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Isolation and characterization of polycyclic aromatic hydrocarbons-degrading *Sphingomonas* sp. strain ZL5

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Abstract

A bacterial strain ZL5, capable of growing on phenanthrene as a sole carbon and energy source but not naphthalene, was isolated by selective enrichment from crude-oil-contaminated soil of Liaohe Oil Field in China. The isolate was identified as a *Sphingomonas* sp. strain on the basis of 16S ribosomal DNA analysis. Strain ZL5 grown on phenanthrene exhibited catechol 2,3-dioxygenase (C23O) activity but no catechol 1,2-dioxygenase, gentisate 1,2-dioxygenase, protocatechuate 3,4-dioxygenase and protocatechuate 4,5-dioxygenase activities. This suggests that the mode of cleavage of phenanthrene by strain ZL5 could be *meta via* the intermediate catechol, which is different from the protocatechuate way of other two bacteria, *Alcaligenes faecelis* AFK2 and *Nocardioides* sp. strain KP7, also capable of growing on phenanthrene but not naphthalene. A resident plasmid (approximately 60 kb in size), designated as pZL, was detected from strain ZL5. Curing the plasmid with mitomycin C and transferring the plasmid to *E. coli* revealed that pZL was responsible for polycyclic aromatic hydrocarbons degradation. The C23O gene located on plasmid pZL was cloned and overexpressed in *E. coli* JM109(DE3). The ring-fission activity of the purified C23O from the recombinant *E. coli* on dihydroxylated aromatics was in order of catechol > 4-methylcatechol > 3-methylcatechol > 4-chlorocatechol $\gg$ 3,4-dihydroxyphenanthrene > 3-chlorocatechol.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are pollutants of concern because of their toxic and carcinogenic potentials (LaVoce et al. 1982; Lotufo 1997). Pollution by PAHs is usually found on the sites of oil field and gas factories. Microbial. degradation is the economically and environmentally attractive solution for cleaning those sites (Kanaly & Harayama 2000; Kastner & Mahro 1996). A large number of microorganisms were isolated from the contaminated environment due to their potential for PAHs degradation. Members of the α-, β- and γ-subclasses of the *Proteobacteria*, especially representatives of the genera *Pseudomonas* and *Sphingomonas* formed a remarkably high fraction of those isolates (Johnsen et al. 2002; Meyer et al. 1999).

Generally, most bacteria capable of growing on phenanthrene could also grow on naphthalene. They degrade phenanthrene *via* salicylate and catechol (the salicylate pathway). Up to now, a very few bacterial strains, for example, *Alcaligenes faecelis* AFK2 (Kiyohara et al. 1982a) and *Nocardioides* sp. strain KP7 (Saito et al. 2000) are found to be capable of growing on phenanthrene but not utilizing naphthalene. They degrade phenanthrene *via* protocatechuate (the

protocatechuate pathway). However, the member of the genus *Sphingomonas* capable of degrading phenanthrene but not naphthalene has not been reported previously.

In this study, we found out that *Sphingomonas* sp. strain ZL5, isolated from crude oil-contaminated soil of Liaohe Oil Field in China, was capable of growing on phenanthrene as the sole carbon and energy source but not naphthalene. Interestingly, the isolate could metabolize phenanthrene *via* the salicylate pathway. The degradative plasmid and the characterization of catechol 2,3-dioxygenase (C23O) of strain ZL5 were also described.

