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Isolation and characterization of phenanthrene-degrading *Sphingomonas paucimobilis* strain ZX4

Ying Xia[1, 2], Hang Min[1*], Gang Rao[3], Zhen-mei Lv[1], Ji Liu[1], Yang-fang Ye[1] & Xue-jun Duan[1]
[1] College of Life Sciences, Zhejiang University, 268 Kaixuan Road, Hangzhou, 310029, China; [2] Microbiology institute, Chinese Academy of Sciences, Beijing 100080,China; [3]State Intellectual Property Office of P.R.China, Beijing, 100080, China (*Corresponding author: Tel: 86-571-86971287; *E-mail* address: Minhang@zju.edu.cn)

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Abstract

Phenanthrene-degrading bacterium strain ZX4 was isolated from an oil-contaminated soil, and identified as *Sphingomonas paucimobilis* based on 16S rDNA sequence，cellular fatty acid composition , mol% G+C and Biolog-GN tests. Besides phenanthrene, strain ZX4 could also utilize naphthalene, fluorene and other aromatic compounds. The growth on salicylic acid and catechol showed that the strain degraded phenanthrene via salicylate pathway, while the assay of catechol 2, 3-dioxygenase revealed catechol could be metabolized through *meta*-cleavage pathway. Three genes, including two of *meta*-cleavage operon genes and one of GST encoding gene were obtained. The order of genes arrangement was similar to S-type *meta*-pathway operons. The phylogenetic trees based on 16S rDNA sequence and *meta*-pathway gene both revealed that strain ZX4 is clustered with strains from genus *Sphingomonas.*

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are major fractions of petroleum mixtures with highly toxic, mutagenic or carcinogenic effects to human and animal (Doddamani & Ninnekar. 2000). The large areas of oil-contaminated soil are posing threats to the ecosystem and human

health in the world. Hence, the remediation of oil-contaminated soils has received an increasing concern. By far, chemical, physical and biological methods have been utilized to remedy PAHs-contaminated soils. The principal processes for the bio-removal of PAHs from the environment，however, are thought to be microbial transformation and degradation (Cerniglia.1992; Harayama. 1997). Bioremediation, based on certain species of microorganisms, is a cheap and effective way to decontaminate PAHs-contaminated soils. By now the framework for studying PAHs metabolism, especially the low-molecular-weight ones such as naphthalene and phenanthrene, has been well established (Balashova et al.1999; Dean-Ross et al.2002), and attention has been turned toward diverse PAHs-metabolizing bacteria and related catabolic genes (Goyal & Zylstra.1996; Laurie& Lloyd-Jones.1999; Saito A et al.2000, Pinyakong et al.2003). Strains that can degrade PAHs completely and rapidly with good adjustment will be more favored although many bacteria capable of degrading PAHs have been isolated. During the aerobic bacterial degradation of PAHs, the first step is dependent on the presence of the initial PAH dioxygenase, catalyzing the hydroxylation of the substrate and subsequent degradation with the stepwise removal of aromatic rings completes the upper pathway finally leading to catechol (or substituted catechols), one of the central intermediates of PAHs degradation. The conversion of catechols could be catalyzed by *meta*-cleavage pathways via 2-hydroxymuconic semi-aldehyde, to pyruvate, acetaldehyde and acetate that could enter the tricarboxylic acid cycle. Therefore, the *meta*-cleavage genes should be critical for the complete degradation of PAHs (Shin et al.1997; Laurie & Lloyd-Jones.1999). In addition, catechol 2,3-dioxygenase (C23O; EC 1.13.11.2)，which is the most important member of *meta*-cleavage pathways, may also mirror the taxonomic grouping of the host bacteria, although horizontal gene transfer often interfers with it’s phylogeny for the gene coding for the enzyme often

lies in plasmid.

In this study, a phenanthrene-degrading bacterial strain was isolated from oil-contaminated soil near factory，and it was identified by analysis of 16S rDNA sequence，cellular fatty acid composition, determination of the mol% G+C and Biolog-GN tests. The catechol *meta*-pathway operon genes and their neighboring glutathione S-transferase gene were also cloned and partially characterized. We anticipated that the strain would be a good model organism for performing bioremediation of PAHs pollution caused by wastes from the factory and obtained detail information on the structure of the *meta*-pathway genes. We also constructed phylogenetic trees based on 16S rDNA and C23O gene sequence to identify the phylogeny of the PAH-degrading strain.

Materials and Methods

Materials

Soil samples were taken from soil contaminated with oil refinery wastes on Huajiachi campus of Zhejiang University，Hangzhou, China. Bushnell-Haas (BH) minimal salt medium was used for enrichment and isolation of phenanthrene-degrading strains. The composition of BH was as follows (g l^{-1}): KH_2PO_4 1, K_2HPO_4 1, NH_4NO_3 1, $MgSO_4.7H_2O$ 0.2, $CaCl_2$ 0.02, $FeCl_3$ 0.05. Liquid Luria-Bertani (LB) medium containing an appropriate antibiotic was used for the cultivation of *E.coli* strains. The LB composition was as follows (g l^{-1}): peptone 10.0, yeast extract 5.0, NaCl 5.0.

Isolation and identification of phenanthrene-degrading bacterium

Soil samples were agitated with Bushnell-Haas minimal salt medium (BH) in presence of 1000 mg l^{-1} of phenanthrene at 200 round per min (r min^{-1})and 30℃ for 7 d. Dilutions of soil slurry were inoculated on BH medium plates supplemented with 0.05% (w/v) yeast extract (BHY). Colonies were picked up from dilution plates based on distinct colony morphology, and transferred onto fresh

BHY plates several times to ascertain culture purity. Each isolate was then tested for its ability to grow in BH liquid medium containing phenanthrene as sole carbon source (Dagher et al.1997).

