**Pseudomonas toyotomiensis** sp. nov., a psychrotolerant facultative alkaliphile that utilizes hydrocarbons

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A psychrotolerant, facultatively alkaliphilic strain, HT-3T, was isolated from a sample of soil immersed in hot-spring water containing hydrocarbons in Toyotomi, Hokkaido, Japan. 16S rRNA gene sequence-based phylogeny suggested that strain HT-3T is a member of the genus *Pseudomonas* and belongs to the *P. oleovorans* group. Cells of the isolate were Gram-negative, aerobic, straight rods, motile by a single polar flagellum. The strain grew at 4–42 °C, with optimum growth at 35 °C at pH 7, and at pH 6–10. It hydrolysed Tweens 20, 40, 60 and 80, but not casein, gelatin, starch or DNA. Its major isoprenoid quinone was ubiquinone-9 (Q-9) and the DNA G+C content was 65.1 mol%. The whole-cell fatty acid profile consisted mainly of C16:0, C16:1ω9c and C18:1ω9c. Phylogenetic analyses based on *gyrB*, *rpoB* and *rpoD* sequences revealed that the isolate could be discriminated from *Pseudomonas* species that exhibited more than 97% 16S rRNA gene sequence similarity and phylogenetic neighbours belonging to the *P. oleovorans* group including the closest relative of the isolate, *Pseudomonas alcaliphila*. DNA–DNA hybridization with *P. alcaliphila* AL15-21T revealed 51 ± 5% relatedness. Owing to differences in phenotypic properties and phylogenetic analyses based on multilocus gene sequence analysis and DNA–DNA relatedness data, the isolate merits classification in a novel species, for which the name *Pseudomonas toyotomiensis* sp. nov. is proposed. The type strain is HT-3T (=JCM 15604T =NCIMB 14511T).

*Pseudomonads* are widely distributed in nature and have been reported as a metabolically and genetically diverse bacterial group (Anzai et al., 2000; Kersters et al., 1996; Moore et al., 1996; Mulet et al., 2010). They are found in agricultural soils and terrestrial and marine animals and plants; they have a variety of functions related to the decomposition of organic matter and the promotion of plant growth and can also act as pathogens (Palleroni, 1993). *Pseudomonas* species of group I based on rRNA–DNA relatedness in the original classification of Palleroni (1984) form the genus *Pseudomonas sensu stricto*. Members of other groups have been transferred to other existing genera or to new genera (Kersters et al., 1996).

Petroleum hydrocarbons are one of the most widespread contaminants not only of terrestrial soils, but also of marine environments. Petroleum hydrocarbons are difficult for natural populations of micro-organisms to decompose over short periods. Therefore, contamination of various environments by petroleum hydrocarbons is a

Abbreviation: MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *gyrB*, *rpoB* and *rpoD* gene sequences of strain HT-3T are AB453701, AB494447, AB548147 and AB548145. Other sequences determined in this study have been deposited as AB494446, AB548144 and AB571150 (*gyrB*), AB548148 and AB571151 (*rpoD*) and AB548146 and AB571152 (*rpoD*).

A supplementary figure and two supplementary tables are available with the online version of this paper.
significant problem. Although C<sub>12</sub>–C<sub>28</sub> n-alkanes are relatively easily biodegraded by micro-organisms, long-chain n-alkanes (over C<sub>30</sub>) and aromatic hydrocarbons are difficult to degrade (Boulton & Ratledge, 1984).

Gram-positive alkaliphilic and psychrotolerant and Gram-negative alkali-tolerant and psychrotolerant micro-organisms capable of degrading petroleum hydrocarbons have been reported (Yumoto et al., 2002; Yamahira et al., 2008). However, there are few reports on the isolation of cold-adapted, Gram-negative, alkaliphilic micro-organisms that can utilize hydrocarbons over a wide pH range including pH 10. In the present study, a psychrotolerant, alkaliphilic bacterium, strain HT-3<sup>T</sup>, that can decompose n-alkanes was isolated from a sample of soil immersed in hot-spring water containing hydrocarbons in Toyotomi hot spring, located in northern Hokkaido, Japan (45° 04′ N 141° 50′ E). Phenotypic and chemotaxonomic characteristics, phylogenetic analyses based on 16S rRNA, gyrB, rpoB and rpoD gene sequences and DNA–DNA hybridization showed that the isolate can be considered as representing a novel species belonging to the genus *Pseudomonas*.

