Novosphingobium pentaromativorans sp. nov., a high-molecular-mass polycyclic aromatic hydrocarbon-degrading bacterium isolated from estuarine sediment

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A Gram-negative, yellow-pigmented, halophilic bacterial strain US6-1^T, which degrades high-molecular-mass polycyclic aromatic hydrocarbons of two to five rings, was isolated from muddy sediment of Ulsan Bay, Republic of Korea. The 16S rRNA gene of the isolate showed high sequence similarity to *Novosphingobium subarcticum* (96.23%) and *Sphingopyxis alaskensis* (96.18%); however, the isolate formed a distinct phyletic line within the genus *Novosphingobium*. DNA–DNA relatedness between US6-1^T and the closest strain *N. subarcticum* revealed that strain US6-1^T was independent from this species. Isolate US6-1^T had ubiquinone 10 and a DNA G + C ratio of 61.1 mol%. Major fatty acids were octadecanoic acid (18:1 ω 7), hexadecanoic acid (16:1 ω 7) and 2-hydroxy-myristic acid (14:0 2-OH). On the basis of polyphasic taxonomic evidence, strain US6-1^T is proposed to represent a novel species in the genus *Novosphingobium* for which the name *Novosphingobium pentaromativorans* sp. nov. is proposed. The type strain is US6-1^T (=KCTC 10454^T=JCM 12182^T).

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants. They have accumulated in the environment as a result of various anthropogenic activities. Elevated PAH concentrations are found in the sediments of coastal embayments, estuaries and the continental shelf, where human pressure is felt most noticeably (Sanger *et al.*, 1999). Contamination of marine sediments by PAHs is of concern because some of these hydrocarbons have toxic, carcinogenic and genotoxic properties. Therefore, removal of PAHs from contaminated environments is of considerable concern. Many micro-organisms have important roles in transformation and degradation of PAHs in the environment and micro-organisms that are able to degrade PAHs have been isolated from various environments (Shin *et al.*, 1999; Juhasz & Naidu, 2000).

We isolated a moderately halophilic, yellow-pigmented

bacterial strain US6-1^T from estuarine sediment at Ulsan Bay, Republic of Korea. This bacterium can degrade PAHs of between two and five rings. Taxonomic characteristics of strain US6-1^T are reported.

About 1 g sediment was inoculated into 10 ml MM2 broth containing [I⁻¹ aged sea water (ZoBell, 1946), pH 7.2]: 18 mM (NH₄)₂SO₄, 1 μ M FeSO₄·7H₂O and 100 μ l 1 M KH₂PO₄/Na₂HPO₄ buffer solution, supplemented with 1000 p.p.m. of a final mixture of PAHs consisting of equal quantities of naphthalene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene. After 2 weeks incubation at 25 °C, 1 ml culture broth was transferred to 10 ml fresh medium. Sequential transfers were performed every 2 weeks on three occasions each. PAH-degrading bacteria were isolated according to the spray-plating method of Kiyohara *et al.* (1982). One of the colonies formed a clear zone in the pyrene-sprayed MM2 agar plate after 20 days incubation at 25 °C. This strain (US6-1^T) was subcultivated on marine agar 2216e (MA; Difco) or trypticase soy agar (TSA; Difco) for morphological and biochemical characterization.

Bacterial cells precultured in marine broth 2216e (MB; Difco) for 1 day at 25 °C were harvested by centrifugation (8000 g, 10 min) and inoculated into MM2 broth containing PAH mixtures (10 p.p.m. each of pyrene, benz[a]anthracene,

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Abbreviations: β -HPCD, 2-hydroxypropyl β -cyclodextrin; PAHs, polycyclic aromatic hydrocarbons.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain US6-1^T is AF502400.

chrysene, benz[b]fluoranthene and benzo[a]pyrene; Aldrich) and 10 % (w/v) 2-hydroxypropyl β -cyclodextrin (β -HPCD). After 8 days incubation, PAHs from culture broth were extracted with *n*-hexane and quantitative analysis was performed using GC/MS (Varian Saturn 2000). After 8 days incubation strain US6-1^T had degraded 88.2–99.9 % of the supplemented PAHs (Fig. 1).