The isolated strain was identified by partial sequence of the 16S rRNA gene, the analysis of the cellular fatty acids (Yabuuchi et al.1990), determination of mol% G+C (Dong X Z & Cai M Y.2001) and Biolog-GN tests (Biolog Inc., USA).

Primers for amplifying and sequencing SSU rDNA sequences were obtained in the European database on SSU rRNA (http://silk.uic.ac.be.primer/ database.html). The following pair of universal primers for eubacteria was applied to amplify nearly full length of 16S rDNA of the isolate: the forward primer BSF8/20 (5- ’AGAGT TTGAT CCTGG CTCAG-3 ’, primering site corresponding to 8-27 of 16S rDNA of *Escherichia coli*) and the reverse primer BSR1541/20 (5 ’-AAGGA GGTGA TCCAG CCGCA-3 ’, primering site corresponding to 1541-1522 of 16S rDNA of *Escherichia coli*). The amplification reaction was performed with the program, which consists of an initial denaturation at 94℃ for 2 min, 29 cycles of denaturation at 94℃ for 1 min, annealing at 56 ℃ for 1 min, and extension at 72℃ for 2 min, with the last cycle followed by a 10 min extension at 72℃, then stored at room temperature. QIAGene DNA purification Kit (Qiagen, Craley, UK) was used to purify PCR products. Nucleotide sequences were determined on Biosystems automated DNA sequencer.

Inoculation of Biolog-GN Microplates

Strain ZX4 was grown on LB agar medium and incubated at 28℃ for 48 h. Colonies were swabbed from the slant medium, suspended in the sterilized water and the inoculation density was adjusted to the recommended turbidity of 0.03. Then 150 ml of this suspension was pipetted into each prefilled, dried well of a Biolog-GN microplate. Microplate was incubated at 20℃ without

shaking and the absorbance (A) at 590 nm on an automated microplate reader was read everyday for a week. The absorbance (A_{590nm}) of the well without carbon source was used as a background control. Data were collected and analyzed by the Biolog bacteria automated identification apparatus.

Growth on other aromatic compounds

The substrates fluorene (1 g l^{-1}), naphthalene (2 g l^{-1}), anthranone (2 g l^{-1}), catechol (1.5 g l^{-1}), salicylate (3 g l^{-1}), toluene(10 g l^{-1}), and diphenylamine (2 g l^{-1}) were added into the BH medium，respectively. The liquid cultures were inoculated with 10^7 ml^{-1} cells induced by phenanthrene and then incubated at 30℃ and 200r min^{-1} for 5d. Cultures were regularly checked for bacterial growth (turbidity) and color (reflecting appearance of intermediate). Non-inoculated media were considered as references.

Measurement of phenanthrene degradation in liquid culture

The flasks with 10ml basal medium containing 1000 mg l^{-1} phenanthrene were inoculated with strain ZX4 at a density of 10^7 ml^{-1} cells and then were incubated at pH 7.0 and 30℃ with 150 r min^{-1}. The remained phenanthrene in the cultures was extracted with 5ml trichloromethane and detected by HP7890 type gas chromatography (Shanghai Equipment Inc., China) equipped with a flame-ionization detector and HP-5 type 25 m long capillary column under the following conditions: the column temperature program，50℃~275℃ at 15℃ min^{-1}, holding at 275℃ for 1min, 275~325 ℃ at 20℃min^{-1} with a flow rate of nitrogen carrier gas of 30 ml min^{-1}.

Enzyme assay

The strain was grown in supplemented minimal medium with 1000 mg l^{-1} phenanthrene or 1 000 mg l^{-1} glucose at 30℃ to an OD_{600nm} 0.5-0.7. After being harvested the cells were disrupted with a process of 99 cycling of sonication for 3s followed by cooling for another 3s in

an ice bath, using an JY92-Ⅱ type ultrasonic oscillator at 200W. Cell debris were removed by a centrifugation at 12 000r min^{-1} (4℃) for 15 min subsequently. The supernatant was used for enzyme assays (Tian et al.2002). The following enzymes were assayed by a spectophotometer, according to the reported method.

(a) Catechol 2,3-dioxygenase activity was determined by measuring the increase in absorbance at 375 nm using 100μl crude lysate in 1ml 20 mM potassium phosphate (pH 7.5) with 1μM catechol (Balashova et al. 2001).

(b) Glutathione S-transferase (GST) activity was assayed with GST Detection Module (Pharmacia Company) and using a molar absorption coefficient for the CDNB ± GSH conjugate at 340 nm.

DNA manipulation

Total DNA was extracted from strain ZX4 and then digested with *Sal* Ⅰ (TaKaRa, Japan). DNA fragments of 2.0-5.0Kb were recovered from 0.7% agarose gel using DNA Recovery Kit (Takara). The target DNA fragments were ligated into plasmid pUC119 (Promega, USA), and then transformed into the *E.coli* JM109 (Promega). Transformants were spread onto LB agar plates supplemented with 50μg ml^{-1} AMP. The *E.coli* strains carrying cloned DNA fragments encoding *meta*-cleavage genes were detected as yellow colonies after 2, 3-dihydroxy biphenyl-diethyl ether solution was sprayed to the plates, revealing the conversion of 2, 3-dihydroxy biphenyl to yellow product (Furukawa K & Miyazaki T.1986; Sambrook et al.1990).

Sequence determination and analysis.

