Thalassospira xianhensis sp. nov., a polycyclic aromatic hydrocarbon-degrading marine bacterium

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A polycyclic aromatic hydrocarbon-degrading marine bacterium, designated strain P-4^T, was isolated from oil-polluted saline soil in Xianhe, Shangdong Province, China. Strain P-4^T was Gram-negative-staining with curved to spiral rod-shaped cells and grew optimally with 3–6 % (w/v) NaCl and at 30 °C. The predominant fatty acids were C_{18:1ω7c} (35.0 %), C_{16:0} (25.0 %), C_{16:1ω7c} (17.9 %), C_{14:0} (6.2 %) and C_{17:0} cyclo (5.2 %). The major respiratory quinone was Q-9 and the genomic DNA G+C content was 61.2 ± 1.0 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain P-4^T belonged to the genus *Thalassospira* of the class *Alphaproteobacteria*. DNA–DNA hybridization with *Thalassospira xiamenensis* DSM 17429^T showed relatedness of 36.0 %, and lower values were obtained with respect to other *Thalassospira* species. Based on physiological and biochemical tests and 16S rRNA gene sequence analysis as well as DNA–DNA relatedness, strain P-4^T should be placed in the genus *Thalassospira* within a novel species. The name *Thalassospira xianhensis* sp. nov. is proposed, with P-4^T (=CGMCC 1.6849^T =JCM 14850^T) as the type strain.

Polycyclic aromatic hydrocarbons (PAHs) are hydrocarbons that consist of two or more fused aromatic rings (Habe & Omori, 2003; Hedlund & Staley, 2001). PAHs are released into the marine environment as a result of various anthropogenic activities such as marine seepage and accidental discharges during the transport and disposal of petroleum products and the use of fossil fuels (Sohn *et al.*, 2004). Some PAHs are highly carcinogenic, genotoxic and cytotoxic to marine organisms and may be transferred to humans through seafood consumption (Menzie *et al.*, 1992). Therefore, removal of PAHs from contaminated marine environments is of considerable importance.

Several PAH-degrading strains have been isolated from PAH-contaminated marine sediments (Hedlund & Staley, 2001; Kwon *et al.*, 2005; Sohn *et al.*, 2004). In this study, we report the characterization of a PAH-degrading marine bacterium that was isolated from saline soil contaminated with crude oil in Xianhe, Shangdong Province, China.

To isolate PAH-degrading bacteria, 5 % sea-salt defined medium (5 % SSDM; Zhao *et al.*, 2009) and 5 % SSDM with 0.5 % yeast extract (5 % SSDMY) were used. Solid 5 % SSDMY medium was prepared with 1.5 % agar. A sample

of oil-polluted saline soil (1 g) was added to 100 ml 5 % SSDM medium supplemented with phenanthrene (100 µg ml⁻¹) in a 300 ml Erlenmeyer flask. The culture was aerobically incubated at 30 °C in darkness on a rotary shaker operating at 200 r.p.m. After 2 weeks, 10 ml culture was transferred to 100 ml 5 % SSDM medium and incubated under the conditions described above. The enrichment was performed five or six times. Next, a culture broth dilution series was spread on 5 % SSDMY agar. After incubation for 2 days, single colonies were picked and cultivated in 5 ml 5 % SSDM using phenanthrene as the sole source of carbon and energy. These isolates developed a yellowish-orange or reddish-brown colour, which is an indication of ring cleavage of PAH compounds (Guerin & Jones, 1988; Kasai *et al.*, 2002) and, thus, phenanthrene-degrading activity. After purification by restreaking, one of the representative isolates, strain P-4^T, was found to have relatively low 16S rRNA gene sequence similarity (98.0 %) to *Thalassospira lucentensis* DSM 14000^T (López-López *et al.*, 2002) in the genus *Thalassospira* of the class *Alphaproteobacteria*, and this strain was selected for further studies.

Cell morphology was examined by scanning electron microscopy as described by Acinas *et al.* (1999). Negatively stained cells with 1.0 % (w/v) phosphotungstic acid were used for the observation of flagella with a Zeiss DSM 960 transmission electron microscope. Gram staining was carried out by using the standard Gram reaction and the result was confirmed by the KOH test (Baron & Finegold, 1990). The NaCl requirement for growth was

Abbreviation: PAH, polycyclic aromatic hydrocarbon.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P-4^T is EU017546.

