Sphingomonas polyaromaticivorans sp. nov., a polycyclic aromatic hydrocarbon-degrading bacterium from an oil port water sample

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A bacterial strain (B2-7^T) capable of degrading a wide range of polycyclic aromatic hydrocarbon compounds (2–4 rings) was isolated from a water sample taken from Botan Oil Port in Xiamen, China. The isolate was Gram-negative, short-rod-shaped, aerobic, non-motile and formed yellow-pigmented colonies on LB medium. Cells of strain B2-7^T were catalase-positive and oxidase-negative. Optimal growth of strain B2-7^T was observed at pH 7.0, at 26 °C and in 0.5 % NaCl. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain B2-7^T grouped with members of the genus *Sphingomonas* and it showed 16S rRNA gene sequence similarity of 95.40 % to *Sphingomonas yunnanensis* YIM 003^T. The major polar lipids were phosphatidylethanolamine, phosphatidylcholine and sphingoglycolipid. Q-10 and sym-homospermidine were the predominant ubiquinone and polyamine components, respectively. The major fatty acids were C_{18:1ω7c} (67.2 %), C_{14:0} 2-OH (10.0 %) and C_{16:0} (9.6 %). The G + C content of the genomic DNA was 61.8 mol%. Based on phenotypic properties, and phylogenetic and genomic data, strain B2-7^T represents a novel species of the genus *Sphingomonas* within the class *Alphaproteobacteria*, for which the name *Sphingomonas polyaromaticivorans* sp. nov. is proposed. The type strain is B2-7^T (=KCCM 42951^T=JCM 16711^T).

The genus *Sphingomonas* was defined by Yabuuchi *et al.* (1990) as a group of Gram-negative, rod-shaped, chemoheterotrophic, strictly aerobic bacteria that possess ubiquinone 10 (Q-10) as the predominant isoprenoid quinone, contain sphingoglycolipid and typically produce yellow pigment. Many members of the genus *Sphingomonas* isolated from different geological areas are able to degrade a wide variety of recalcitrant natural and anthropogenic compounds, such as polycyclic aromatic hydrocarbons (PAHs) (Pinyakong *et al.*, 2003; Stolz, 2009). In this study, we report a *Sphingomonas*-like bacterium isolated from a water sample of an oil port.

Abbreviation: PAHs, polycyclic aromatic hydrocarbons.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain B2-7^T is EF467848.

Four supplementary figures are available with the online version of this paper.

A mixed culture from a water sample of Botan Oil Port in Xiamen, China, was enriched under selective pressure for PAH-degrading ability, then serially diluted and spread on Luria–Bertani (LB) agar plates and incubated at 30 °C for 24 h. Using the sublimation method (Huang *et al.*, 2008), a visible layer of phenanthrene was deposited onto the agar surface. The plates were then incubated for 5 days. Colonies forming clear zones around them were picked as phenanthrene-degrading bacteria. A yellowish gleaming colony was isolated using this method and designated B2-7^T. The ability of B2-7^T to degrade PAHs with 2 to 4 rings, e.g. naphthalene, fluorene, anthracene, phenanthrene and fluoranthene, was confirmed by HPLC when strain B2-7^T was grown on mineral salt medium [containing (l⁻¹): (NH₄)₂SO₄, 1 g; Na₂HPO₄, 0.8 g; KH₂PO₄, 0.2 g; MgSO₄ · 7H₂O, 0.2 g; FeCl₃ · 3H₂O, 0.005 g; CaCl₂ · 2H₂O, 0.1 g; and (NH₄)₆Mo₇O₂₄ · 4H₂O, 0.001 g] utilizing PAHs as the sole carbon and energy source. The strain showed a particularly high ability to degrade phenanthrene; 100 p.p.m. phenanthrene

was mineralized to undetectable levels within 72 h (see Fig. S1 in IJSEM Online).