Materials and methods

Media, bacterial strains and vectors

All chemicals, of the highest purity available were obtained from Fluka, Merck or Sigma. Mineral salts basal (MSB) solution contained 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCl_2 \cdot 2H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.02 g of $MnSO_4 \cdot H_2O$, 0.4 g of KH_2PO_4, 0.6 g of Na_2HPO_4, 1.0 g of NH_4NO_3 in 1 l of distilled water, and the solution pH was adjusted to 8.0. To prepare phenanthrene-MSB medium, filter-sterilized stock concentration of phenanthrene dissolved in acetone was delivered to the sterile empty flask, and then the sterile MSB solution was added after the acetone was removed by evaporation giving a final phenanthrene concentration of 2000 mg l^{-1}. Similar procedures were used for preparing pyrene–MSB and naphthalene-MSB medium. The final pyrene concentration was 100 mg l^{-1}. The final concentrations of naphthalene were 2000, 1000, 500, 100, 50, and 10 mg l^{-1}, respectively. To prepare MSB agar medium supplemented with individual PAH, the spray–plate method was used as described previously (Kiyohara et al. 1982b). Luria–Bertani (LB) medium and LB agar plates containing appropriate antibiotics were used for culturing *E. coli* and the mutants of strain ZL5.

E. coli DH5α was used as the host for transformation of plasmid pZL. *E. coli* JM109(DE3) was used as the host for expression of plasmid pET-C23O. pGEM T-easy vector was purchased from Promega Co. and pET-30a(+) used as the expression vector was obtained from Novagen Co. Ni-NTA Purification System (Invitrogen) was used to purify C23O from the recombinant *E. coli*.

Isolation and identification of the strain

One gram of soil from Liaohe Oil Field in China was added to a 250-ml shaking flask containing 50 ml of phenanthrene–MSB medium. Cultivation was conducted at 30 °C with shaking at 150 rpm and after 5 days of incubation, 10 ml of enrichment culture was transferred to the fresh phenanthrene-MSB medium and incubated for additional 5 days. After five times of continuous transferring, serial dilutions of the culture were prepared and spread on MSB agar plates, and immediately phenanthrene in acetone was uniformly sprayed over the surface of the plates. The plates were then incubated at 30 °C for 3 days after the acetone vaporized from the surface. Colonies surrounded with a clear zone were isolated. Isolates were individually inoculated to phenanthrene-MSB media and incubated at 150 rpm and 30 °C. The fastest-growing strain, designated as ZL5, was selected for further study. All cultures were incubated in the dark for avoiding photo-destruction of PAHs.

In order to identify strain ZL5, 16S rDNA was amplified by PCR (Weisburg et al. 1991) and sequenced by the dideoxynucleotide termination method using a ABI 377 DNA sequencer. Additionally, cells of ZL5 were viewed with a transmission electron microscope and Gram-staining was performed. Physiological and biochemical properties were examined according to the procedures as described (Yrjala et al. 1998).

Antibiotic resistance determination

Strain ZL5 was inoculated in LB broth containing serially diluted antibiotics. The optical density at 600 nm was measured after 2 days of cultivation. The antibiotics tested were chloramphenicol (CAM), rifamycin (RIF), ampicillin (AMP), gentamicin (GEN), tetracycline (TET) and streptomycin (STR).

Degradation of PAHs by stain ZL5

The potential for naphthalene, phenanthrene and pyrene degradation by strain ZL5 was exam-

ined by reversed-phase high pressure liquid chromatography (HPLC) quantifying remaining each PAH and simultaneously monitoring bacterial growth in cultures. One milliliter of strain ZL5 culture was transferred to 50-ml flasks containing 10 ml of PAH-MSB medium and incubated at 150 rpm and 30 °C. Uninoculated flasks and flasks without PAH served as controls. PAH and bacterial protein concentrations were measured at 24 h intervals over 5 days. For naphthalene degradation, the incubation time was prolonged for 14 days. The remaining PAH concentration was determined using duplicate cultures by HPLC analysis of total organic extracts (Bezalel et al. 1996). Bacterial protein concentrations were measured by the Bradford method (Bradford 1976), with bovine serum albumin as the standard. Protein and PAH concentrations at sampling intervals are expressed as the average of those obtained for duplicate flasks.

For the biodegradation versatility test, several monocyclic aromatic hydrocarbons were tested. They were toluene, *m*-, and *p*-xylene, benzene. Each compound was added to sterile MSB solution (final conc. 100 mg l^{-1}) and then strain ZL5 was inoculated to the medium. After incubation for 1 week at 30 °C in the dark, the ability of the strain to utilize a compound as its sole carbon and energy source was determined.