Air-dried smears from MA cultures were stained using the Bio-Rad Gram-staining kit according to the manufacturer's instructions to determine the Gram reaction. Motility was determined with an optical microscope (Zeiss Axioplan) using the hanging drop technique (Skerman, 1967). For scanning electron microscopy (SEM), strain US6-1^T was fixed with 4 % glutaraldehyde. After washing with 50 mM HEPES buffer (pH 7.5) and post-fixation with 2 % OsO₄ solution, the material was dehydrated through a series of graded ethanol solutions. Freeze-dried material was coated with gold and examined at 10 kV using SEM (model S-2500; Hitachi). Growth was tested at 20, 25, 30 and 37 °C in MB. The pH range for optimal growth was determined in MB adjusted to pH 3–10. Salinity requirements were tested using modified MB (5 g peptone, 1 g yeast extract, 0.01 g FePO₄, 5 g MgCl₂·6H₂O, 1 g CaCl₂·2H₂O, 1 l distilled water) supplemented with 0, 1, 2, 3, 4, 5, 6 or 7 % (w/v) NaCl. Growth under anaerobic conditions was tested using MB after purging with nitrogen gas. Presence of oxygen was monitored using resazurin as an indicator of redox potential.

Strain US6-1^T was Gram-negative, non-motile, rod-shaped and formed yellow colonies within 2 days on MA and TSA plates at 30 °C. Cell diameter was 0.36–0.45 μ m and length 0.97–1.95 μ m. Optimal growth occurred at 30 °C; below 20 °C, growth was retarded. The organism tolerated pH values from 6 to 9 (optimum 6.5). Strain US6-1^T showed essential requirements for NaCl, as no growth was observed in medium without NaCl. US6-1^T grew at NaCl

concentrations of 1–6 %, with optimal growth at 2.5 % NaCl. The isolate could grow under anaerobic conditions but growth was retarded.

API 20NE (bioMérieux) and Microlog GN2 plates (Biolog) were used for physiological and biochemical characterization according to the manufacturers' instructions, except that the solution for bacterial suspension was our modified solution (5 g MgCl₂·6H₂O, 1 g CaCl₂·2H₂O, 25 g NaCl in 1 l distilled water) rather than saline solution. Enzyme activity for strain US6-1^T was positive for catalase, but negative for oxidase. From the tests using the API 20NE identification kit, strain US6-1^T gave positive results for aesculin hydrolysis, nitrate reduction and assimilation of glucose, maltose and phenylacetate and negative results for β -galactosidase, indole production, arginine dehydrogenase, urease, gelatin hydrolysis, acidification from glucose and assimilation of adipate, caprate, citrate, gluconate, arabinose, malate, mannose and *N*-acetylglucosamine. Some phenotypic differences were observed between strain US6-1^T and other species in the genus *Novosphingobium* (Table 1). Based on the Microlog system, strain US6-1^T could oxidize cyclodextrin, dextrin, Tween 40, Tween 80, α -D-glucose, maltose, D-trehalose, sucrose, psicose, methyl

Table 1. Phenotypic characteristics that differentiate strain US6-1^T from other *Novosphingobium* species

Strains: 1, US6-1^T; 2, *N. subarcticum* JCM 10398^T; 3, *N. stygium* ATCC 700280^T; 4, *N. tardagens* JCM 11434^T; 5, *N. hassiacum* DSM 14552^T; 6, *N. capsulatum* ATCC 14666^T; 7, *N. rosa* ATCC 51837^T; 8, *N. subterraneum* ATCC 700279^T; 9, *N. aromaticivorans* ATCC 700278^T. Data in columns 2–9 were taken from Fujii *et al.* (2003) and Kämpfer *et al.* (2002). All strains show yellow-coloured colonies and are positive for catalase and nitrate reductase activity. All strains were negative for oxidase, arginine dihydrogenase and urease activities, production of indole, acid production from glucose and assimilation of *n*-capric acid and citrate.

Fig. 1. Degrading activities towards five different polycyclic aromatic hydrocarbons (PAHs) by strain US6-1^T.

Characteristic	1	2	3	4	5	6	7	8	9
Assimilation of:									
Glucose	+	+	+	+	+	+	+	+	+
L-Arabinose	–	+	+	–	–	+	+	+	+
D-Mannose	–	–	+	–	–	+	+	+	+
D-Mannitol	–	+	+	–	–	+	–	–	+
N-Acetylglucosamine	–	+	–	–	–	+	–	–	–
Maltose	+	+	+	–	–	+	+	+	+
Gluconate	–	–	–	–	–	+	+	–	–
Adipic acid	–	–	–	–	–	–	–	–	+
DL-Malate	–	+	–	–	–	+	–	–	–
Phenyl acetate	+	–	–	–	–	–	–	–	–
Enzyme activity:									
β -Glucosidase	+	+	+	–	–	+	+	+	+
Gelatinase	–	–	–	–	–	+	–	–	–
β -Galactosidase	–	+	+	–	–	+	+	+	+

pyruvate, β -hydroxybutyric acid, α -ketobutyric acid, propionic acid, acetic acid, quinic acid, L-alanine, L-alanyl glycine, L-aspartic acid, L-glutamic acid, L-proline, L-threonine and L-phenylalanine. These traits clearly differentiate US6-1^T from *Novosphingobium subarcticum* (formerly *Sphingomonas subarctica*; Nohynek *et al.*, 1996).