Nucleotide sequences were determined directly from plasmid on Biosystems automated DNA sequencer. Using DNA-STAR package, sequence analysis was performed. Multiple alignments were

carried out on a computer using the Clustal W 1.8. Both of the translation of nucleotide sequences and phylogenetic analysis were performed with DNAMAN 4.0 software.

Results

Isolation and characterization of phenanthrene-degrading strain

A bacterial isolate capable of degrading phenanthrene，(strain ZX4)，was isolated from oil-contaminated soil. This strain forms yellow，smooth and wet colonies, which could be easily scraped off on BHY plates at 30℃ within 1 to 2 d，and was Gram-negative, non-spore-forming rods with polar flagellum. It was positive in tests for catalase, phenylalanine ammonia-lyase, glucose fermentation, glycerol fermentation and rhamnose fermentation but negative for starch hydrolysis, fructose fermentation, indole test, Voges-prokauer test, gelatin hydrolysis and nitrate reduction.

The partial 16S rDNA sequence (position 32 to position 1440 at *Escherichia coli* numbering) from strain ZX4 was obtained. Sequence alignment revealed that strain ZX4 was most closely related to the species in genus *Sphingomonas*. Furthermore, the whole cell fatty acid composition in strain ZX4 was shown as following: octadecenoic acid (C18: 1) 56%, hexadecenoic acid (C16: 1) 23%, hexadecanoic acid (C16:0) 16% ，2-hydroxymyristicacid (2-OH C14:0) 2% and the others 3%, which accord with the characteristic of genus *Spingomonas*. All the results indicated that the isolated strain should be classed into genus *Spingomonas*.

Strain ZX4 oxidized 55 of the 95 different carbon sources, tested with the Biolog identification systems (data shown in Table 1). According to the results, the strain was identified as *Sphingomonas paucimobilis* with the Biolog bacteria automated identification apparatus. The G+C content of the DNA of the strain was 63.5mol%, which fell within the range of values found for strains of the species *Sphingomonas paucimobilis* (Yabuuchi et al.1990). Strian ZX4 also exhibited the highest

similarity to *Sphingomonas paucimobilis* strain UT26(98%).Therefore, the isolated strain was identified as the species *Sphingomonas paucimobilis.*

Table 1 Utilization of 95 carbon substrates by strain ZX4 using Biolog microplate

Carbon substrate	OD_{590}	Carbon substrate	OD_{590}	Carbon substrate	OD_{590}
Water	0.084	Turanose	0.835	D-Alanine	1.103
α-Cyctodextrin	0.116	Xylitol	0.353	L-Alanine	0.452
Destrin	0.080	Methyl Pyruvate	0.084	L-Alanylglycine	0.077
Glycogen	0.100	Mono-Methyl-Succinate	0.090	L-Asparagine	1.453
Tween 40	0.096	Acetic Acid	0.082	L-Aspartic Acid	0.066
Tween 80	1.068	Cis-Aconitic Acid	0.898	L-Glutamic Acid	1.103
N-Acetyl-D-galactosamina	0.064	Citric Acid	0.082	Glycyl-L-Aspartic Acid	0.080
N-Acetyl-D-glucosamine	1.091	Formic Acid	0.062	Glycyl-L-Glutamic Acid	0.073
Adonitol	0.076	D-Galactonic Acid Lactone	0.121	L-Histidine	0.064
L-Arabinose	0.063	D-Galacturonic Acid	0.061	Hydroxy-L-Proline	0.077
D-Arabitol	0.085	D-Gluconic Acid	0.856	L-Leucine	0.106
D-Cellobiose	0.086	D-Glucosaminic Acid	1.245	L-Ornithine	0.106
L-Erythritol	0.081	D-Glucuronic Acid	0.094	L-Phenylalanine	0.057
D-Fructose	0.056	α-Hydroxy Butyric Acid	0.074	L-Proline	0.070
L-Fucose	0.105	β- Hydroxy Butyric Acid	0.068	L-Pyroglutamic Acid	0.086
D-Galactose	0.089	γ- Hydroxy Butyric Acid	0.970	D-Serine	0.104
Gentiobiose	0.120	p-Hydroxy Phenylactic Acid	0.098	L-Serine	0.065
α-D-Glucose	0.105	Itaconic Acid	0.097	L-Threonine	0.085
m-Inositol	0.075	α-Keto Butyric Acid	0.090	D, L-Carnitine	0.080
α-D-Lactose	0.101	α-Keto Glutaric Acid	0.089	γ-Amino Butyric Acid	0.117
Lactulose	0.177	α-Keto Valeric Acid	0.086	Urocanic Acid	0.070
Maltose	0.102	D, L-Lactic Acid	0.067	Inosine	0.078
D-Mannitol	0.111	Malonic Acid	0.101	Uridine	0.117
D-Mannose	0.116	Propionic Acid	0.122	Thymidine	0.128
D-Melibiose	0.109	Quinic Acid	0.102	Phenyethylamine	0.184
β-Methyl-D-Glucoside	0.085	D-Saccharic Acid	0.079	Putrescine	0.369
D-Psicose	0.089	Sebacic Acid	0.067	2-Aminoethanol	0.102
D-Raffinose	0.066	Succinic Acid	0.112	2,3-Butanediol	0.107
L-Rhamnose	0.171	Bromo Succinic Acid	0.107	Glycerol	0.124
D-Sorbitol	0.085	Succinamic Acid	0.100	D, L-α-Glycerol Phosphate	0.098
Sucrose	0.107	Glucuronamide	0.112	Glucose-1-Phosphate	0.135
D-Trehalose	0.102	L-Alaninamide	0.130	Glucose-6-Phosphate	0.157