Strain B2-7^T was cultivated for 1–3 days at 30 °C on LB agar or liquid medium for morphological and biochemical characterization. Motility was determined by an optical microscope (Zeiss Axioplan) using the hanging-drop technique (Skerman, 1967). Gram-staining was performed by the Hucker method (Gerhardt *et al.*, 1994). Catalase activity was determined by bubble production in a 3 % (v/v) H₂O₂ solution. Oxidase activity was tested using oxidase reagent (bioMérieux) according to the manufacturer's instructions. Production of the extracellular enzymes lipase, protease, amylase and cellulase was tested on LB agar containing 1 % (v/v) glycerol tributyrates, 1 % skim milk, 0.5 % starch mixture or 0.5 % carboxymethyl-cellulose (sodium salt), respectively. DNase activity was detected on Difco DNase test agar with methyl green. Results were examined twice after growth on agar plates for 2 and 5 days. Cell morphology was examined by transmission electron microscopy (H600; Hitachi).

The NaCl concentration, temperature and pH ranges for growth were determined in 0–15 % (w/v) NaCl, at 10–50 °C and at pH 4.0–11.0. Cells of strain B2-7^T were Gram-negative, short-rod-shaped, aerobic and non-motile, forming yellowish gleaming colonies on LB medium. Cells were catalase-positive and oxidase-negative. Strain B2-7^T showed lipase activity but not protease, amylase, DNase or cellulase activities. Cells were 1.04–1.90 µm in length and 0.52–0.75 µm in width (see Fig. S2 in IJSEM Online). Strain B2-7^T was able to grow at 20–35 °C, at pH 4.0–7.5 and in 0.5–3.0 % (w/v) NaCl. Optimal growth was observed at 26 °C, at pH 7.0 and in 0.5 % (w/v) NaCl.

16S rRNA gene amplification, cloning and sequence analysis were carried out as described previously (Huang *et al.*, 2008). The 16S rRNA gene sequence of strain B2-7^T was amplified by PCR using the eubacterial universal primers P27F and P1492R. The PCR product was cloned into vector pMD18-T and sequenced. The 16S rRNA gene sequence of strain B2-7^T was aligned with those of members of closely related *Sphingomonas* species (with similarities of more than 94 %) and type strains of the type species of other genera in the family *Sphingomonadaceae* obtained from the GenBank/EMBL/DDJB database using CLUSTAL_X software (Thompson *et al.*, 1997). Alignment was manually verified and adjusted prior to the reconstruction of a phylogenetic tree. The phylogenetic tree was reconstructed using the neighbour-joining (Jukes–Cantor correction) (Saitou & Nei, 1987) algorithms implemented in ARB software package (Ludwig *et al.*, 2004). The robustness of the inferred tree topologies was evaluated after 1000 bootstrap replicates of the neighbour-joining data. *Rhodospirillum rubrum* ATCC 11170^T was used as out-group. The values for sequence similarity among the closest 100 strains were further determined using the Eztaxon server 2.1 (Chun *et al.*, 2007).

A nearly complete 16S rRNA gene sequence (1450 bp) for strain B2-7^T was obtained and subjected to a comparative analysis. Strain B2-7^T shared sequence similarities of 92.0–95.4 % with other members of the genus *Sphingomonas*. Its closest relatives were *Sphingomonas yunnanensis* YIM 003^T (95.40 %) and *Sphingomonas desiccabilis* CP1D^T (95.08 %). Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain B2-7^T formed a distinct phyletic line within the genus *Sphingomonas* (Fig. 1). Similar results were obtained with the maximum-parsimony tree reconstructed using the PHYLIP 3.69 package. However, the position of strain B2-7^T differed in the tree generated with the maximum-likelihood method in the PHYLIP 3.69 package (see Figs S3 and S4 in IJSEM Online). 16S rRNA gene sequence analysis demonstrated that B2-7^T contained the signature nucleotides C:G (52:359), G (134), G (593), G:C (987:1218) and U:G (990:1215) (*Escherichia coli* numbering; Brosius *et al.*, 1978) for the genus *Sphingomonas sensu stricto* according to Takeuchi *et al.* (2001). Phylogenetic analysis indicated that strain B2-7^T represents a novel member of the genus *Sphingomonas* but its phylogenetic position within the genus remains unclear.

