Thalassospira tepidiphila sp. nov., a polycyclic aromatic hydrocarbon-degrading bacterium isolated from seawater

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A Gram-negative, mesophilic bacterial strain, designated 1-1B^T, which degrades polycyclic aromatic hydrocarbons, was isolated from petroleum-contaminated seawater during a bioremediation experiment. A 16S rRNA gene sequence analysis indicated that the isolate was affiliated with the genus *Thalassospira* in the *Alphaproteobacteria*; the sequence was found to be most similar to those of *Thalassospira profundimaris* WP0211^T (99.8%), *Thalassospira xiamenensis* M-5^T (98.2%) and *Thalassospira lucentensis* DSM 14000^T (98.1%). However, the levels of DNA–DNA relatedness between strain 1-1B^T and these type strains were 50.7 ± 17.2, 35.7 ± 17.8 and 32.0 ± 21.1%, respectively. In addition, strain 1-1B^T was found to be distinct from the other described species of the genus *Thalassospira* in terms of some taxonomically important traits, including DNA G+C content, optimum growth temperature, salinity tolerance, utilization of carbon sources and fatty acid composition. Furthermore, strain 1-1B^T and *T. profundimaris* were also different with regard to motility and denitrification capacities. On the basis of physiological and DNA–DNA hybridization data, strain 1-1B^T represents a novel species within the genus *Thalassospira*, for which the name *Thalassospira tepidiphila* sp. nov. is proposed. The type strain is 1-1B^T (=JCM 14578^T =DSM 18888^T).

The genus *Thalassospira* was proposed by López-López *et al.* (2002). At the time of writing, the genus includes three species: *Thalassospira lucentensis*, the type species of the genus (López-López *et al.*, 2002), *T. xiamenensis* and *T. profundimaris* (Liu *et al.*, 2007). *T. xiamenensis* M-5^T and *T. profundimaris* WP0211^T were isolated from a bacterial consortium growing on hydrocarbons as carbon sources, but neither strain was able to degrade hydrocarbons in pure culture (Liu *et al.*, 2007). We recently isolated several bacterial strains affiliated with the genus *Thalassospira* from oil-contaminated seawater and found that they could degrade several polycyclic aromatic hydrocarbons (PAHs), including naphthalene, dibenzothiophene, phenanthrene and fluorene (unpublished). We now consider that these strains play important roles in marine spilled-oil bioremediation. In this communication, we report on the

polyphasic taxonomic characterization of one of our PAH-degrading isolates, strain 1-1B^T, which is affiliated with the genus *Thalassospira*.

Strain 1-1B^T was isolated from petroleum-contaminated seawater during a bioremediation experiment described previously (Kasai *et al.*, 2002). Seawater was collected from a bioremediation tank, serially diluted and spread onto 1% (w/v) Gelrite plates containing the artificial seawater medium ONR7a (Dyksterhouse *et al.*, 1995). The plates were then coated with heat-treated Arabian light crude oil (0.2%, w/v) (Kasai *et al.*, 2002) and incubated at 20 °C. After incubation for 3 weeks, small colonies appearing on these plates were picked and streaked onto solid plates containing marine broth 2216 (MB; Difco) and 1% (w/v) Gelrite for purification. MB was used for routine cultivation. To monitor growth, cell concentrations were determined from the direct counting of 4',6-diamino-2-phenylindole-stained cells under an epifluorescence microscope, as described by Kodama & Watanabe (2003). Cells of strain 1-1B^T were stored at –80 °C in MB supplemented with 15% (v/v) glycerol.

Abbreviation: PAH, polycyclic aromatic hydrocarbon.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 1-1B^T is AB265822.