Cellular lipids extracted from cells cultivated in MB at 25 °C according to Folch *et al.* (1957) were transesterified into methyl esters by using strong acid methanolysis (Carreau & Dubacq, 1978) for GC analysis. Detected peaks were identified by the fatty acid methyl-ester standard (Supelco) and equivalent chain-length values (Christie, 1988). Mass spectral verification was additionally performed using a model HP5971 mass-selective detector interfaced with a model HP5890 series II GC equipped with a HP ultra-1 capillary column (30 m $\times$ 0.25 mm internal diameter) and the CHEMSTATION program. Oven temperature was held at 200 °C. The dominant fatty acids in strain US6-1^T were octadecanoic acid (18:1 ω 7, 62.6%), hexadecanoic acid (16:1 ω 7, 6.7%) and 2-hydroxy-myristic acid (14:0 2-OH, 19.7%), the last being the dominant hydroxy fatty acid. Fatty acid profiles of strain US6-1^T and other *Novosphingobium* species are given in Table 2. Respiratory quinones were analysed from 100 mg lyophilized cells according to the method of Kim *et al.* (2000). Ubiquinone standards were purchased from Sigma (Q-9, Q-10) and also prepared from *Serratia proteamaculans* (Q-8). Strain US6-1^T contained ubiquinone Q-10. Sphingolipids were analysed according to the method of Christie (1988) and the presence of sphingosine was confirmed on TLC plates (Silicagel G-60; Merck).

16S rDNA was prepared and purified as described by Lee *et al.* (1999). Sequencing was performed using a BigDye terminator cycle sequencing kit (PE Applied Biosystems) and an Applied Biosystems model 3100 automatic DNA sequencer (PE Applied Biosystems). The resultant sequence (1481 bp) of strain US6-1^T was compared against the 16S rRNA gene sequences held in the GenBank and Ribosomal Database Project (RDP; Maidak *et al.*, 1994), and indicated that the organism was placed between the genera *Novosphingobium* and *Sphingopyxis*. The closest relatives are *N. subarcticum* KF1^T (96.23% 16S rRNA gene sequence similarity), *Sphingopyxis alaskensis* AF 01^T (96.18%) and *Sphingopyxis witflariensis* W-50^T (95.54%). Therefore, the sequence of strain US6-1^T was aligned manually with representative sequences of the *Sphingomonadaceae* obtained from the RDP and GenBank databases, using known 16S rRNA secondary structure information (Gutell, 1994). Distances (options according to Jukes & Cantor, 1969) and clustering with Fitch–Margoliash (Fitch & Margoliash, 1967) methods were determined using bootstrap values based on 1000 replications (Felsenstein, 1985). The phylogenetic trees were rooted using *Rhodospirillum rubrum* and *Acetobacter aceti* as outgroups. The PHYLIP package (Felsenstein, 1993) was used for all analyses. Phylogenetic analysis was carried out using 1370 unambiguously aligned nucleotide positions. Strain US6-1^T was placed in the genus *Novosphingobium* and is most closely related to *N. subarcticum* (Fig. 2). Sequence similarity to *N. subarcticum* (96.42%) indicates that strain US6-1^T represents a novel *Novosphingobium* species. However, DNA–DNA relatedness between US6-1^T and *N. subarcticum* KF-1^T using the dot-blot direct binding assay

Table 2. Cellular fatty acid compositions (%) of strain US6-1^T and other *Novosphingobium* species

Strains: 1, US6-1^T; 2, *N. subarcticum* JCM 10398^T; 3, *N. stygium* ATCC 700280^T; 4, *N. tardaugens* JCM 11434^T; 5, *N. hassiacum* DSM 14552^T; 6, *N. capsulatum* ATCC 14666^T; 7, *N. rosa* ATCC 51837^T; 8, *N. subterraneum* ATCC 700279^T; 9, *N. aromaticivorans* ATCC 700278^T. Data were taken from Fujii *et al.* (2003) and Kämpfer *et al.* (1997, 2002). Values are percentages of total fatty acids.