Samples of surface soil immersed in hydrocarbon-containing hot-spring water (cold mineral spring, 33 °C; pH 7.4) and of the hot-spring water were obtained. To isolate novel alkali-tolerant or alkali-tolerant strains, approximately 2 g soil and 1 ml hot-spring water were inoculated into 100 ml synthetic medium (AT medium; Yumoto et al., 2002) [5 g KNO<sub>3</sub>, 0.5 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.01 g FeSO<sub>4</sub> · 7H<sub>2</sub>O, 0.02 g CaCl<sub>2</sub> · 7H<sub>2</sub>O, 0.011 g MnSO<sub>4</sub> · nH<sub>2</sub>O and 0.0005 g ZnSO<sub>4</sub> · 7H<sub>2</sub>O in 1 l NaHCO<sub>3</sub>/NaCO<sub>3</sub> buffer (100 mM in deionized water; pH 9)] supplemented with 3% n-tetradecane as the sole carbon source and incubated at 27 °C with shaking at 110 r.p.m. for 9 days. This cultivation was performed once more by transferring 5 ml culture to 100 ml fresh AT medium and incubating under the same conditions and for the same period. Part of the resulting culture was spread on a plate of AT medium containing 1.5% agar supplemented with vaporized n-tetradecane as the sole carbon source. n-Tetradecane-soaked filter paper was placed on the lid of the plate, which was then inverted to allow vaporization onto the surface of the agar. Inoculated plates were incubated at 27 °C for 1 week. After incubation, a colony that appeared was transferred to Luria–Bertani (LB) agar [10 g tryptone (Difco), 5 g yeast extract (Difco), 5 g NaCl and 20 g agar in 1 l distilled water, pH 7.2; Sambrook et al., 1989] and reisolated five times and the same medium was then used for subinoculation. Although hydrocarbons were present in the hot-spring water, the origin of strain HT-3<sup>T</sup> is considered to be the soil, since the isolate is aerobic. Cell suspensions were prepared in 20% (w/v) glycerol in distilled water for long-term storage at −85 °C.

Three strains were isolated as hydrocarbon-degrading strains and one of them, strain HT-3<sup>T</sup>, was used in this experiment as it possibly represented a novel species. *Pseudomonas alcaliphila* AL15-21<sup>T</sup> and *Pseudomonas mendocina* JCM 5966<sup>T</sup> were used as reference strains for DNA–DNA hybridization, determination of fatty acid composition and phenotypic characterization, and *Pseudomonas aeruginosa* JCM 5962<sup>T</sup> was also included as a reference for determination of fatty acid composition and phenotypic characterization. Reference strains were cultivated in nutrient broth [5 g peptone (Kyokuto), 3 g meat extract (Kyokuto) and 5 g NaCl in 1 l deionized water] at pH 7.0 and 25–37 °C.

For phenotypic characterization of strain HT-3<sup>T</sup>, LB broth or agar was used as the basal medium. The strain was incubated at 27 °C for 2 weeks, and experiments were performed three times to confirm the reproducibility of results. Acid production from carbohydrates was determined by the method of Hugh & Leifson (1953) and by using the API 50 CH strip (bioMérieux) in accordance with the manufacturer’s instructions. Utilization of substrates as sole carbon and energy sources was determined using US medium (Yumoto et al., 2001) [2 g NH<sub>4</sub>Cl, 2 g Na<sub>2</sub>PO<sub>4</sub>, 1 g KH<sub>2</sub>PO<sub>4</sub>, 0.1 g MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.05 g CaCl<sub>2</sub> · 2H<sub>2</sub>O and 1 ml metal mixture in 1 l NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>PO<sub>4</sub> buffer (100 mM; pH 7.0) instead of NaHCO<sub>3</sub>/NaCO<sub>3</sub> buffer (100 mM; pH 10)] with the substrate added at 0.2%. The metal mixture included (per 100 ml distilled water) 1.8 g disodium EDTA, 5.0 g ZnSO<sub>4</sub> · 7H<sub>2</sub>O, 5.0 g FeSO<sub>4</sub> · 7H<sub>2</sub>O, 1.5 g MnSO<sub>4</sub> · 4H<sub>2</sub>O, 0.4 g CuSO<sub>4</sub> · 5H<sub>2</sub>O, 0.25 g Co(NO<sub>3</sub>)<sub>2</sub> · 6H<sub>2</sub>O and 0.1 g H<sub>3</sub>BO<sub>3</sub>. NaCl tolerance for growth was examined using a medium containing 1 g peptone (Kyokuto), 0.1 g yeast extract (Kyokuto) and 0–100 g NaCl in 1 l distilled water (pH 7.0). Other physiological and biochemical characteristics were examined according to the methods described by Barrow & Feltham (1993) and the API 20E and API ZYM strips (bioMérieux) in accordance with the manufacturer’s instructions. The optimum temperature, NaCl concentration and pH for growth of strain HT-3<sup>T</sup> were determined using a temperature-gradient incubator (model TN-2612; Advantec) for automatic growth monitoring. L-shaped tubes were used, each containing 10 ml LB broth (pH 7). The temperature range used was 5–50 °C. Growth below 5 °C was examined using a conventional incubator. Growth experiments at pH 6–10 were performed using LB broth containing 100 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>PO<sub>4</sub> (pH 6–8) or 100 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>CO<sub>3</sub> (pH 9–10). Growth dependence on pH at 27 °C was estimated over the range pH 7–10. Hydrocarbon utilization was examined by liquid culture using AT medium containing 1% (v/v) hydrocarbon at pH 10 (100 mM NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> buffer) at 27 °C with shaking at 120 r.p.m.