Phylogenetic analysis based on 16S rDNA sequence

To identify the phylogeny of strain ZX4 among PAHs-degrading strains, strains from different genera were chosen to construct the phylogenetic tree based on 16S rDNA sequences. Four main

clusters were formed in the phylogenetic tree of PAHs degrading strains based on 16S rDNA sequence (Figure 1). Strain ZX4 was clustered into α- *Proteobacteria* with other strains of genus *Sphingomonas.*

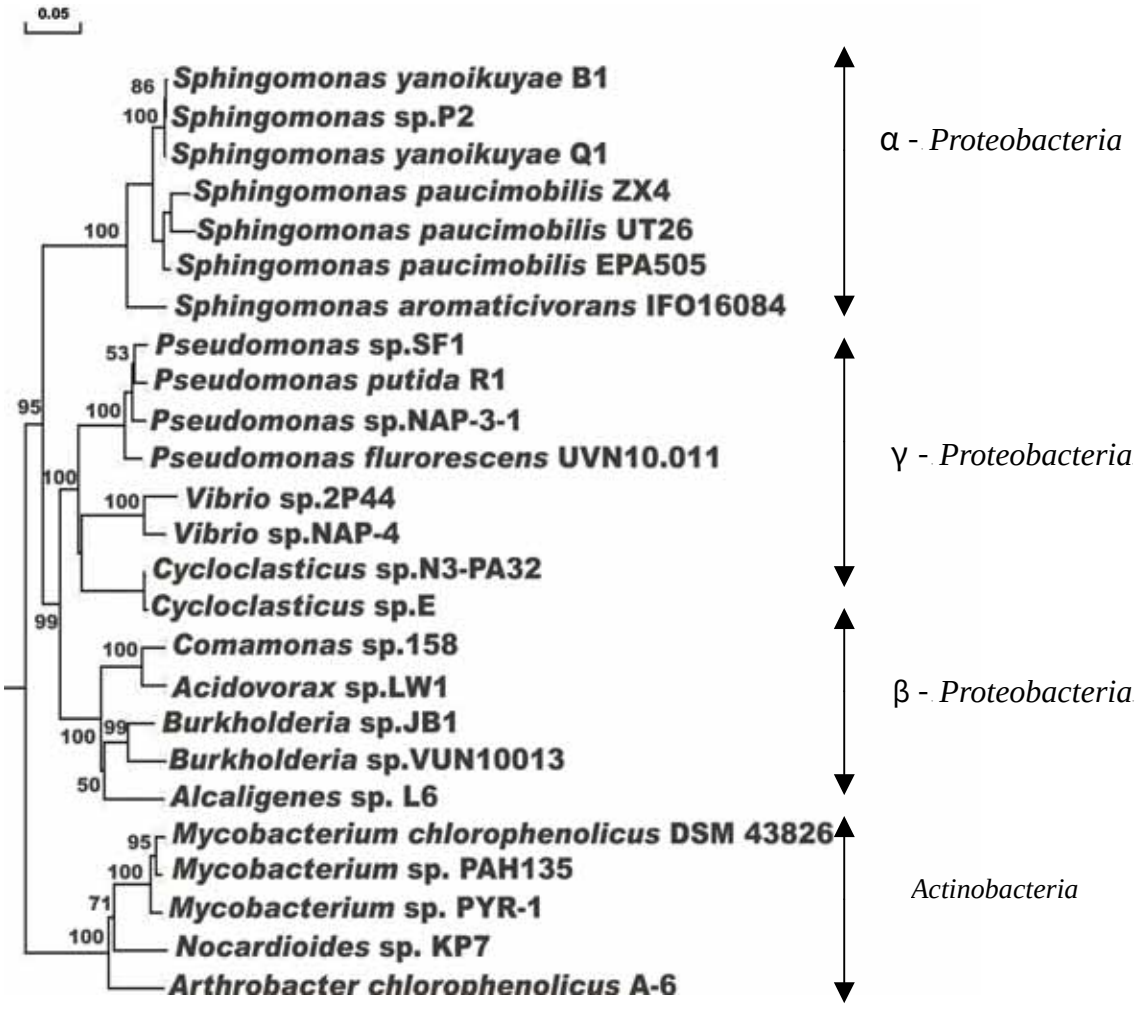


Figure 1. Phylogenetic tree based on a distance matrix analysis of the 16S rDNA sequences. Numbers at the nodes indicate the percentages of bootstrap samplings, derived from 1000 samples, supporting the internal branches.

Growth on various aromatic compounds

Strain ZX4 was able to utilize low molecular weight PAHs，like phenanthrene, fluorene and naphthalene as sole sources of carbon and energy. Some other aromatic compounds, such as toluene

and anthranone, could also be used. In addition, the strain could utilize catechol and salicylic acid which were considered intermediate in phenanthrene metabolism via salicylate way. Phthalic acid and diphenylamine could not support growth of ZX4.

Shaking flask batch fermentation experiments showed that the strain could grow well at pH 5.5 to pH8.0 on phenanthrene as sole carbon source, with an optimal growth condition of pH 7.0 and 35℃. Biomass yield was found to increase with increasing initial phenanthrene concentration (from 100 mg l^{-1} to 2500 mg l^{-1}). When ZX4 was incubated at pH 7.0 and initial concentration of 1000 mg l^{-1} phenanthrene for 14d, 98.74% of the initial phenanthrene was degraded (Figure 2).