Enzyme assay of crude cell extracts of strain ZL5

To screen for the presence of ring-cleavage dioxygenase activity in crude cell extracts of strain ZL5 grown in phenanthrene–MSB medium, the colorimetric reaction was performed. C23O activity was measured as described previously (Kojima et al. 1961). Catechol 1,2-dioxygenase (C12O) activity was measured by an increase in absorbance at 260 nm (Cain 1966), and gentisate 1,2-dioxygenase activity was measured by an increase in absorbance at 334 nm (Crawford et al. 1975). Protocatechuate 3,4-dioxygenase activity was determined by measuring the decrease in absorbance at 290 nm (Stanier & Ingraham 1954). Protocatechuate 4,5-dioxygenase was monitored by an increase in absorbance at 410 nm (Wheelis et al. 1967). Cells of strain ZL5 grown in glucose-MSB medium (final conc. 100 mg l^{-1}) worked as a non-induced control.

Identification of the plasmid and location of C23O gene

The plasmid of strain ZL5, designated as pZL, was prepared as described by Kado & Liu (1981), and recovered from low-melting-point agarose. Digestion of different restriction endonucleases was performed to measure the size of pZL. To elucidate the relationship between pZL and PAH-biodegradability, both curing and transferring the plasmid were performed. Curing the plasmid with mitomycin C was conducted as described (Chakrabarty 1972). Mutants of strain ZL5 capable of growing on LB agar but not on phenanthrene-MSB plates were screened. Transferring the plasmid to *E. coli* DH5α was performed as described previously (Sambrook et al. 1989), and *E. coli* colonies capable of growing on phenanthrene-MSB plates were screened. C23O activity of crude cell extracts from *E. coli* DH5α introduced the plasmid was detected. *E. coli* DH5α served as controls.

The primers SPHCA23St/SPHCA23OR (Meyer et al. 1999) were used for specific amplification of the partial C23O gene of strain ZL5 with plasmid pZL as a template. The PCR product was cloned into pGEM T-easy vector and sequenced. A 700 bp *Eco*RI fragment of C23O gene was radiolabeled with ^{32}P, using as a probe. DNA fragments for Southern blotting were derived from the recovered plasmid pZL and the introduced plasmid of the recombinant *E. coli* DH5α digested with *Bam*HI, *Bgl*II, *Hin*dIII and *Kpn*I, *respectively*.

Overexpression of C23O gene and substrates specificity analysis

A pair of primers C23OF [5′-CGCCATATGGCTTTGACTGGTGTAATTCG-3′] (the underlined sequence is the *Nde*I site) and C23OR [5′-GAGAAGCTTCCGTCCATGATAAGGTGTTC-3′] (the underlined sequence is the *Hin*dIII site) was used to amplify C23O gene of strain ZL5. PCR product was cloned between the *Nde*I and *Hin*dIII site of pET-30a(+) to yield pET-C23O. pET-C23O was transformed into *E. coli* JM109-(DE3). The recombinant *E. coli* was grown in LB broth with 100 μg ml^{-1} kanamycin to an optical density of 0.6 at 600 nm and then induced with 1 mM IPTG for 6 h at 37 °C. C23O protein from the recombinant *E. coli* was separated by 15%

SDS-PAGE and then scanned at 560 nm with a thin layer scanner. Induced cells of *E. coli* JM109 (DE3) with pET-30a(+) served as controls.

C23O from the recombinant *E. coli* was purified using Ni-NTA resin according to the procedures of the manual. C23O activity assays were performed at 23 °C in 3.0 ml of 100 mM phosphate buffer (pH 7.5) containing 1 μmol substrate. Catechol, 3-methylcatechol, 4-methylcatechol, 3-chlorocatechol, 4-chlorocatechol, and 3,4-dihydroxyphenanthrene were served as the substrates individually. One unit of the enzyme activity was defined as the amount of enzyme that produces 1 μmol *meta*-cleavage compound from each substrate per minute. Aromatic ring-cleavage activities were determined by the molar extinction coefficient of the respective ring-fission product (Kang et al. 1998).