Biochemical and physiological characteristics of the isolate and reference bacterial strains were determined using the API 20E, API 20NE, API ZYM (bioMérieux) and Microlog GN2 (Biolog) systems inoculated with bacterial suspensions that were prepared in 1 % NaCl solution. Reference strains *S. yunnanensis* YIM 003^T (=KCTC 12346^T), *S. desiccabilis* CP1D^T (=DSM 16792^T), *Sphingomonas koreensis* JSS26^T (=KCTC 2882^T) and *Sphingomonas pseudosanguinis* G1-2^T (=CIP 109431^T) were obtained from the Korean Collection for Type Cultures (KCTC), the German Resource Centre for Biological Material (DSMZ) and the Collection de L'Institut Pasteur (CIP), France. They were grown under the same conditions as the isolate. Differential phenotypic characteristics of strain B2-7^T and these reference strains are given in Table 1.

To determine the DNA G + C content, genomic DNA was extracted from cells in late exponential phase grown on LB at 30 °C and analysed by HPLC (Mesbah *et al.*, 1989); the DNA G + C content was 61.8 mol%, which is in the range (60.7–69.9 mol%) for members of the genus *Sphingomonas*. For analysis of fatty acids, cells were grown on tryptic soy agar (TSA) at 28 °C for 24 h. Cellular fatty acids were extracted and analysed by GC (Hewlett Packard 6890) according to the standard protocol of the Sherlock Microbial Identification System (version 4.5; MIDI database TSBA40 4.10). The major non-polar fatty acids in strain B2-7^T (Table 2) were C_{18:1ω7c} (67.2 %) and C_{16:0} (9.6 %). 2-Hydroxy fatty acids were present, with C_{14:0} 2-OH (10.0 %) predominating, but 3-hydroxy fatty acids were absent. These are common characteristic features of members of the genus *Sphingomonas*. However, the presence of C_{12:0} 2-OH and unknown ECL 19.689 distinguished strain B2-7^T from closely related strains.

Fig. 1. Rooted neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences showing the relationships between strain B2-7^T and strains of other related species. The tree was constructed using the ARB software package (Ludwig *et al.*, 2004). Numbers at nodes indicate the level (%) of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets. Only values above 50 % are shown. *Rhodospirillum rubrum* ATCC 11170^T was used as the outgroup in the analysis. Bar, 0.02 nt substitutions per nucleotide position.

Cells were harvested in the late-exponential phase and freeze-dried for respiratory quinone and polar lipid extraction as described by Collins *et al.* (1980). Polar lipids were separated by two-dimensional TLC (Minnikin *et al.*, 1977) and sprayed with specific reagents [molybdophosphoric acid (for all lipids), ninhydrin (for amino groups), molybdenum blue (for phosphorus-containing lipids), α -naphthol (for sugar-containing lipids) and Dragendorff's reagent (for choline-containing lipids)]. Polar lipids of strain B2-7^T comprised diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidyl-dimethylethanolamine, sphingoglycolipid, phosphatidylcholine, phosphatidyl-*N*-methylethanolamine, unknown aminophosphoglycolipids, unknown phospholipids, unknown aminolipids and unknown lipids (Fig. 2). The presence of abundant sphingoglycolipid in strain B2-7^T indicates that the isolate is a member of the family *Sphingomonadaceae*. The isoprenoid quinone was determined to be ubiquinone 10 (Q-10) by HPLC analysis according to Collins (1985). *S. yunnanensis* YIM 003^T was used as a reference in the analysis of polar lipids and isoprenoid quinones.

For polyamine analysis, strain B2-7^T was grown in PYE broth (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2) and harvested in the late-exponential phase. Polyamines were extracted as described by Busse & Auling (1988) and analysed as described by Busse *et al.* (1997) using the HPLC equipment reported by Stolz *et al.* (2007). The polyamine pattern of strain B2-7^T consisted

of predominantly *sym*-homospermidine [35.9 $\mu\text{mol (g dry mass)}^{-1}$], with spermidine [0.5 $\mu\text{mol (g dry mass)}^{-1}$] and putrescine [0.3 $\mu\text{mol (g dry mass)}^{-1}$] present in smaller amounts, and traces [$<0.1 \mu\text{mol (g dry mass)}^{-1}$] of cadaverine and spermine. The polyamine pattern is in agreement with assignment of strain B2-7^T to the genus *Sphingomonas sensu stricto* (Busse *et al.*, 1999; Geueke *et al.*, 2007; Takeuchi *et al.*, 2001).