Tables showing fatty acid contents and DNA–DNA relatedness values between strain P-4^T and the type strains of *Thalassospira* species are available as supplementary material with the online version of this paper.

tested using 5 % SSDMY medium with NaCl concentrations ranging from 0 to 20 % (w/v) (pH 7.2, 30 °C). The effects of pH and temperature on growth were determined using 5 % SSDMY medium at pH 4.0–10.0 (30 °C) and by incubation at 4–50 °C (pH 7.2). After incubation at 30 °C for 2 days on 5 % SSDMY agar, strain P-4^T formed small, cream–yellow, opaque, circular and convex colonies with flat edges. Cells were Gram-negative-staining, curved- to spiral-shaped rods, 1.0–1.6 µm long and 0.3–0.6 µm wide (Fig. 1). No flagellum or motility was observed. Strain P-4^T grew in 5 % SSDMY broth containing 0.1–17.0 % NaCl (optimum 3.0–6.0 %). The temperature range for growth was 10–42 °C (optimum 30 °C). The pH range for growth was pH 5.5–9.0 (optimum pH 7.2).

Catalase activity was tested by assessing bubble production in a 3 % hydrogen peroxide solution. Oxidase activity was determined by the oxidation of 1 % *p*-aminodimethylaniline oxalate. Production of urease was tested as described by Cowan & Steel (1965). Hydrolysis of starch, Tween 80 and DNA was determined on 5 % SSDMY agar with concentrations of substrates described by Cowan & Steel (1965) and Lányi (1987). Facultatively anaerobic growth was tested with pyruvate (20 mM) as the carbon source and nitrate (10 mM), nitrite (5 mM), sulfate (20 mM), fumarate (20 mM), ferric iron (50 mM) or MnO₂ (10 mM) as the electron acceptor. Nitrate and nitrite reduction were assessed as described by Lányi (1987). Denitrification was tested by growing strain P-4^T anaerobically in the presence of nitrate (Zumft, 1992). For acid production from carbohydrates, bromocresol purple was added to a final concentration of 0.03 g l⁻¹. The ability of strain P-4^T to use various organic substrates as sole carbon sources at a concentration of 0.4 % (w/v) was investigated in 5 ml 5 % SSDM medium. The strain was also characterized using Biolog GN plates according to the method described by Ivanova *et al.* (1998). Susceptibility of the strain to antibiotics was tested by using 5 % SSDMY agar and 6 mm sensitivity discs (Tiantan) for the following antibiotics (µg per disc unless otherwise stated): ampicillin (30), ceftriaxone (30), cefotaxime (30), cephalothin (30), ciprofloxacin (5), clarithromycin (15), clindamycin (2), erythromycin (15), gentamicin (10), penicillin (10 IU), streptomycin (300), tetracycline (30) and vancomycin (30). Strain P-4^T was

positive for catalase, oxidase and urease and hydrolysis of Tween 80 and was negative for DNase and hydrolysis of starch. The strain was facultatively anaerobic and grew under anaerobic conditions by reducing nitrate and nitrite, but did not grow with fumarate, sulfate, ferric iron or MnO₂. Other characteristics of strain P-4^T are listed in the species description. Some features of strain P-4^T are in accordance with those of recognized members of the genus *Thalassospira*, whereas other features differentiate strain P-4^T from closely related *Thalassospira* species. The physiological properties of strain P-4^T and those of the type strains of *Thalassospira* species are summarized in Table 1.

Respiratory lipoquinones were extracted and purified as described by Komagata & Suzuki (1987). The purified ubiquinones were dissolved in acetone and separated by reversed-phase HPLC (Shim-pack VP-OD; Shimadzu). Cellular fatty acid methyl esters were prepared and analysed using gas chromatography according to the instructions of the Microbial Identification System (MIDI). Peaks were integrated automatically and fatty acid names and percentages were determined using the Microbial Identification software (Sasser, 1990). The predominant isoprenoid quinone was Q-9. The results of the cellular fatty acid content analysis are given in Supplementary Table S1 (available in IJSEM Online). The fatty acids making up more than 5 % of the total in strain P-4^T were C_{18:1ω7c} (35.0 %), C_{16:0} (25.0 %), C_{16:1ω7c} (17.9 %), C_{14:0} (6.2 %) and C_{17:0} cyclo (5.2 %). The predominant fatty acids of strain P-4^T were similar to those of other recognized species of *Thalassospira*, although the proportions were different.

The capacity of strain P-4^T to degrade naphthalene, phenanthrene, anthracene, fluoranthene, pyrene and benzo[a]pyrene was tested in 5 % SSDM culture supplemented with single PAH (100 µg ml⁻¹) using HPLC as described previously (Zhao *et al.*, 2009). After 8 days of incubation, strain P-4^T could degrade naphthalene, phenanthrene and pyrene whereas no degradation occurred on anthracene, fluoranthene or benzo[a]pyrene.