Nucleotide sequence accession number

The 16S rDNA sequence of strain ZL5 has been deposited in GenBank (accession no. AY460123).

Results

Isolation and identification of Sphingomonas *sp. strain ZL5*

Strain ZL5 grown on phenanthrene as the sole source of carbon and energy was isolated from the contaminated soil by culture enrichment technique. The bacterium was gram-negative with yellow pigmentation and produced oxidase, catalase and urease. Nitrate was reduced, and both gas and acid were produced from carbohydrates. Observation of electron microscopy showed that the cell of the strain was a straight rod, 1.5–2.5 μm long and 0.7–0.9 μm in diameter, without flagella. 16S rDNA sequence analysis showed that strain ZL5 belonged to the genus *Sphingomonas* and was most closely related to *Sphingomonas herbicidovorans* (similarity, 98%). The bacterium was, therefore, tentatively identified as a *Sphingomonas* sp. strain. The experiments of antibiotic susceptibility showed strain ZL5 was resistant to 100 $\mu g\,ml^{-1}$ of AMP, STR and RIF, but was sensitive to 25 $\mu g\,ml^{-1}$ of CAM, 2.5 $\mu g\,ml^{-1}$ of GEN and 7.5 $\mu g\,ml^{-1}$ of TET.

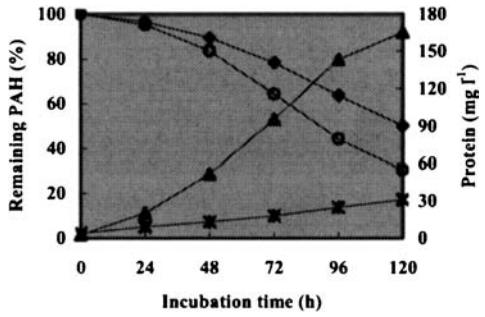

Figure 1. Curves of growth and degradation of *Sphingomonas* sp. strain ZL5 in phenanthrene-MSB and pyrene-MSB media. Remaining phenanthrene (○) or pyrene (◆) was determined by HPLC analyses; Growth on phenanthrene (▲) or pyrene (∗) was shown as an increase in the level of bacterial cell protein in cultures.

Aromatic hydrocarbons degradation by strain ZL5

In phenanthrene-MSB medium, after 120 h of incubation, approximately 70% of added phenanthrene was degraded, and the average degradation rate was 11.5 $mg\,l^{-1}\,h^{-1}$. The culture reached the exponential-phase after incubating for 24 h and the stationary-phase after 96 h. Concomitantly, bacterial biomass increased to 165.3 $mg\,l^{-1}$ at 120 h. Strain ZL5 grew slowly in pyrene-MSB medium. The average degradation rate was 0.6 $mg\,l^{-1}\,h^{-1}$ and accumulated biomass reached 31.3 $mg\,l^{-1}$ at 120 h (Figure 1). Colonies of strain ZL5 showed no change on naphthalene-MSB plates, which remained covered with a white layer of the solid hydrocarbon. When the strain was inoculated in naphthalene–MSB media, there was no reduction of naphthalene and increase in bacterial protein concentrations over 14 days. This demonstrated that strain ZL5 could not grow on naphthalene as a sole carbon and energy source. After incubation for 1 week at 30 °C in the dark, MSB media supplemented with benzene, toluene, *m-*, and *p*-xylene turned turbid, showing that strain ZL5 was able to utilize those monocyclic aromatic hydrocarbons.

Enzyme assay

In order to determine whether the ring-cleavage intermediate was catechol or protocatechuate and whether phenanthrene was degraded through an *ortho-* or a *meta-* cleavage pathway, enzyme assays were performed on crude cell extracts of strain

ZL5 grown on phenanthrene. Strain ZL5 exhibited C23O activity (1.0 U mg^{-1}) and there was not any C23O activity detected when ZL5 was grown on glucose. This indicated the C23O activity was induced by phenanthrene and catechol was the ring-cleavage intermediate. No C12O, gentisate 1,2-dioxygenase, protocatechuate 3,4-dioxygenase, and protocatechuate 4,5-dioxygenase activities were detected, indicating that gentisate and protocatechuate were not the intermediates of phenanthrene degradation.

