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Characterization of a cytochrome P450 monooxygenase capable of high molecular weight PAHs oxidization from *Rhodococcus* sp. P14

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ABSTRACT

Rhodococcus sp. P14 is able to degrade a wide range of polycyclic aromatic hydrocarbons (PAHs). By analyzing its whole genome sequence, a gene cluster encoding cytochrome P450 monooxygenase (CYP108J1) with ferredoxin (fdx) and ferredoxin reductase (hcaD) relating to polycyclic aromatic hydrocarbons degradation was predicted. Protein sequence analysis of CYP108J1 showed 47.9% and 36.4% identity to the CYP108A1 and CYP108D1 from *Pseudomonas* sp. and *N. aromaticivorans* DSM12444 in CYP108 family, respectively. The transcriptional level of gene *cyp108j1* was up-regulated when the strain was grown within the medium containing benz[a]anthracene, pyrene, phenanthrene and anthracene as the sole carbon source, and the increment was detected to be 2.4, 8.0, 16.0 and 11.3-fold, respectively, by comparing to that grown with glucose. Further investigation on the recombinant protein CYP108J1 in *E. coli* also indicates that CYP108J1 was capable of degrading a series of PAHs compounds (from low to high molecular weight), including biphenyl, phenanthrene, anthracene and benz[a]anthracene.

1. Introduction

Recalcitrant polycyclic aromatic hydrocarbons (PAHs) are toxic environmental pollutants in the list of priority pollutants of the US Environmental Protection Agency (USEPA) [1,2]. Microbial biodegradation of PAHs has been extensively studied, in which the hydroxylation process of the aromatic rings by dioxygenases or cytochrome P450 monooxygenases is one of the common initial degradation steps [3].

Cytochrome P450 monooxygenases (P450s) are hemoproteins which show extraordinary diversity in their catalytic reaction process, including C—H bond hydroxylation, O-dealkylation and S-oxidation because P450 s can accept a various range of substrates [4]. Generally, P450 s have a highly conserved to topography structure related to the reacting mechanism for oxygen activation; however, the protein sequences from different P450 families are species-specific, and the similarity is even less than 20% [5]. In contrast to those conserved sequences, P450 s have many flexible substrate recognition regions, which enable P450 s to accept a wide range of substrates [6,7]. To initialize the catalytic reaction, P450 s usually are associated with one or two redox partner proteins as the component of electron transport chain [8,9], and P450 s catalyze the initial degradation step of PAHs to epoxide, which are converted to dihydrodiols by epoxide hydrolase, and further converted via dihydrodiol dehydrogenases into PAH catechols, the substrate for ring cleavage dioxygenase [10,11].

Rhodococcus sp. P14 was isolated from crude oil-contaminated sediments and it can utilize a wide range of PAHs as the sole source of carbon and energy [12]. Four different hydroxyphenanthrene products (1, 2, 3, or 4-hydroxyphenanthrene) were detected during *Rhodococcus* sp. P14 metabolism of phenanthrene [12], which suggested that monooxygenases might play an important role in the process of phenanthrene oxidative degradation in *Rhodococcus* sp. P14. Previous studies indicated that cytochrome P450 monooxygenases from fungal species can epoxidize low and high molecular weight PAHs [13–15] and cytochrome P450 monooxygenases from bacterial strains, such as CYP151 from *Mycobacterium vanbaalenii* PYR-1, CYP108D1 from *Novosphingobium aromaticivorans* DSM12444 and cytochrome P450 monooxygenase from *Rhodococcus* ruberDSM44319, can catalyze the epoxidation of low molecular weight PAHs (e.g., naphthalene) [16–18]; however, information of P450 from bacteria on catalyzing the epoxidation of high molecular weight PAHs (e.g., benz[a]anthracene) is extremely rare. In this study, one cytochrome P450 monooxygenase (CYP108J1) from *Rhodococcus* sp. P14, which is involved in the first step of low and high molecular weight PAHs catabolism, was identified and characterized. Real-time quantitative PCR (qPCR) was performed to compare the transcriptional differences induced by PAHs. The recombinant CYP108J1 expressed from *E. coli* BL21 (DE3) was also assessed on its ability to initialize the PAHs oxidization.