Genomic DNA was extracted and purified by the method of Marmur (1961) and DNA purity was assessed by the

Fig. 1. Electron micrographs of strain P-4^T showing a scanning electron micrograph (a) and electron micrograph of negatively stained cells (b). Bars, 1 µm.

Table 1. Differential phenotypic characteristics of strain P-4^T and related type strains of the genus *Thalassospira*

Strains: 1, *Thalassospira xianhensis* sp. nov. P-4^T; 2, *T. xiamenensis* DSM 17429^T; 3, *T. profundimaris* DSM 17430^T; 4, *T. lucentensis* DSM 14000^T; 5, *T. tepidiphila* DSM 18888^T. Data were taken from this study unless indicated. All strains are positive for catalase and oxidase activities, utilization of L-arabinose, cellobiose, D-fructose, D-mannose, D-glucosaminic acid, γ -hydroxybutyric acid, DL-lactic acid, L-alanyl glycine, L-alanine, L-glutamic acid and glycerol and acid production from D-galactose and mannose. All strains are negative for utilization of melibiose and D-sorbitol and acid production from arabinose and lactose. +, Positive; w, weakly positive; –, no reaction or growth; ND, no data available.

Characteristic	1	2	3	4	5
Cell shape*	CR–S	CR†	CR†	CR–S†	V–S†
Flagella‡	–	MS†	–†	MS†	MS†
Pigment§	C–Y	~Y	OP	~Y	SY
Oxygen requirement	FA	FA†	FA†	SA†	FA†
Growth at:					
4 °C	+	+	–	+	–
40 °C	+	+	–	+	+
NaCl concentration for growth (%)					
Range	0.1–17	0.5–10	2–8	2–10	2–14
Optimum	3–6	2–4	3–4	ND	3.0
Reduction of nitrate	+	+	+	–	+†
Denitrification	+	+	+	–	–†
Hydrolysis of Tween 80	+	–	+	–	w
Substrate utilization					
Acetic acid	–	–	–	+	+
N-Acetyl-D-glucosamine	+	+	–	+	–
cis-Aconitic acid	+	+	–	w	+
γ -Aminobutyric acid	+	–	–	+	+
L-Asparagine	+	+	–	–	+
L-Aspartic acid	+	–	–	+	+
Citric acid	+	–	–	+	+
D-Galactose	+	+	–	+	+
D-Galacturonic acid	–	–	w	w	w
D-Glucuronic acid	+	–	–	+	+
L-Gluconic acid	+	+	+	+	–
L-Histidine	+	+	w	+	+
Hydroxy-L-proline	+	+	–	–	+
p-Hydroxyphenylacetic acid	–	–	–	+	–
myo-Inositol	+	+	–	+	+
Lactose	+	–	–	–	+
L-Leucine	–	–	–	w	–
Maltose	+	+	–	+	–
D-Mannitol	+	+	+	–	–
L-Ornithine	+	+	–	–	–
Putrescine	+	–	–	+	–
D-Saccharic acid	+	–	–	+	–
L-Serine	–	+	–	+	+
Sucrose	+	+	–	–	–
L-Threonine	–	w	–	–	–

Table 1. cont.

Characteristic	1	2	3	4	5
Thymidine	–	+	–	–	–
Trehalose	+	+	–	–	–
Acid production from:					
D-Fructose	+	+	+	–	–
Glucose	+	+	–	+	+
Maltose	+	+	+	+	–
Mannitol	+	+	+	–	+
Sucrose	+	+	–	–	–
Xylose	+	+	+	+	–
DNA G+C content (mol%)¶	61.2 ± 1.0	52.9 ± 0.8	46.8 ± 1.2	54.4 ± 0.9	53.5 ± 1.6

*CR, Curved rods; s, spirilla; v, vibrioid.

†Data from Kodama *et al.* (2008), Liu *et al.* (2007) and López-López *et al.* (2002).

‡MS, Monopolar single.

§C, Cream; OP, opalescent; SY, slightly yellow; Y, yellow; ~Y, yellowish.

||FA, Facultative anaerobe; SA, strict aerobe.

¶Values are means ± SEM (n=3).