Degradative plasmid and location of C23O gene

The resident plasmid pZL was estimated to be about 60 kb by digestion with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Xho*I, *Xba*I, *Sal*I and *Not*I, respectively. The phenanthrene-negative (phn^-) derivatives, which could grow on LB agar plates, but not phenanthrene-MSB plates, were obtained from strain ZL5 treated with 5 $\mu g\,ml^{-1}$ mitomycin C. Plasmid preparation showed that the phn^- mutants had lost plasmid pZL. It suggests the plasmid could be involved in PAHs biodegradation that strain ZL5 cured plasmid pZL lost the capability to degrade phenanthrene. Several transformants were screened which could grow on phenanthrene-MSB plates after plasmid pZL was introduced into *E. coli* DH5α. The transformants were proved to obtain such a large plasmid and exhibited C230 activities (about 0.3 U mg^{-1}). The results of introduction of plasmid pZL into *E. coli* demonstrated that plasmid pZL could be responsible for PAHs degradation.

A 900 bp DNA fragment as predicted size was produced by PCR amplification from strain ZL5 with the primers SPHCA23St/SPHCA23OR and sequenced. The incomplete open reading frame (ORF) showed extensive similarity to the C23O genes from *Sphingomonas* sp. It was 99% identical to *cmp*E from *Sphingomonas* sp. HV3 (GenBank accession no. Z84817) and *phn*E from *Sphingomonas chungbukensis* sp. nov (GenBank accession no. U83882). The C23O gene of strain ZL5 was found by Southern hybridization to reside on the 7 kb *Bam*HI fragment, the 25 kb *Bgl*II fragment, the 20 kb *Hin*dIII fragment and the 6 kb *Kpn*I fragment of plasmid pZL, respectively (Figure 2). There was no positive signal when the chromosomal DNA of strain ZL5 was subjected to Southern blotting under the same conditions (data not shown). When *Eco*RI fragment of C23O gene was used as a probe to hybridize with the fragments of the introduced plasmid from the recombinant *E. coli* DH5α digested by the endonucleases above mentioned, the same hybridization pattern appeared as plasmid pZL did. Plasmid pZL is, therefore, a degradative plasmid responsible for PAHs degradation.

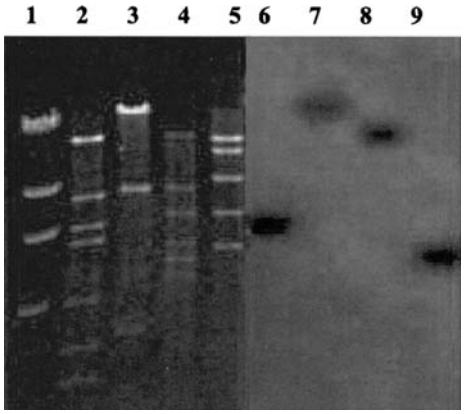

Figure 2. Localization of C230 gene on plasmid pZL of *Sphingomonas* sp. strain ZL5 by Southern hybridization. Lane 1, Lambda DNA/*Hin*dIII marker; Lanes 2–5, Plasmid pZL DNA was digested by *Bam*HI, *Bgl*II, *Hin*dIII and *Kpn*I, respectively; Lanes 6–9, The digested plasmid DNA was subjected to hybridization with *Eco*RI fragment of C23O gene as a probe.