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Table 1
Primers used in this study.

Primer name	Primer sequence (5′ → 3′)
cyp108j1-RT-F	GGGCGACCGCTTCATGCTGCTGTAC
cyp108j1-RT-R	TAGCCGAAGGCGATGTGCTTGTTGG
16s-RT-F	GCACAAGCGGTGGAGCAT
16s-RT-R	AACCCAACATCTCACGACACGA
cyp108j1-F	CGCGGATCCGGTGAGCGCCGCAACGTACGGCGCTA
cyp108j1-R	CCCAAGCTTTTACCCGGTGAACTCGATCTTGACG
fdx-F	GGAATTCCATATGGTGACCGGCGACACAATGTGGAC
fdx-R	CCGGATATCACGGCGTCCGAAGGTGGCACGGTCACC
hcad-F	CGCGGATCCGGTGGTGATCGTCGGGGCCGGGCATG
hcad-R	CCCAAGCTTTCACGTACTCCATGTCTTGTGGGCC

2. Materials and methods

2.1. Chemicals and reagents

Biphenyl, phenanthrene, pyrene, anthracene, benz[a]anthracene, benzo[a]pyrene and other PAHs were purchased from Sigma-Aldrich (USA). Methanol and dichloromethane (HPLC grade) were purchased from Honeywell Inc (USA). RNAiso Plus, random primers, ribonuclease inhibitor, dNTP mixture, recombinant DNase I, SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus), T4 DNA ligase and the restriction enzymes were purchased from Takara Biotechnology Co. Ltd. (Dalian, China).

2.2. Bacteria strains, plasmids, and growth conditions

Rhodococcus sp. P14 (CGMCC NO.2343) used in this study was isolated from crude oil-contaminated sediments and maintained in our laboratory. *E. coli* BL21 was used for heterogonous expression of target protein. The plasmid T-Vector pMD™19 (TaKaRa, Dalian, China) was used for DNA cloning. The plasmids pETDuet™-1 and pACYCDuet™-1 (Novagen, WI, USA) were used for gene co-expression. Recombinant *E.coli* strains were cultivated at 37 °C in Luria-Bertani (LB) medium supplemented with the appropriate antibiotics. *Rhodococcus* sp. P14 was grown aerobically at 25 °C in 2216E medium [12] or in mineral basal medium (MBM) with 10 μg ml^{-1} PAHs.

2.3. RNA isolation and quantitative real-time PCR (qPCR)

Cells were grown on MBM supplemented with 10 μg ml^{-1} anthracene, benz[a]anthracene, pyrene, phenanthrene or glucose (negative control) as the sole carbon source at the mid-exponential phase (OD_{600nm} = 0.5). Total RNA was isolated by using the RNAiso Plus. RNase-free DNase I was used to remove the residual DNA. The purified RNA was determined by using a NanoDrop 2000c UV–vis spectrophotometer (Thermo Scientific, USA). Reverse transcription of RNA samples were performed according to the manufacturer's instructions.

qPCR was performed in 96-well plate on the ABI 7300 real-time PCR system (Perkin-Elmer Applied Biosystems, USA) using SYBR Premix Ex-Taq II (Takara Bio, Shiga, Japan) according to manufacturer's directions. Two primer sets were used for qPCR (Table 1). Primer *cyp108j1*-RT-F and *cyp108j1*-RT-R amplified the intergenic 114 bp sequence of *cyp108j1*, and the other primer set, *16s*-RT-F and *16s*-RT-R, was used to amplify 16S rDNA as a control. The final volume of PCR mixture was 20 μL, and the cycle threshold C_t of *cyp108J1* was normalized according to the C_t of 16S rDNA. Relative gene transcription levels were calculated using the $2^{-\triangle\triangle Ct}$ method by comparing to that using glucose as the sole carbon source [19]. Blank control without template was also set-up in all assays and each sample was analyzed in triplicate.