A_{280}/A_{260} ratio (Johnson, 1994). The 16S rRNA gene sequence of strain P-4^T was amplified by PCR as described by Rainey *et al.* (1992) with universal bacterial primers corresponding to *Escherichia coli* position 8F (5'-AGAGTTTGTATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTACGACTT-3'). The sequences obtained were compared with reference 16S rRNA gene sequences available in the GenBank database. 16S rRNA gene sequence alignments were performed with the CLUSTAL X program (version 1.64b; Thompson *et al.*, 1997). A phylogenetic tree was constructed using the neighbour-joining and maximum-parsimony methods with MEGA version 3 (Kumar *et al.*, 2004; Saitou & Nei, 1987). An almost-complete 16S rRNA gene sequence (1456 bp) of strain P-4^T was determined and analysed. Strain P-4^T showed highest levels of sequence similarity to *Thalassospira xiamenensis* M-5^T (99.2%), *T. profundimaris* WP0211^T (98.9%), *T. tepidiphila* 1-1B^T (98.5%) and *T. lucentensis* DSM 14000^T (98.0%). The phylogeny of 16S rRNA gene sequences revealed that strain P-4^T had closest affinity with the recognized species of *Thalassospira*, with high levels of bootstrap support (Fig. 2).

The G+C content of genomic DNA was determined by the thermal denaturation method (Gerhardt *et al.*, 1994) using DNA from *E. coli* K-12 as a control. DNA–DNA hybridization was performed in triplicate by the thermal denaturation and renaturation method (Zakrzewska-Cerwinska *et al.*, 1988). The genomic DNA G+C content of strain P-4^T was 61.2 ± 1.0 mol%. Strain P-4^T exhibited levels of DNA–DNA relatedness of 36.0 ± 5.2, 28.3 ± 4.8, 8.3 ± 2.3 and 5.3 ± 1.2 % with *T. xiamenensis* DSM 17429^T, *T. tepidiphila* DSM 18888^T, *T. profundimaris* DSM 17430^T and *T. lucentensis* DSM 14000^T, respectively (Supplementary Table S2), which are clearly below the 70 % threshold generally accepted for

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the members of the genus *Thalassospira* and related genera within the family *Rhodospirillaceae*. *Azorhizobium caulinodans* LMG 6465^T was used as the out-group. Bootstrap values (>50%) based on 1000 replicates are shown at branch nodes. Bar, 1% sequence divergence.

species delineation (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

On the basis of phenotypic, chemotaxonomic and phylogenetic data and genomic distinctiveness, strain P-4^T should be placed in the genus *Thalassospira* as a representative of a novel species, for which we propose the name *Thalassospira xianhensis* sp. nov.

Description of *Thalassospira xianhensis* sp. nov.

Thalassospira xianhensis sp. nov. (xian.hen'sis. *xianhensis* pertaining to Xianhe of Shangdong Province, China, where the type strain was isolated).

Cells are facultatively anaerobic, Gram-negative-staining, curved to spiral rods (0.3–0.6 × 1.0–1.6 µm). Colonies on 5% SSDMY agar are cream–yellow, circular, smooth, opaque and approximately 1.5–2 mm in diameter after incubation at 30 °C for 2 days. Growth occurs at temperatures between 10 °C and 42 °C (optimum 30 °C), with saline concentrations between 0.1 and 17.0% (optimum 3.0–6.0%) and at pH 5.5–9.0 (optimum pH 7.2). Positive for catalase, oxidase and urease and hydrolysis of Tween 80, but negative for DNase and hydrolysis of starch. Facultative anaerobic growth with nitrate and nitrite as electron acceptors. Sensitive to ampicillin, cephalothin, clarithromycin, clindamycin, erythromycin, penicillin and vancomycin, but resistant to ceftriaxone, cefotaxime, ciprofloxacin, gentamicin, streptomycin and tetracycline. Among the 95 carbon sources in the Biolog system, the following are utilized: adonitol, L-arabinose, D-arabitol, cellobiose, i-erythritol, D-fructose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, D-mannose, raffinose, D-sorbitol, turanose, xylitol, pyruvate methyl ester, D-galactonic acid lactone, D-gluconic acid, D-glucosaminic acid, β-hydroxybutyric acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, quinic acid, D- and L-alanine, L-alanyl glycine, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, bromosuccinic acid, L-proline, L-pyroglyutamic acid, urocanic acid and glycerol. Acid is

produced from D-fructose, D-glucose, D-galactose, D-xylose, D-mannitol, maltose, mannose and sucrose, but not from lactose or arabinose. The predominant respiratory quinone is Q-9. The major fatty acids are C_{18:1}ω7c, C_{16:0}, C_{16:1}ω7c, C_{14:0} and C_{17:0} cyclo, with smaller amounts of C_{16:0} 3-OH, C_{18:0} and C_{14:0} 3-OH. The genomic DNA G + C content of the type strain is 61.2 ± 1.0 mol%.

The type strain, P-4^T (=CGMCC 1.6849^T =JCM 14850^T) was isolated from a saline soil contaminated by crude oil, collected from Xianhe, Shangdong Province, China.

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