The ability of strain 1-1B^T to degrade PAHs was examined in ONR7a medium supplemented with a mixture of heat-treated Arabian light crude oil (100 mg l⁻¹) and three compounds, namely phenanthrene, pyrene and benzo[a]pyrene (1 mg l⁻¹ in each case). To prepare an inoculum, cells of strain 1-1B^T were grown in MB for 2 days at 30 °C and harvested by centrifugation (8000 g for 10 min). Duplicate aliquots of the medium (10 ml) were prepared in 50 ml vials, inoculated with cells and sealed with Teflon-coated butyl rubber septa and crimped aluminium caps and then incubated at 30 °C with shaking. Aliquots of non-inoculated medium were incubated similarly and served as controls. After incubation for 11 days, oil components were extracted with chloroform and quantitatively analysed using GC-MS (GC-MS-QP5000; Shimadzu) as described elsewhere (Kasai *et al.*, 2002). The proportion (%) of the initial amount that was biodegraded was calculated as described by Dutta & Harayama (2000). Strain 1-1B^T degraded 92.0 ± 3.1 % (mean ± SD) of C₀-alkylnaphthalene, 97.4 ± 0.4 % of C₁-alkylnaphthalene, 28.0 ± 4.6 % of C₂-alkylnaphthalene, 84.5 ± 9.0 % of C₀-alkyldibenzothiophene, 38.5 ± 11.6 % of C₀-alkylphenanthrene and 73.0 ± 10.6 % of C₀-alkylfluorene within 11 days, whereas significant degradation (more than 10 % of initial amounts) was not observed for n-alkanes, C₃₋₅-alkylnaphthalenes, C₁₋₄-alkyldibenzothiophenes, C₁₋₃-alkylphenanthrenes, C₁₋₃-alkylfluorenes, pyrene or benzo[a]pyrene (C₀–C₅ represent carbon numbers of the alkyl side chains in alkylated PAHs). When the medium was supplemented with naphthalene, dibenzothiophene, phenanthrene or fluorene as the sole carbon source, strain 1-1B^T degraded naphthalene (44.6 ± 20.2 %) and fluorene (17.1 ± 3.5 %), but did not degrade phenanthrene or dibenzothiophene within 11 days.

Cell morphology was examined by means of transmission and scanning electron microscopy (Beveridge *et al.*, 1994). Motility was checked using phase-contrast microscopy. Gram staining and oxidase and catalase tests were conducted according to standard procedures (Smibert & Krieg, 1994). The optimal growth temperature and pH were determined in MB and salinity requirements were examined using modified MB (Sohn *et al.*, 2004). Nitrate reduction and denitrification were examined using ONR7a supplemented with NaNO₃ (5 mM) as an electron acceptor and sodium acetate (10 mM) as an electron donor. After the medium had been autoclaved, the following sterile solutions were added (l⁻¹) under an oxygen-free N₂ atmosphere: 5 ml resazurin solution (0.2 g l⁻¹) as an oxidation–reduction indicator and 10 ml 4 % TiCl₃ solution as a reducing agent (Kodama *et al.*, 2007). Consumption of nitrate and nitrite was measured by ion chromatography using an ICA-2000 ion analyser equipped with an electrolytic conductivity detector and a spectrophotometer (Toa DKK). For physiological and biochemical characterizations, API 20NE (bioMérieux) and Biolog GN2 microplates (Biolog) were used according to the manufacturers' instructions.

Strain 1-1B^T comprised cells that were Gram-negative, motile and vibrioid to spiral (0.17–0.33 µm wide and 0.9–2.0 µm long) in shape (Fig. 1). Cells were motile by means of single polar flagella (Fig. 1). The oxidase and catalase tests gave positive results. Growth of strain 1-1B^T was observed between 8 and 45 °C, with an optimum at 43 °C. Strain 1-1B^T grew at pH 5–10, with an optimum at pH 7. NaCl was essential for the growth of strain 1-1B^T: no growth was observed in medium lacking NaCl. Growth occurred at NaCl concentrations of 2–14 %, with an optimum at 3 %. Nitrate was reduced to nitrite under anaerobic conditions, but nitrite was not reduced to nitrogen. The API 20NE test showed that strain 1-1B^T was positive for nitrate reduction but negative for indole production, glucose fermentation, arginine dihydrolase, urease, aesculin hydrolysis, gelatinase and β-galactosidase. The results from the Biolog test are given in the species description (see below).

The cellular fatty acids were analysed in the TechnoSuruga Laboratory (Shizuoka, Japan) using cells grown in MB for 24 h. The fatty acids present in strain 1-1B^T (≥0.1 %) were as follows: C_{18:1}ω7c (41.73 %), C_{16:0} (20.25 %), C_{17:0} cyclo (9.30 %), C_{14:0} (4.81 %), C_{16:1}ω7c and/or iso-C_{15:0} 2-OH (4.67 %), C_{19:0} cyclo ω8c (4.10 %), C_{16:0} 3-OH (3.22 %), C_{18:0} (2.09 %), C_{14:0} 3-OH and/or iso-C_{16:1} I (1.38 %), C_{18:1} 2-OH (1.22 %), C_{13:1} (1.14 %), C_{16:1}ω5c (0.69 %), C_{18:0} 3-OH (0.63 %), 11-methyl C_{18:1}ω7c (0.50 %), C_{12:0} (0.42 %), C_{18:1}ω5c (0.41 %), C_{17:0} (0.37 %), C_{17:1}ω8c (0.28 %), C_{15:0} (0.27 %), 10-methyl C_{19:0} (0.14 %), anteiso-C_{17:1}ω9c (0.11 %), C_{20:1}ω7c (0.11 %) and C_{17:0} 3-OH (0.10 %). The major fatty acids of strain 1-1B^T were similar to those of members of the genus *Thalassospira* (López-López *et al.*, 2002; Liu *et al.*, 2007). However, the presence of a relatively large proportion of the fatty acid C_{17:0} cyclo in 1-1B^T serves to distinguish the strain from *Thalassospira* species (Table 1).