2.4. Construction of CYP108J1 monooxygenase co-expression system

Total DNA from *Rhodococcus* sp. P14 cells was extracted according to the method of Desomer [20]. Gene *cyp108j1*, *fdx* and *hcad*, encoding cytochrome P450 monooxygenase, ferredoxin and ferredoxin reductase, respectively, were amplified by primer sets *cyp108j1*-F/*cyp108j1*-R, *fdx*-F/*fdx*-R and *hcad*-F/*hcad*-R, which are listed in Table 1.

pETDuet-1 and pACYCDuet-1 expression system (Novagen, Madison, WI) were used for target gene cloning. Genes of *fdx* and *hcad* were both ligated into the plasmid pACYCDuet-1 to form plasmid pACYCDuet-*fdx-hcad*, and gene *cyp108j1* was ligated into the plasmid pETDuet-1 to form plasmid pETDuet-*cyp108j1*. The constructed plasmids then were transformed into *E. coli* BL21 (DE3) to establish two kinds of transformed cells, which were named as BL21-C (with pETDuet-*cyp108j1* only) and BL21-CFH (with both pACYCDuet-*fdx-hcad* and pETDuet- *cyp108j1*).

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) assays for recombinant protein expression

The transformed strains BL21-C and BL21-CFH were both activated overnight in 5 mL LB medium with ampicillin and chloramphenicol, respectively. *E. coli* BL21(DE3) containing pETDuet-1 and pACYCDuet-1 without inserts was used as the control. The overnight culture was transferred to 20 mL LB medium (0.1% inoculum) and cultivated at 37 °C to reach an OD_{600nm} of 0.5. Then the incubation temperature was lowered to 25 °C, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added into the culture with a final concentration of 0.5 mM for induction. The cells were harvested by centrifugation after incubation for 12 h at 25 °C. After washed with phosphate buffer (pH 7.4), the pellet was mixed with gel loading buffer, and analyzed with 12% SDS-PAGE. Protein bands were visualized by Coomassie brilliant blue R-250 staining [21].

2.6. Western blotting

Proteins located on the PAGE gel were electro-blotted onto 0.45 μm polyvinylidene fluoride (PVDF) membranes (Merck, Germany). The membranes were blocked by 5% skimmed milk powder for 1 h at 37 °C followed washed by 20 mM Tris base solution containing 0.1% Tween-20 and incubating with His-Tag (CYP108J1, hcaD) (0.1%) or S-Tag (fdx) (0.1%) for 2 h at 37 °C. After washed by Tris base-Tween 20 solution again, the membranes were further incubated with horseradish peroxidase (HRP) labeled goat anti-rat IgG or conjugated HRP-labeled goat anti-rabbit IgG. With the final washing step, the specific protein bands on the membranes were visualized with 0.03% (w/v) 3,3′-diaminobenzidine (DAB) substrate.

2.7. PAH metabolites analysis by GC–MS

Analysis of PAH oxidation metabolites was performed according to the method from Kim [22] with minor revision. The transformed cells were incubated, inducted by IPTG (0.5 mM) and harvested by centrifugation as described above. The collected pellets were washed by M9-glucose (M9) [22] medium for three times, and re-suspended in 25 mL of sterilized M9 medium supplemented with proper antibiotics. After adding biphenyl, phenanthrene, anthracene, benz[a]anthracene (10 μg ml^{-1} each) as the substrates, the cultures were incubated for 12 h at 25 °C. Cultures without cells were also set as the control. To evaluate the PAH metabolites, the supernatant was extracted with dichloromethane and dried by a termovap sample concentrator, and then the dried residues were dissolved in dichloromethane. The extracts were analyzed by GCMS-QP2010Plus (Shimadzu, Japan) with a DB-17MS

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