3. Oxidase- and catalase-positive. In the API 20 NE system, arginine dihydrolase and urease are negative. Indole and β-galactosidase production as well as nitrate reduction and aesculine hydrolysis are negative. Assimilation of glucose, mannose, mannnitol, caprate, adipate and malate is...
positive. Negative results are obtained for assimilation of N-acetylg glucosamine, maltose, gluconate, citrate and phenylacetaldehyde. In Biolog GN2 plates, the assimilation of Tween 40, Tween 80, D-fructose, α-D-glucose, D-mannitol, D-mannose, D-sorbitol, sucrose, trehalose, methyl pyruvate, monomethyl succinate, acetate, cis-aconitate, citrate, D-galacturonate, D-glucuronate, D-glucosamine, D-glucuronate, β-hydroxybutyrate, γ-hydroxybutyrate, α-ketoglutarate, D,L-lactate, quinate, D-saccharate, succinate, bromosuccinate, L-alaninamide, D-alanine, L-alanine, L-alanlyglycine, L-asparagine, L-aspartate, L-glutamate, glycyl-L-glutamate, L-histidine, L-proline, D,L-carnitine, γ-aminobutyrate, urocanate and putrescine is positive. Negative results obtained for α-cyclodextrin, dextrin, glycollen, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, adonitol, L-arabinose, L-erythritol, L-fucose, D-galactose, myo-inositol, α-lactose, lactulose, maltose, melibiose, methyl β-D-galactoside, D-psicose, raffinose, L-rhamnose, turanose, xylitol, formate, α-hydroxybutyrate, itaconate, α-ketobutyrate, α-ketovalerate, malonate, sebacate, succinamate, glucuronamide, glycyll-L-aspartate, L-ornithine, L-phenylalanine, D-serine, L-threonine, inosine, uridine, thymidine, phenylethylamine, 2-aminoethanol, 2,3-butanediol, glycerol and glucose 6-phosphate. Finally, assimilation of D-arabitol, cellobiose, gentiobiose, D-galactonate lactone, β-hydroxyphenylacetate, propionate, hydroxy-L-proline, L-leucine, L-pyroglutamate, L-serine, D,L-α-glycerol-phosphate and glucose 1-phosphate was weakly positive.

The type strain is IB1.1^T (=VKM B-2935^T, CECT 9091^T), isolated from oil-contaminated soil in the Turukhansk District, East Siberia, Russia. The DNA G+C content of the type strain is 58.5 mol%.

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We are grateful to Garafutdinov Ravil (IBG USC RAS, Ufa) for his help carrying out Scanning Probe Microscopy (SPM) and to Sergei Salazar and Zuleica García Palacios (IRNASA-CSIC) for technical support.

References