Fig. 1. Transmission electron micrograph of a cell of strain 1-1B^T. Bar, 1 µm.

Table 1. Characteristics that differentiate strain 1-1B^T from type strains of *Thalassospira* species

Strains: 1, 1-1B^T (data from this study); 2, *T. profundimaris* WP0211^T (unless indicated otherwise, data were taken from Liu *et al.*, 2007); 3, *T. xiamenensis* M-5^T (Liu *et al.*, 2007); 4, *T. lucentensis* QMT2^T (López-López *et al.*, 2002). +, Positive; –, negative; w, weakly positive; ND, no data available.

Characteristic	1	2	3	4
Morphology	Vibrioid to spiral	Curved rods	Curved rods	Vibrioid to spiral
Motility	+	–	+	+
DNA G + C content (mol%)	55.1	47.0	52.6	54.7
Temperature range (optimum) (°C)	8–45 (43)	10–37 (22)	4–40 (22)	4–40 (ND)
NaCl range (optimum) (%)	2–14 (3)	2–8 (3–4)	0.5–10 (2–4)	2–10 (ND)
Reduction of nitrate to nitrite	+	–*	+	+
Denitrification	–	–*	+	–*
Substrate utilization:†				
Tween 40	+	+	–	+
Tween 80	w	+	–	w*
<i>N</i> -Acetyl-D-glucosamine	–	–	+	+
L-Rhamnose	–	–	–	+
D-Saccharic acid	–	–	–	+
Major fatty acids (%)	C _{18:1} ω7c (41.73), C _{16:0} (20.25), C _{17:0} cyclo (9.30)	C _{18:1} ω7c (43.13), C _{16:0} (17.79), C _{18:0} (6.95)	C _{18:1} ω7c (44.34), C _{16:0} (14.89), C _{18:0} (8.58)	C _{18:1} ω7c (45.25), C _{16:1} ω7c (15.95), C _{16:0} (15.77)

*Determined in this study.

†Based on the Biolog system.

A phylogenetic analysis based on 16S rRNA gene sequences was conducted as described by Watanabe *et al.* (2000), with the following modifications: amplification was performed using a PCR thermal cycler (Takara) and primers B27f and U1492r (Orphan *et al.*, 2001) in a 50 µl mixture containing 1.25 U KOD Dash DNA polymerase (Toyobo), 5 µl 10 × PCR buffer, 0.2 mM each dNTP, 50 pmol each primer and 10 ng DNA. The PCR conditions used were as follows: 2 min activation of the polymerase at 96 °C, followed by 35 cycles consisting of 15 s at 95 °C, 15 s at 50 °C and 30 s at 74 °C, ending with 2 min extension at 74 °C. The nucleotide sequences of the PCR products were determined using a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems) and a model 3730 DNA sequencer (Applied Biosystems). The software used to align the sequences was CLUSTAL W, version 1.7 (Thompson *et al.*, 1994), and the alignments were refined by visual inspection. The phylogenetic dendrogram was constructed using the NJPlot program in CLUSTAL W, version 1.7. Phylogenetic analysis based on 16S rRNA gene sequences showed that 1-1B^T was affiliated with the genus *Thalassospira* (Fig. 2) and closely related to *T. profundimaris* WP0211^T (99.8 % sequence similarity), *T. xiamenensis* M-5^T (98.2 %) and *T. lucentensis* DSM 14000^T (98.1 %). It has been reported that strains with 16S rRNA gene sequence similarity of above 99 % sometimes exhibit a DNA–DNA reassociation value below 70 % (Stackebrandt & Ebers, 2006). In this case, although strain 1-1B^T and *T. profundimaris* WP0211^T are fairly similar in their 16S rRNA gene sequences (99.8 % identical), they showed some differences in phenotypic properties (e.g. in the ability to degrade PAHs, motility,

growth temperature and salinity tolerance), prompting us to examine the DNA–DNA relatedness. Genomic DNA was extracted as described by Murray & Thompson (1980) and then purified using caesium chloride density-gradient ultracentrifugation. DNA–DNA relatedness was determined in triplicate by the fluorometric hybridization method on a microdilution plate (Ezaki *et al.*, 1989) in the TechnoSuruga Laboratory. Reverse hybridization was also conducted, again in triplicate. The levels of DNA–DNA relatedness between strain 1-1B^T and the type strains of *Thalassospira* species (*T. profundimaris* WP0211^T, *T. xiamenensis* M-5^T and *T. lucentensis* CECT 5390^T) were 50.7 ± 17.2, 35.7 ± 17.8 and 32.0 ± 21.1 %, respectively (means ± SD, *n* = 6). The DNA G + C content of strain 1-1B^T, as determined using the method of Katayama-Fujimura *et al.* (1984), was 55.1 ± 0.4 mol% (mean ± SD, *n* = 3).