Strain B2-7^T shared similar chemotaxonomic characteristics with members of the genus *Sphingomonas sensu stricto* in terms of the DNA G+C content, polar lipids, major polyamine, predominant ubiquinone, major fatty acids and 16S rRNA signature nucleotides. However, the 16S rRNA gene sequence similarities between strain B2-7^T and the reference type strains were below 95.4 %, indicating that strain B2-7^T represents a novel species. In addition, strain B2-7^T could be distinguished from other closely related species based on some chemotaxonomic and phenotypic features, such as presence of C_{12:0} 2-OH and unknown ECL 19.689, esterase activity, and assimilation of *N*-acetyl-D-galactosamine, monomethyl succinate, L-asparagine, L-histidine and L-phenylalanine.

In conclusion, based on phenotypic and chemotaxonomic characteristics, and phylogenetic analysis, strain B2-7^T should be categorized as a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas polyaromaticivorans* sp. nov. is proposed.

Table 1. Differential phenotypic characteristics of strain B2-7^T and members of closely related species

Strains: 1, B2-7^T; 2, *Sphingomonas yunnanensis* YIM 003^T; 3, *Sphingomonas desiccabilis* CP1D^T; 4, *Sphingomonas koreensis* JSS26^T; 5, *Sphingomonas pseudosanguinis* G1-2^T. All data were generated in this study. All strains formed yellow colonies, were positive for catalase, alkaline phosphatase, esterase lipase (C8), leucine and valine arylamidases, acid phosphatase, and oxidation of dextrin, L-arabinose, gentiobiose, maltose, sucrose, trehalose, methyl pyruvate, β -hydroxybutyric acid and L-proline. All strains were negative for Gram-staining, nitrate reduction, indole production, glucose acidification, arginine dihydrolase, protease, assimilation of mannitol, N-acetylglucosamine, caprate, adipate, malate, citrate, phenylacetate, phosphohydrolase, α -galactosidase, β -glucuronidase, α -mannosidase, α -fucosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, gelatinase, mannitol, inositol, sorbitol, rhamnose, melibiose, adonitol, D-arabitol, erythritol, L-fucose, *myo*-inositol, D-mannitol, D-sorbitol, xylitol, *cis*-aconitic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, malonic acid, quinic acid, D-saccharic acid, succinamic acid, glucuronamide, L-ornithine, DL-carnitine, γ -aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol and 2,3-butanediol.

Characteristic	1	2	3	4	5
Esterase (C4)	–	+	+	+	+
β -Galactosidase	–	+	+	–	–
Gelatinase	–	–	+	–	–
Lipase	+	–	–	+	+
Oxidase	–	–	+	–	+
Assimilation of:					
N-Acetyl-D-galactosamine	+	–	–	–	–
Amygdalin	+	–	+	–	–
Glycerol	+	–	–	–	+
L-Asparagine	+	–	–	–	–
L-Histidine	+	–	–	–	–
L-Phenylalanine	+	–	–	–	–
Monomethyl succinate	+	–	–	–	–
Sucrose	+	–	–	–	+
Trypsin	+	–	–	+	+

Description of *Sphingomonas polyaromaticivorans* sp. nov.

Sphingomonas polyaromaticivorans [po.ly.a.ro.ma.ti.ci.vo'r-ans. Gr. adj. *polu* many; L. adj. *aromaticus* aromatic, fragrant; L. part. adj. *vorans* devouring; N.L. part. adj. *polyaromaticivorans* devouring many aromatic (compounds)].