To observe the shape of cells and flagellation, transmission electron microscopy of negatively stained cells was performed after growth on a PYA agar slant (Yumoto et al., 2001). Procedures for transmission electron microscopy preparation and observation were described previously (Yumoto et al., 2001). Cell motility was observed by light microscopy using cells suspended in physiological saline. The morphological, physiological and biochemical characteristics of the isolate are given in the species description and Table 1. Transmission
electron and light microscopy showed that the cells were straight rods (0.4–0.6 × 1.0–2.5 μm), motile with one polar flagellum (see Supplementary Fig. S1, available in IJSEM Online).

Utilization of hydrocarbons by strain HT-3T was tested at pH 10 at 27 °C and compared with that of P. alcaliphila AL15-21T. Utilization of hydrocarbons was determined on the basis of the turbidity of the culture broth in medium containing a hydrocarbon as the sole carbon source. The isolate utilized C12–C16 and C28–C30 n-alkanes and the branched-chain hydrocarbon pristine. The hydrocarbon-utilization range of strain HT-3T was very similar to that of P. alcaliphila AL15-21T (Supplementary Table S1).

Table 1. Differential characteristics of strain HT-3T and related type strains

<table>
<thead>
<tr>
<th>Characteristic</th>
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<td>Hydrolysis of:</td>
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<tr>
<td>Gelatin</td>
<td>–</td>
<td>+</td>
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<td>+</td>
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<td>Casein</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>Aesculin</td>
<td>+</td>
<td>–</td>
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<td>–</td>
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<td>Tryptophan deaminase</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Lipase (C14)</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Trypsin</td>
<td>–</td>
<td>–</td>
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<td>+</td>
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<td>Acid produced from:</td>
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<tr>
<td>Mannitol</td>
<td>–</td>
<td>+</td>
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<td>D-Ribose</td>
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<tr>
<td>D-Galactose</td>
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<td>–</td>
<td>+</td>
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<tr>
<td>D-Mannose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Gentiobiose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Growth at 42 °C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>DNA G + C content (mol%)</td>
<td>65.1</td>
<td>63.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.8–64.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.2&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

*Data taken from: a, Yumoto et al. (2001); b, Palleroni (1984).