Features of strain 1-1B^T that differentiate it from recognized *Thalassospira* species are shown in Table 1. The ability of the *Thalassospira* type strains to reduce nitrate was examined in this study, and the Biolog test was applied to *T. lucentensis* CECT 5390^T. Although strain 1-1B^T and *T. profundimaris* WP0211^T are fairly similar in terms of their 16S rRNA gene sequences (99.8 % similarity), they differ with regard to some physiological and taxonomic properties, i.e. growth temperature, salinity tolerance, nitrate reduction and G + C content (Table 1). Strain 1-1B^T differed from *T. xiamenensis* M-5^T in its denitrification abilities and substrate utilization, and it differed from *T. lucentensis* CECT 5390^T in its substrate-utilization pattern and major fatty acids. In addition, strain

Fig. 2. Neighbour-joining phylogenetic dendrogram, based on 16S rRNA gene sequence comparisons, showing the position of strain 1-1B^T and related genera within the family Rhodospirillaceae. The sequence of *Azorhizobium caulinodans* LMG 6465^T was used as the outgroup. Accession numbers of sequences are given in parentheses. Numbers at branch nodes are bootstrap values (per 100 trials); only values greater than 50 are shown. Bar, 0.01 substitutions per site.

1-1B^T is the only one of these strains that can degrade PAHs. The DNA–DNA relatedness values for strain 1-1B^T with respect to the three *Thalassospira* type strains were significantly below 70% ($P < 0.01$, as assessed with a *t*-test). On the basis of these physiological and chemotaxonomic properties, together with the DNA–DNA relatedness values (Wayne *et al.*, 1987), strain 1-1B^T is distinguishable from the recognized *Thalassospira* species and represents a novel species of the genus *Thalassospira*, for which the name *Thalassospira tepidiphila* sp. nov. is proposed.

Description of *Thalassospira tepidiphila* sp. nov.

Thalassospira tepidiphila (te.pi.di.phi'la. L. adj. *tepidus* lukewarm; Gr. adj. *philos* loving; N.L. fem. adj. *tepidiphila* lukewarm-loving, mesophilic).

Cells are Gram-negative, vibrioid to spiral, 0.17–0.33 µm wide and 0.9–2.0 µm long. Cells are motile by means of single polar flagella. Colonies on MB Gelrite are smooth, convex, opaque and slightly yellow. Oxidase- and catalase-positive. In the API 20NE test, negative for glucose fermentation, arginine dihydrolase and gelatinase activity. NaCl is required for growth; optimum growth occurs with 3% NaCl and the range is 2–14%. The optimum growth temperature is 43 °C (range, 8–45 °C) and the optimum pH is 7.0 (range, pH 5.0–10.0). Facultatively anaerobic; nitrate is reduced to nitrite under anaerobic conditions, but nitrite is not reduced to nitrogen (nitrite is the final product of nitrate reduction). In Biolog GN2 plates, the following are utilized as carbon sources: Tween 40, L-arabinose, D-arabitol, cellobiose, D-fucose, D-galactose, α-D-glucose, myo-inositol, D-mannitol, D-mannose, methyl pyruvate, acetic acid, *cis*-aconitic acid, citric acid, D-glucosaminic acid, D-glucuronic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketoglutaric acid, DL-lactic acid, quinic acid, succinic acid, bromosuccinic acid,

D-alanine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-proline, L-pyrogutamic acid, L-serine, γ-aminobutyric acid, urocanic acid and glycerol. The following are utilized weakly: glycogen, Tween 80, D-psicose, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, malonic acid, propionic acid, succinamic acid and L-alanyl glycine. Degrades naphthalene, phenanthrene, dibenzothiophene and fluorene (mixed into crude oil). The major cellular fatty acids are C_{18:1ω7c}, C_{16:0} and C_{17:0} cyclo. The genomic DNA G + C content of the type strain is 55.1 ± 0.4 mol%.

The type strain, 1-1B^T (=JCM 14578^T =DSM 18888^T), was isolated from petroleum-contaminated seawater in a beach-simulation tank during a bioremediation experiment.

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