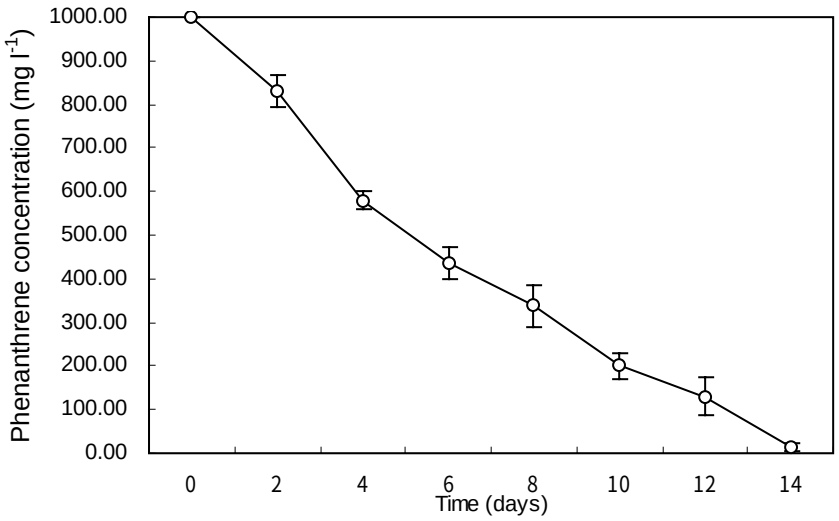


Figure 2. Phenanthrene degradation by strain ZX4. Error bar shows standard deviation.

(Three replicates were factored into the standard deviation)

Enzyme assay

When ZX4 was grown in medium with indole, blue indigo appeared, indicating aromatic ring dioxygenase activity.

Lower C23O activity was observed when ZX4 was grown on glucose as a sole source of carbon than on phenanthrene. The growth on salicylic acid and catechol showed that the strain

degrades phenanthrene via salicylate pathway, while the assay of catechol 2,3-dioxygenase revealed catechol could be metabolized through *meta*-cleavage pathway.

The detection of GST revealed it was CDNB (1-chloro-2，4-dinitrobenzene)-accepting type. The activity of the GST increased after phenanthrene induction.

Functional genes in strain ZX4

The sequence inserted into plasmid pUC119 containing two complete ORFs (*phnH* and *phnI*) and one partial ORF (*phnG*) has been determined (Figure.3 and Figure.4). The properties were summarized in Table 2.

Table 2 Summary of predicted polypeptides identified on the *Sal*I fragment

ORF	Gene	No. of Nucleotide sequence	No. of aa	Molecular weight	mol% G+C content	Protein feature	Similarity to analogous enzymes
1	*phnG*	450bp	149	-	-	GST	94% GST from EPA505[a]
2	*phnH*	852bp	283	31KDa	62.09%	HMSH	81% PhnD from DJ77[b]
3	*phnI*	927bp	308	35KDa	60.30%	C23O	94.4% C23O from B1[c]

Abbreviation: GST, glutathione S-transferase; HMSH, 2-hydroxymuconic semialdehyde hydrolase. C23O catechol 2,3-dioxygenase. a: *Sphingomonas paucimobilis* EPA505 (Lloyd-Jones & Lau.1997)); b: *Sphingomonas chungkuensis* DJ77 (Shin et al.1997); c: *Sphingomonas yanoikuyae* B1 (Kim & Zylstra1995).

An 181bp sequence space was detected between *phnG* and *phnH* while only 19bp sequence space was found between *phnH* and *phnI*. Putative promoter sequences TTGCAA(-35 region) and TGCAAT（-10 region） were found in the inserted sequence between *phnG* and *phnH*. Shine-Dalgarno-type sequences GGGAG and AGGAG were found at 4-bp upstream from the start codon of *phnH* and 7-bp upstream from the start codon of *phnI*，respectively (Figure.3 and Figure. 4).

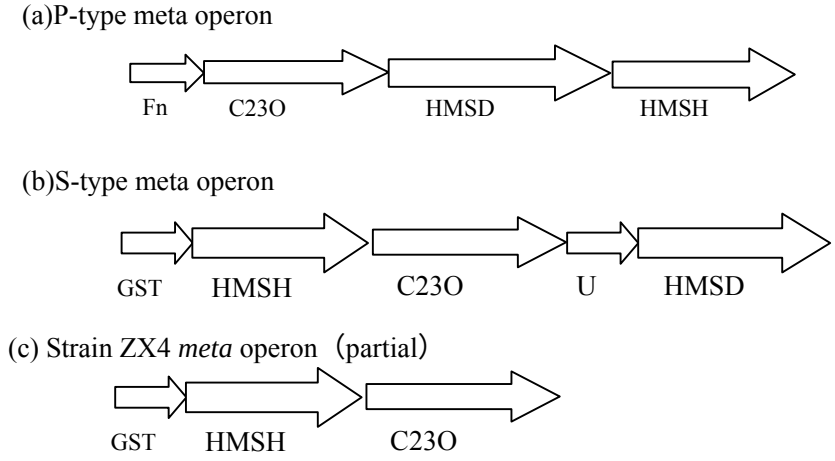


Figure.3 Gene organization of *meta*-operons.(a) P-type (*Pseudomonas*) *meta* pathway operons,(b)S-type (Sphingomonas) meta-pathway operons, and (c) Gene organization of the meta-pathway of strain ZX4.Gene abbreviation are:Fn,chloroplast-like ferredoxin; C23O, catechol 2,3-dioxygenase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; U, gene of unknown function.