The 16S rRNA gene was amplified by PCR using primers 9F (5′-GAGTTTGATCCTGGCTCAG) and 1541R (5′-AAGGAGGTGATCCAGCC). The approximately 1.5 kb PCR product was sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer (PRISM 3100; Applied Biosystems) with BigDye Termination RR mix version 3.1 (Applied Biosystems) according to the manufacturer’s instructions. Primers 9F, 515F (5′-GTGCGCGAGCCCCGTATG), 907F (5′-AAACTCAAGGAAATTCAG), 357R (5′-CTGCTGCCTCCGGTAG) and 1541R were used for sequencing. Sequences were aligned and the consensus sequence was determined using the program CLUSTAL W (Thompson et al., 1994). Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Felsenstein, 1981) and minimum-evolution (Fitch, 1971) methods in MEGA 4 (Tamura et al., 2007). For neighbour-joining analysis, the distance between sequences (K<sub>sub</sub> value) was calculated using Kimura’s two-parameter model (Kimura, 1980). The similarity between sequences was calculated using the GENETYX computer program (Software Development). A 1490 bp 16S rRNA sequence was obtained from strain HT-3T and analysed. 16S rRNA gene sequence similarity to previously reported strains was determined, and a neighbour-joining phylogenetic tree of the strain together with its closely related neighbours is shown in Fig. 1. The maximum-parsimony tree was similar to the neighbour-joining tree, whereas ‘Pseudomonas indoloydans’ IPL-1 was the closest neighbour in the minimum-evolution tree (not shown). However, the bootstrap values for branches around strain HT-3T were lower than 50% in all the phylogenetic trees.

16S rRNA gene sequence phylogeny suggested that strain HT-3T is a member of the genus Pseudomonas and belongs to the Pseudomonas oleovorans group. The strain showed the highest similarities to P. alcaliphila AL15-21T (99.7% similarity) and P. oleovorans subsp. lubricantis RS1<sup>1</sup> (99.7%) among strains belonging to named species. It also showed high similarity to its phylogenetic neighbours Pseudomonas mendocina LMG 1223<sup>T</sup> (98.8%), ‘P. indoloydans’ IPL-1 (98.8%) and P. oleovorans LMG 1225 (type strain of Pseudomonas pseudalkaligenes) (98.5%). From the results of phylogenetic analyses based on the 16S rRNA gene sequence, it was difficult to determine whether the isolate represents a novel species. Therefore, multilocus sequence analysis (MLSA) was attempted. The gyrB gene was amplified by PCR using primers UP-1 and UP-2r as described previously (Yamamoto & Harayama, 1995) and the rpoB and rpoD genes were amplified by PCR using the primer sets LAPS and LAPS27 and 70F and 70R, respectively (Ait Tayeb et al., 2005; Yamamoto et al., 2000). Sequences and sequence similarity were determined, and phylogenetic trees were reconstructed as described above. Pseudomonas strains that exhibited more than 97% 16S rRNA gene sequence similarity were considered for the reconstruction of phylogenetic trees in MLSA. Phylogenetic trees reconstructed on the basis of the gyrB, rpoB and rpoD genes.
Whole-cell fatty acids and isoprenoid quinones were analysed as described previously (Yumoto et al., 2001). The type strains of the most closely related species, **P. alcaliphila**, and the type species of the genus **Pseudomonas**, **P. aeruginosa**, were used as reference strains in analysis of the fatty acid composition. The results of GLC analysis of fatty acids of strain TH-3\(^\text{T}\) grown at pH 7 are shown in Supplementary Table S2. The major fatty acid was \(\text{C}_{18:1}\) \(\omega_{9}\)c. Although the fatty acid composition of strain HT-3\(^\text{T}\) is similar to that of its closest neighbour, **P. alcaliphila** AL15-21\(^\text{T}\), differences were observed in this study or in the contents of cyclopropyl fatty acids. Cyclopropyl fatty acids were not detected in **P. alcaliphila** in this study or in the study of Saha et al. (2010). The major isoprenoid quinone in strain HT-3\(^\text{T}\) was Q-9.

Bacterial DNA was prepared according to the method of Marmur (1961) and the DNA base composition was determined by the method of Tamaoka & Komagata (1984). The DNA G+C content of strain HT-3\(^\text{T}\) was 65.1 mol%.

Levels of DNA–DNA relatedness were determined fluorometrically by the method of Ezaki et al. (1989) using photobiotin-labelled DNA probes and black microplates. The hybridization temperature was 48.6 °C. According to the sequence similarities and phylogenetic analysis based on the 16S rRNA gene sequence, strain HT-3\(^\text{T}\) is most closely related to **P. alcaliphila** AL15-21\(^\text{T}\) among strains of named species. DNA–DNA hybridizations were performed between strain HT-3\(^\text{T}\) and **P. alcaliphila** AL15-21\(^\text{T}\) and **P. mendocina** JCM 5966\(^\text{T}\), used as a reference. Results of DNA–DNA hybridization using DNA of strain HT-3\(^\text{T}\) as the probe indicated that strain HT-3\(^\text{T}\) is distinct from **P. alcaliphila** AL15-21\(^\text{T}\) (51 ± 5%; \(n = 3\)) and **P. mendocina** JCM 5966\(^\text{T}\) (50 ± 6%; \(n = 3\)). Reciprocal values of DNA–DNA hybridization with DNA of **P. alcaliphila** AL15-21\(^\text{T}\) and **P. mendocina** JCM 5966\(^\text{T}\) as the probe were 63 ± 8% (\(n = 3\)) and 50 ± 0% (\(n = 3\)), respectively.