Cells are aerobic, Gram-negative, non-motile rods. Forms yellow-pigmented colonies (2–3 mm in diameter) after incubation at 30 °C for 2–3 days on LB medium. Catalase-positive and oxidase-negative. Cells are 1.04–1.90 $\times$ 0.52–0.75 μ m. Utilizes a variety of PAHs with 2 to 4 rings, including naphthalene, fluorene, anthracene, phenanthrene

Table 2. Cellular fatty acid compositions of strain B2-7^T and members of closely related species

Strains: 1, B2-7^T; 2, *Sphingomonas yunnanensis* YIM 003^T; 3, *Sphingomonas desiccabilis* CP1D^T; 4, *Sphingomonas koreensis* JSS26^T; 5, *Sphingomonas pseudosanguinis* G1-2^T. All data were generated in this study. Values are percentages of total fatty acids. tr, Trace (<1%); –, not detected.

Fatty acid	1	2	3	4	5
Saturated fatty acids					
C _{14:0}	2.3	tr	tr	1.9	tr
C _{15:0}	tr	–	tr	tr	–
C _{16:0}	9.6	11.1	12.7	13.0	12.4
C _{18:0}	tr	–	tr	tr	1.8
Unsaturated fatty acids					
C _{16:1ω5c}	1.0	1.2	1.6	1.3	–
C _{17:1ω6c}	tr	tr	6.8	1.5	1.2
C _{18:1ω5c}	3.3	–	3.7	1.8	2.0
C _{18:1ω7c}	67.2	51.7	37.8	62.5	71.5
11-methyl C _{18:1ω7c}	–	6.0	–	–	–
Hydroxy fatty acids					
C _{12:0} 2-OH	1.6	–	–	–	–
C _{14:0} 2-OH	10.0	14.3	4.5	9.4	4.9
ECL 19.689	2.6	–	–	–	–

Fig. 2. Two-dimensional TLC of polar lipids of strain B2-7^T, detected with molybdophosphoric acid (for all lipids), ninhydrin (for amino groups), molybdenum blue (for phosphorus-containing lipids), α -naphthol (for sugar-containing lipids) and Dragendorff's reagent (for choline-containing lipids). DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PDE, phosphatidyl-N-methylethanolamine; PME, phosphatidyl-N-methylethanolamine; PC, phosphatidylcholine; SGL, sphingoglycolipid; APGL, unknown aminophosphoglycolipid; AL, unknown aminolipid; L, unknown lipid; P, pigments.

and fluoranthene, as the sole carbon and energy sources. Positive for *N*-acetyl- β -glucosaminidase, acid phosphatase, alkaline phosphatase, amygdalin, arabinose, creatine, cystine arylamidase, esterase lipase, β -glucosidase, glucose, α -glucosidase, β -glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, sucrose, trypsin and valine arylamidase (API 20NE kit). Positive for utilization of acetic acid, alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, *N*-acetyl-D-galactosamine, L-arabinose, α -ketobutyric acid, cellobiose, dextrin, formic acid, D-fructose, D-galactose, gentiobiose, α -D-glucose, L-glutamic acid, glucose 1-phosphate, glucose 6-phosphate, glycerol, L-histidine, β -hydroxybutyric acid, α -lactose, L-leucine, maltose, D-mannose, methyl β -D-glucoside, L-phenylalanine, hydroxy-L-proline, L-proline, methyl pyruvate, monomethyl succinate, L-serine, sucrose, trehalose, L-threonine, turanose, Tween 40, Tween 80 and α -ketovaleric acid using the Biolog system. Grows at 20–35 °C, at pH 4.0–7.5 and in 0.5–2.5 % NaCl. Optimal growth is at 26 °C and pH 7.0 and requires the presence of 0.5 % (w/v) NaCl. The non-polar cellular fatty acid profile is composed mainly of C_{18:1}ω7c, C_{16:0} and C_{18:1}ω5c. Hydroxy-fatty acids are C_{14:0} 2-OH and C_{12:0} 2-OH. An unknown fatty acid ECL 19.689 is present. The predominant isoprenoid quinone is ubiquinone Q-10. The major polar lipids detected are phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine and sphingoglycolipid. Major polyamine is *sym*-homospermidine.

The type strain, strain B2-7^T (=KCCM 42951^T=JCM 16711^T), was isolated from a water sample taken from Botan Oil Port in Xiamen, PR China. The genomic DNA G+C content of the type strain is 61.8 mol%.

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