***PhnG* (partial)**

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1       GTCGACACCCTGGACAGCGGCGAGACCCTGACCGAGAACCCCGCCATCCTGCTGTACATA
1        V  D  T  L  D  S  G  E  T  L  T  E  N  P  A  I  L  L  Y  I

61      GCCGACCAGAACCCCGCGTCTGGCCTGGCGCCGGCCGAAGGCAGCCTCGATCGTTACCGA
21       A  D  Q  N  P  A  S  G  L  A  P  A  E  G  S  L  D  R  Y  R

121     CTGCTGAGCCGTCTCAGCTTCCTCGGTTCCGAATTCCACAAGGCATTCGTGCCGTTGTTC
41       L  L  S  R  L  S  F  L  G  S  E  F  H  K  A  F  V  P  L  F

181     GCCCCCGGCACTTCCGACGAAGCGAAGGCGGCGGCCGCGGAATCGGTCAAGAACCACCTC
61       A  P  G  T  S  D  E  A  K  A  A  A  A  E  S  V  K  N  H  L

241     GCCGCGCTCGATAAGGAATTGGCCGGGCGGGACCACTATGCTGGCAATGCTTTCAGTGTT
81       A  A  L  D  K  E  L  A  G  R  D  H  Y  A  G  N  A  F  S  V

301     GCCGACATCTACCTGTTCGTGATGCTGGGTTGGCCAGCCTACGTCGGTATCGACATGGCC
101      A  D  I  Y  L  F  V  M  L  G  W  P  A  Y  V  G  I  D  M  A

361     GCCTACCCCTCGCTCGGCGCCTATGTCGGCAAGATCGCGCAGCGTCCCGCCGTGGGTGCC
121      A  Y  P  S  L  G  A  Y  V  G  K  I  A  Q  R  P  A  V  G  A

421     GCGCTCAAGGCCGAAGGCTTGGCGTGA
141      A  L  K  A  E  G  L  A  *
        GCCGGACAGTCCGACTTCCCGCATTATGCGATGGAGTTCAACCATTCTCCATTTGCAAAT

        GCGTTCTATTTAGTGCAATAGTAACGAGAAATCGGATGGACGCGAAAGCTGTGTGCATGA

        CCTCGCGCGAACACAGGCAATGCTAGCGTCAAAAAACAGTTGAATATCGATTGGGAGAGCC
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PhnH

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1     ATGACTGCCGTAGCCAACGACATCGCACGCCCGGAGATTGGCAAGTCCATCACGGTTGAT
1      M  T  A  V  A  N  D  I  A  R  P  E  I  G  K  S  I  T  V  D

61    GGCAGCGTCACGAATTATCACGATTTGGGCGATGGCGCACCGGTCCTGCTGATCCACGGA
21     G  S  V  T  N  Y  H  D  L  G  D  G  A  P  V  L  L  I  H  G

121   TCGGGACCCGGGGTGACTGCCTGGGCCAATTGGCGGCTCAACATGCCCGAGCTAGCGAAG
41     S  G  P  G  V  T  A  W  A  N  W  R  L  N  M  P  E  L  A  K

181   CGCTTCAGGGTCATCGCGCCCGACATGTTCGGGTTTGGCTATTCGGCTTCGAAGGGCCGG
61     R  F  R  V  I  A  P  D  M  F  G  F  G  Y  S  A  S  K  G  R

241   ATCGAAGATAAGCGGGTGTGGGTCGATCAGGTCGCATCGCTGCTCGATAGCCTCGGGATC
81     I  E  D  *K     V  W  V  D  Q  V  A  S  L  L  D  S  L  G  I

301   GACAAGGTCTCGATGGTCGGCAATTCCTTCGGCGGCGGCATAACCCTGGCGTTCATGATC
101    D  K  V  S  M  V  G  N  S  F  G  G  G  I  T  L  A  F  M  I

361   GCGCATCCCGATCGAGTCGAAAGGGCGGTGCTGATGGGTCCGGCGGGGCTAGATTTCCCG
121    A  H  P  D  R  V  E  R  A  V  L  M  G  P  A  G  L  D  F  P

421   ATCACGCCTGCGCTCGACCTGGTCTGGGGCTATCAGCCTTCGCTAGAGGAAATGCGCACC
141    I  T  P  A  L  D  L  V  W  G  Y  Q  P  S  L  E  E  M  R  T

481   TCGCTCAAGTACCTCGCCTGGGATCACAGCCGGCTGACCGAAGACCTGGTCCAGTCGCGC
161    S  L  K  Y  L  A  W  D  H  S  R  L  T  E  D  L  V  Q  S  R

541   TATGAAGCGAGTGCGCGTCTGGAAGCGCATGAGCCGTATCACGCGACGTTCGGCGGGGCT
181    Y  E  A  S  A  R  L  E  A  H  E  P  Y  H  A  T  F  G  G  A

601   GACCGGCAGCGCAACATCGCGATGCTGGCAAGCCGCGAGGAAGACGTGGCGGCGCTAAAG
201    D  R  Q  R  N  I  A  M  L  A  S  R  E  E  D  V  A  A  L  K

661   CACGAAACGCTCATCCTGCATGGCCTGGACGACCAGGTGATTCCGCTGGAATCGACCGTT
221    H  E  T  L  I  L  H  G  L  A  D  Q  V  I  P  L  E  S  T  V

721   CGCCTGGCCAGCCTGTTGCCGCGCGCCGGACCTGCACGTGTTCGCGGAATGCGGCCACTGG
421   GAGCCGCGCGGTATGCGCGCGACCAGGTTCGATCACTGCGCCCTCAATGGTGTCGATATT
141    E  P  R  G  M  R  A  T  R  F  D  H  C  A  L  N  G  V  D  I

481   TCGGCGAGCGCGAAGATTTTTGTCGAGGCTCTCGATTTTTCGGTGGCCGAGGAATTGGTG
161    S  A  S  A  K  I  F  V  E  A  L  D  F  S  V  A  E  E  L  V