The DNA–DNA relatedness levels obtained were in agreement with the results of MLSA. These results discriminate strain HT-3\(^\text{T}\) from the closely related species **P. alcaliphila**. Therefore, it can be concluded that strain HT-3\(^\text{T}\) represents a novel species.

Comparison of phenotypic characters was performed using the type strain of the most closely related species, **P. alcaliphila**, under the same experimental conditions. The type strains of the type species of the genus, **P. aeruginosa**, and the phylogenetically neighbouring species **P. mendocina** were used as references. Strain HT-3\(^\text{T}\) can be differentiated from **P. alcaliphila** AL15-21\(^\text{T}\) on the basis of several phenotypic and chemotaxonomic characteristics. For example, it was negative for hydrolysis of gelatin and casein.
Fig. 2. Neighbour-joining phylogenetic trees derived from sequences of gyrB (a), rpoB (b) and rpoD (c) showing the positions of *P. toyotomiensis* sp. nov. HT-3\(^T\) and related organisms. Numbers at branch points are bootstrap percentages based on 1000 replicates. Bars, 0.02 (a, c) and 0.01 (b) changes per nucleotide position.
and positive for acid production from D-galactose, D-mannose and D-arabitol and growth at 42 °C and its DNA G+C content was different (Table 1). Furthermore, the combination of growth at pH 6.0–10.5 and at 4–42 °C and the absence of protease production in strain HT-3T is unique in comparison with all Pseudomonas species with validly published names.

On the basis of the above results, strain HT-3T is assigned to a novel species, for which the name Pseudomonas toyotomiensis sp. nov. is proposed.

**Description of Pseudomonas toyotomiensis sp. nov.**

Pseudomonas toyotomiensis (to.yo.to.mi.en’sis. N.L. fem. adj. toyotomiensis pertaining to Toyotomi, where the type strain was isolated).

Grows at pH 6.0–10.5, with optimum growth at pH 6–9. Gram-negative, aerobic, straight rods (0.4–0.6 × 1.0–2.5 μm), motile by a single polar flagellum. Colonies are circular and white. Does not produce a fluorescent pigment. Grows in the presence of 0–9 % NaCl, with optimum growth at 1–3 % NaCl, and at 4–42 °C, with optimum growth at 35 °C at pH 7. Positive for oxidase, catalase, indole production, nitrate reduction and arginine dihydrolase and negative for the Voges–Proskauer, methyl red and ONPG tests, lysine decarboxylase and ornithine decarboxylase. Positive for hydrolysis of asculin and Tween 20, 40, 60 and 80, and negative for hydrolysis of casein, gelatin, starch, DNA, lipid and alginic acid. Acid, but no gas, is produced from D-xylene, D-glucose, D-mannose, D-galactose, melibiose and D-mannitol when grown at pH 7. No acid is produced from fructose, maltose, sucrose, raffinose, myo-inositol, sorbitol, rhamnose or trehalose. Utilizes D-glucose, fructose, glycerol, D-mannose, L-alanine, L-histidine, L-glutamate, L-lysine, L-asparagine, L-glycine and L-proline, but not lactose, maltose, raffinose, sucrose, sorbitol, L-rhamnose, cello-βiose, inulin, L-tryptophan, L-threonine, L-valine or L-leucine. Other characteristics are listed in Table 1. The major isoprenoid quinone is Q-9. The cellular fatty acids are C12:0, C14:0, C16:0, C18:0, C16:1ω6c, C16:1ω7c, C17:0, C13:0 3-0H, C18:0 9c, cyclo-C17:0, C18:0, C18:1ω7c and C19:0 cyclo. The DNA G+C content of the type strain is 65.1 mol%.

The type strain is HT-3T (=JCM 15604T =NCIMB 14511T).

**References**