Fatty acid	1	2	3	4	5	6	7	8	9
Saturated fatty acids									
12:0 2-OH	0.8	—	—	—	—	—	—	—	1.0
13:0 2-OH	—	—	—	—	—	—	—	—	2.6
14:0	0.6	—	—	0.2	—	0.6	1.5	0.9	0.5
14:0 2-OH	19.7	7.6	14.9	12.0	17.2	1.9	12.8	13.0	16.8
15:0	—	—	—	—	—	—	—	—	0.9
15:0 2-OH	0.3	—	2.5	—	19.2	—	—	3.3	4.5
16:0	1.0	10.2	2.5	3.1	—	21.0	10.9	4.7	2.4
16:0 2-OH	2.5	2.1	—	—	—	—	—	—	—
18:0	—	—	—	—	—	—	—	3.2	—
Unsaturated fatty acids*									
16:1 ^a	8.8	15.5	0.6	15.6	16.7	5.1	22.8	20.1	20.7
17:1 ^b	2.0	3.3	4.9	2.6	19.3	0.5	—	7.4	12.8
18:1 ^c	64.0	61.1	57.3	55.0	23.6	66.0	52.0	48.2	37.7

*Contained one or more of the following fatty acids: a, 16:1 ω 5c and/or 16:1 ω 7c; b, 17:1 ω 6c and/or 17:1 ω 8c; c, 18:1 ω 7c, 18:1 ω 5c, 18:1 ω 9t and/or 18:1 ω 12t. *cis* and *trans* isomers are indicated by the suffixes *c* and *t*.

Fig. 2. Rooted Fitch–Margoliash tree based on nearly complete 16S rRNA gene sequences (1370 aligned positions) showing relationships between strain US6-1^T, *Novosphingobium* species and some other members of the *Sphingomonadaceae*. The numbers at the nodes indicate the levels of bootstrap support based on Fitch–Margoliash analyses of 1000 resampled datasets. Bar, 0.1 nucleotide substitutions per nucleotide position.

described by Kim *et al.* (2000) was only 20.4%. The G + C content of the genomic DNA was determined using the thermal denaturation method of Kim *et al.* (2000). The DNA G + C content of US6-1^T was 61.1 mol%.

On the basis of morphological, physiological and chemotaxonomic properties, together with DNA–DNA hybridization and 16S rDNA sequence comparison data, strain US6-1^T is proposed as a novel species of the genus *Novosphingobium*, *Novosphingobium pentaromativorans* sp. nov.

Description of *Novosphingobium pentaromativorans* sp. nov.

Novosphingobium pentaromativorans (pen'ta.ro.ma'ti.vo.rans. Gr. numeral *penta* five; L. gen. n. *aromatis* of spice; L. v. *voro* devour; N.L. neut. adj. *pentaromativorans* degrading/devouring aromatic compounds with five rings).

Cells are Gram-negative, non-motile rods, 0.36–0.45 × 0.97–1.95 µm. Colonies on solid MA and TSA are yellowish. Catalase-positive, oxidase-negative and facultative anaerobic growth. Positive results for aesculin hydrolysis, nitrate reduction, assimilation of glucose, maltose and phenyl acetate are obtained from API 20NE kit. Oxidizes cyclodextrin, dextrin, Tween 40, Tween 80, α-D-glucose, maltose, D-trehalose, sucrose, psicose, methyl pyruvate, β-hydroxybutyric acid, α-ketobutyric acid, propionic acid, acetic acid, quinic acid, L-alanine, L-alanyl glycine, L-aspartic acid, L-glutamic acid, L-proline, L-threonine and L-phenylalanine in the Microlog GN2 plate. Optimal growth at 30 °C, pH 6.5 and 2.5% NaCl. The strain obligately requires NaCl for growth. Degrades fluorene, phenanthrene, fluoranthene, anthracene, pyrene, benz[a]anthracene,

chrysene, benz[b]fluoranthene and benzo[a]pyrene. The major respiratory quinone is ubiquinone 10. Genomic DNA G + C content is 61.1 mol%. Dominant fatty acids are octadecanoic acid (18:1ω7), hexadecanoic acid (16:1ω7) and 2-hydroxy-myristic acid (14:0 2-OH); sphingosine is present.

The type strain is US6-1^T (= KCTC 10454^T = JCM 12182^T).

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