541   GACGAAGGGTCCGGCACTCGGCTGGGCATCTTCCTGTCATGCAGCAACAAGGCGCATGAC
181    D  E  G  S  G  T  R  L  G  I  F  L  S  C  S  N  K  A  H  D

601   GTGGCGTTTCTGGGCTATCCCGAGGACGGACGGATCCATCACACATCGTTCAACCTCGAA
201    V  A  F  L  G  Y  P  E  D  G  R  I  H  H  T  S  F  N  L  E

661   TCCTGGCACGATGTCGGTCATGCGGCGGACATCATCAGCCGCTACGACATTTCGCTCGAT
221    S  W  H  D  V  G  H  A  A  D  I  I  S  R  Y  D  I  S  L  D

721   ATCGGCCCGACCCGCCATGGGATCACACGCGGTCAGACAATCTACTTCTTCGACCCCTCG
241    I  G  P  T  R  H  G  I  T  R  G  Q  T  I  Y  F  F  D  P  S

781   GGCAACCGCAACGAGACGTTCAGCGGCGGCTACACCTATTATCCCGATAATCCGCGCCGG
261    G  N  R  N  E  T  F  S  G  G  Y  T  Y  Y  P  D  N  P  R  R

841   ATGTGGCAGGCCGAGAATGCCGGCAAGGCGATCTTCTATTACGAGAAGGCGCTCAACGAC
281    M  W  Q  A  E  N  A  G  K  A  I  F  Y  Y  E  K  A  L  N  D

901   CGCTTCATGACGGTAACACCTGA
301    R  F  M  T  V  T  P  *
101    P  G  V  G  R  K  I  R  F  N  T  P  T  A  H  V  F  D  L  Y