Overexpression of C23O gene in E. coli *JM109 (DE3)*

A pair of primers C23OF and C23OR was designed to amplify the gene encoding C23O from strain ZL5. The resulting DNA fragment was cloned into pET-30a(+) to construct pET-C23O and sequenced. Nucleotide sequence analysis of the C23O gene revealed a complete ORF of 924 bp, which was the same sequence with the C230 gene in *Kpn*I fragment of plasmid pZL. The ORF encodes a polypeptide chain containing 307 amino acid residues with molecular mass of 34 kDa. The deduced amino acid sequence of the C23O exhibited high (more than 80%) homology with that of C23O from other *Sphingomonas* sp. strains, while it exhibited lower than 60% sequence identity with that of C23O from the other genus. For example, it was 52% identical to that of C23O from *Pseudomonas aeruginosa* (GenBank accession no. AAM54735) and *Alcaligenes eutro-*

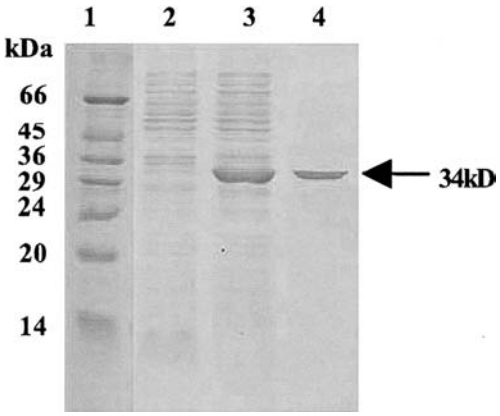

Figure 3. Coomassie-stained SDS-PAGE of expression of the C23O gene. Lane 1, Molecular weight standards; lane 2, pET-30a(+) in JM109(DE3); lane 3, pET-C230 in JM109 (DE3); lane 4, purified C23O.

phus (GenBank accession no. JE0112). The results indicated that the C23O of strain ZL5 belonged to '*Sphingomonas* group' classified by Meyer et al. (1999).

E. coli JM109(DE3) harboring pET-C23O was cultured with IPTG for induction. On SDS-PAGE, C23O was overexpressed with the appearance of a specific band of approximate 34 kDa, similar to the predicted size. Thin layer scanning analysis showed that the expressed protein accounted for 42% of the total protein in the crude extracts. C23O specific activity of the crude extract was 25.4 U mg^{-1}. Induced cells of *E. coli* JM109(DE3) with pET-30a(+) did not exhibit any C23O activity. The purified C23O from the recombinant *E. coli* exhibited a single band of about 34 kDa on the SDS-PAGE (Figure 3). The aromatic ring-fission activity of the purified enzyme on dihydroxylated aromatics and analogs as substrate was in the order catechol > 4-methylcatechol > 3-methylcatechol > 4-chlorocatechol $\gg$ 3,4-dihydroxyphenanthrene > 3-chlorocatechol (Table 1).

Table 1. Aromatic ring-fission activity of the purified C23O from *E. coli* JM109(DE3) harboring pET-C23O

Substrate	Specific activity (U mg^{-1})
Catechol	87.6
3-Methylcatechol	45.1
4-Methylcatechol	68.6
3-Chlorocatechol	1.8
4-Chlorocatechol	25.2
3,4-Dihydroxyphenanthrene	4.5

Discussion

The aerobic degradation of phenanthrene and naphthalene has been extensively studied. Bacteria degrade phenanthrene by one of two distinct routes. Bacteria utilizing both phenanthrene and naphthalene metabolize phenanthrene *via* the salicylate pathway (Grimm & Harwood 1999), while those utilizing phenanthrene but not naphthalene degrade phenanthrene *via* the protocatechuate pathway (Saito et al. 2000). On the salicylate pathway, phenanthrene is converted to salicylate which is further transformed to catechol, and the latter is further degraded *via ortho*-cleavage pathway by C12O or *via meta*-cleavage pathway by C23O. In some cases, salicylate could be oxidized to gentisate which is further converted to maleylpyruvate by gentisate 1,2-dioxygenase (Fuenmayor et al. 1998). On the protocatechuate pathway, phenanthrene is converted to protocatechuate which is further transformed to 2-carboxy-*cis*, *cis*-muconate by protocatechuate 3,4-dioxygenase or 4-carboxy-2-hydroxymuconate semialdehyde by protocatechuate 4,5-dioxygenase. In this study, strain ZL5 exhibited C23O activity and no other enzyme activity above mentioned. The results suggested that *Sphingomonas* sp. strain ZL5 could degrade phenanthrene *via* the salicylate way, which differed from the protocatechuate way of *Alcaligenes faecelis* AFK2 and *Nocardioides* sp. strain KP7, also capable of growing on phenanthrene but not naphthalene. In most cases, the bacteria degrading phenanthrene could also convert naphthalene to salicylate by a shared catabolic pathway (Takizawa et al. 1994). The reason that strain ZL5 could not degrade naphthalene perhaps is due to the limitation of substrate specificity of initial dioxygenase, which is different from other ones involved in phenanthrene and naphthalene degradation.