361   GCCGAGATGGAGCTGTCCGATACGGGTCCGGCCGTGCGCAATCCGGACGTCTGGATCGCC
121    A  E  M  E  L  S  D  T  G  P  A  V  R  N  P  D  V  W  I  A
```

Figure.4 Sequence of the region encoding *PhnG* (partial), *PhnH* and *PhnI*. Asterisks indicate stop codons. Potential Shine-Dalgarno-type sequences (RBS) were marked by gray color. Putative promotor sequences were boxed. The hydrolase motif was underlined.

Phylogenetic analysis based on C23O gene sequences

To study the phylogeny based on 16S rDNA sequence and C23O gene sequence, we also constructed phylogenetic tree based on C23O sequence from PAHs degrading strains. The phylogenetic tree showed that *PhnI* from strain ZX4 was clustered with C23O gene from *Sphingomonas* species (Figure 5).

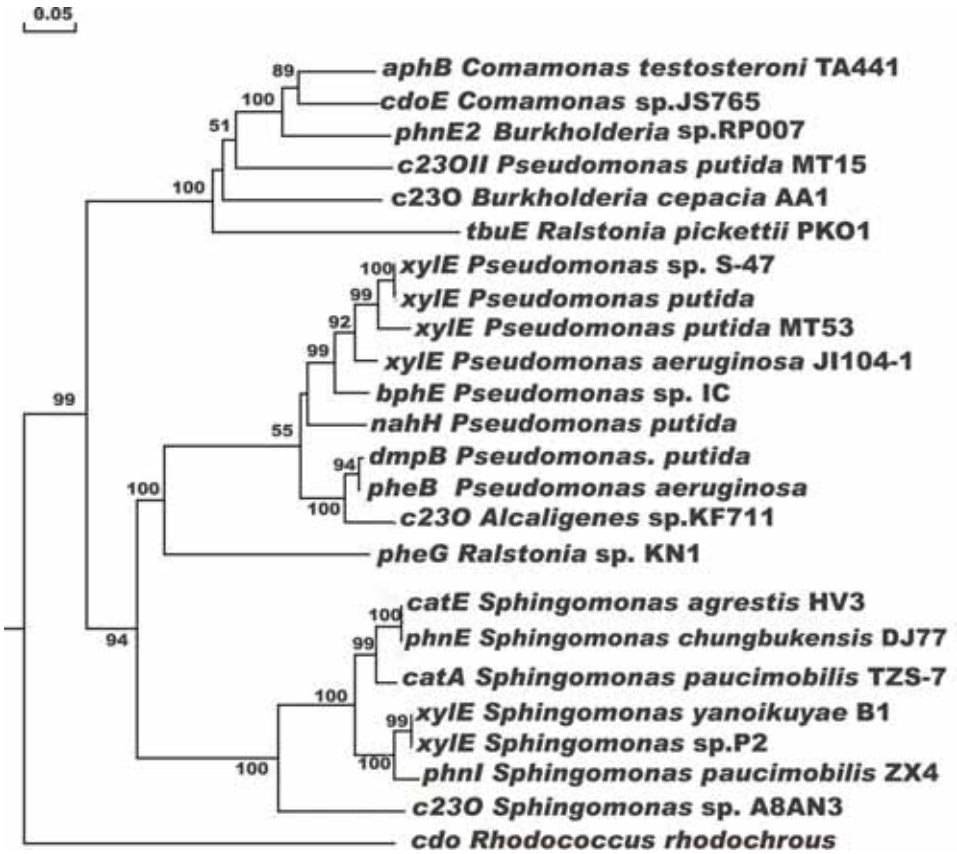


Figure 5. Phylogenetic tree based on a distance matrix analysis of the C23O sequences. Numbers at the nodes indicate the percentages of bootstrap samplings, derived from 1000 samples, supporting the internal branches.

Discussion

In this study, the isolated strain ZX4 was high effective for degrading phenanthrene. The strain could degrade phenanthrene of high concentration within a wide pH range. It was also showed that the strain ZX4 possess the ability of degrading phenanthrene thoroughly, through successfully cloning of *meta*-pathway genes from it. It was indicated that the strain could be a potential one used for bioremediation of oil-contaminated environments. Furthermore, strain ZX4 could transform indole to blue indigo, suggesting it could also be an engineering bacterium used to produce indigo.

Based on analysis of 16S rDNA sequence, fatty acid composition, mol% G+C and Biolog-GN tests, the strain should be classified into genus *Sphingomonas* (Yabuuchi et al.1990; Nohynek et al.1996). The results of mol% G+C and Biolog-GN test indicated that the strain was mostly similar to *Sphingomonas paucimobilis*. Likewise, 16S rDNA sequence alignment also demonstrated the strain exhibited the highest similarity to *Sphingomonas paucimobilis* UT26 (Nalin et al.1999). Therefore, ZX4 should be identified as *Sphingomonas paucimobilis*.

Sequence alignment revealed *phnG*, *phnH* and *phnI* were GST encoding gene, HMS hydrolase encoding gene and C23O encoding gene，respectively. Sequence analysis showed that the *phnH* and *phnI* genes were closely spaced with only 19-bp between two encoding regions. However, analysis of region between *phnG* and *phnH* genes revealed a 181-bp spacer with putative promotor sequences. This indicated that *phnH* and *phnG* should be included in the same *meta*-cleavage pathway operon，in which *phnH* gene was the first gene (Shin et al.1997). Obviously, GST gene was not located in this operon though GST gene adjoined hydrolase-C23O gene. This arrangement of *meta*-pathway genes and its neighboring gene is similar to S-type *meta*-operons (a characteristic of *Sphingomonas* spp.) and different from P-type *meta*-operons (a characteristic of *Pseudomonas* spp.)(Laurie & Lloyd-Jones.1999)（Figure 3）

GST could catalyze the addition of glutathione to endogenous or xenobiotic, often toxic electrophilic chemicals (Vuilleumier S & Pagni M.2002). The GST activity of strain ZX4 could be induced by phenanthrene, indicating that it might play important roles in detoxification of phenanthrene and intermediates in phenanthrene metabolism. GST from ZX4 is CDNB-active, which is a characteristic of general GST though it is often absent in bacteria GST. Sequence alignment showed that GST encoding gene of ZX4 was more similar to that from *Sphingomonas paucimobilis* EPA505, *Sphingomonas aromaticivorans* F199, and *Cycloclasticus oligotrophus* RB1. These homologues of GST are all CDNB-active. By contrast, GST of strain ZX4 showed a low similarity to that from *Sphingomonas paucimobilis* SYK-6 and *Sphingomonas* sp.RW5，which are CDNB-inactive.

Though the first gene of *meta*-cleavage operon was *phnH gene*, the initial step involved in the *meta*-cleavage of catechol should be catalyzed by PhnI (C23O), which was a kind of extradiol dioxygenase. Phylogenetic tree based on C23O gene sequences showed that strain ZX4 was clustered with other strains of *Sphingomonas*, similar to that based on 16S rDNA.

In the phylogenetic tree based on C23O gene sequences, it was also found that C23O gene sequences from the same species *Pseudomonas putida* were scattered to different clusters, though those from the genus *Sphingomonas* were clustered to the same group. Perhaps it is the horizontal gene transfer and evolutionary pressures within the different *Proteobacterial* subclasses that caused the results.

PhnH hydrolase (HMS hydrolase) is necessary for conversion of 2-hydroxymuconic semialdehyde, *meta*-cleavage product of catechol, to 2-hydroxypent-2, 4-dienoate. Analysis of deduced protein sequence pointed out the PhnH hydrolase should belong to α/β hydrolase fold

enzyme family with motif Gly-Xaa-Ser-Xaa-Gly-Gly and Ser^{109}- Asp^{232}- His^{259} catalytic triad (Diaz & Timmis.1995). The phylogeny of *phnH* gene appears to follow the same tread as that of the C23O sequence.

Sphingomonas paucimobilis, a model species in genus *Sphingomonas*, is also a versatile bacteria. Among the known strains of *Sphingomonas paucimobilis,* many could degrade xenobiotic compounds, such as strain UT26 and strain SYK-6 degrading lindane, and strain EPA505 degrading PAHs. Some genes encoding catabolic pathway in those strains have already been cloned and sequenced (Miyauchi et al.1998; Masai et al.1999). However, there is no report on *meta*-pathway genes in *Sphingomonas paucimobilis* capable of degrading PAHs by far. Since the *meta*-pathway plays important function roles in degrading PAHs completely, it is believed that obtaining detailed information on the structure of the *meta*-pathway operon from *Sphingomonas paucimobilis* ZX4 would be available for understanding the metabolic capacities of the strain. What's more, it would consequentially facilitate the analysis and improvement of the PAHs degradation capability of the strain through genetic modification.

Conclusion

A high effective phenanthrene degrading strain was isolated from oil-contaminated soil. The strain was identified as *Sphingomonas paucimobilis* based on analysis of fatty acids content, mol% G+C, Biolog-GN tests and 16S rDNA sequence. Besides degrading phenanthrene, this strain could also degrade other aromatic compounds like fluorene, naphthalene and toluene. Phenanthrene was metabolized by the strain via salicylate pathway. The arrangement of catechol *meta*-cleavage operon genes and their neighboring gene was similar to that of S-type *meta*-operon. It is a first report about *meta*-cleavage operon genes in *Sphingomonas paucimobilis.*

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