Catabolic genes which encode PAHs degradation pathways are frequently located on plasmids, although some pathways can be located on chromosome. Many degradative plasmids responsible for PAHs degradation were isolated, such as plasmid NAH7 in *Pseudomonas putida* G7 (Dunn & Gunsalus 1973), the plasmid in *Alcaligenes fa-*

ecelis AFK2 (Kiyohara et al. 1982a), plasmid pNL1 in *Sphingomonas aromaticivorans* F199 (Romine et al. 1999), etc. Plasmid NAH7 encodes catabolic enzymes required for oxidative degradation of naphthalene *via* the salicylate pathway. The upper pathway operon converts naphthalene to salicylate, and the lower operon converts salicylate to acetyl-CoA (Grimm & Harwood 1999). In addition, NAH7 and NAH7-like plasmids could mediate metabolism of phenanthrene (Sanseverino et al. 1993). Genes for phenanthrene degradation *via* protocatechuate by *Alcaligenes faecelis* AFK2 were also located on its plasmid. Mitomycin C is an effective reagent for curing plasmid and has been used to cure 500 kb plasmid successfully (Cho & Kim 2001). A 60 kb plasmid pZL of strain ZL5 was cured by mitomycin C and the mutants lost the ability to degrade phenanthrene. It was a hint that pZL could be a degradative plasmid. *E. coli* DH5α could not grow on phenanthrene, however, it obtained the ability to grow on phenanthrene after plasmid pZL was introduced into it. Pattern of Southern blotting of the introduced plasmid is the same as that of plasmid pZL. The results further indicated that genes responsible for phenanthrene degradation *via* the salicylate pathway by strain ZL5 were located on plasmid pZL.

Extradiol dioxygenases play a key role in the metabolism of aromatic rings by the bacteria. C23O, one of extradiol dioxygenases, cleaving aromatic C—C bond at *meta* position of dihydroxylated aromatic substrates, catalyses the convention of catechol to 2-hydroxymuconic semialdehyde. In as much as C23O was extremely sensitive to oxygen, it was difficult to purify C23O from cultures of the wild-type strain (Kojima et al. 1961). It will be appropriate for enzymological study to overexpress the C23O gene in *E. coli* through gene engineering technology. We used pET-30a(+) as an expressing vector and achieved overexpression of C23O gene from strain ZL5 in *E. coli* JM109(DE3). The purified C23O from the recombinant *E. coli* exhibited the highest aromatic ring-fission activity to catechol as the substrate and much lower activity to 3,4-dihydroxyphenanthrene and 3-chlorocatechol. Its activity to catechol was about 20-fold higher than that to 3,4-dihydroxyphenanthrene. This indicated that the C23O preferentially cleaved dihydroxylated monocyclic substrates, and therefore, strain ZL5 contained other extradiol-type dioxygenases involved in aromatic ring-fission of dihydroxyphenanthrene formed as an intermediate of the initial catabolic pathway of phenanthrene. The deduced amino acid sequence of the C23O of strain ZL5 exhibited high homology with that of C23O from other *Sphingomonas* sp., while the homology is not more than 52% with that of C23O from *Pseudomonas* sp. This observation implied that genes for the catechol oxidative enzymes of *Sphingomonas* sp. and *Pseudomonas* sp. varied largely during the evolution or perhaps derived from different ancestral sequences although the two species belong to the class *Proteobacteria*.

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