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Characterization of a polycyclic aromatic ring-hydroxylation dioxygenase from *Mycobacterium* sp. NJS-P

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HIGHLIGHTS

- A dioxygenase PdoAB capable of oxidizing benzo[*a*]pyrene.
- *In silico* molecular modeling analysis to characterization of PdoAB structure.
- Binding energy may predict the substrate conversion toward HMW PAHs.

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ABSTRACT

Ring-hydroxylating dioxygenases (RHDs) play a critical role in the biodegradation of polycyclic aromatic hydrocarbons (PAHs). In this study, genes *pdoAB* encoding a dioxygenase capable of oxidizing various PAHs with up to five-ring benzo[*a*]pyrene were cloned from *Mycobacterium* sp. NJS-P. The α-subunit of the PdoAB showed 99% and 93% identity to that from *Mycobacterium* sp. S65 and *Mycobacterium* sp. py136, respectively. An *Escherichia coli* expression experiment revealed that the enzyme is able to oxidize anthracene, phenanthrene, pyrene and benzo[*a*]pyrene, but not to fluoranthene and benzo[*a*]anthracene. Furthermore, the results of *in silico* analysis showed that PdoAB has a large substrate-binding pocket satisfying for accommodation of HMW PAHs, and suggested that the binding energy of intermolecular interaction may predict the substrate conversion of RHDs towards HMW PAHs, especially those may have steric constraints on the substrate-binding pocket, such as benzo[*a*]pyrene and benzo[*a*]anthracene.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) have become a matter of great concern due to the potential toxicity, mutagenicity and carcinogenicity (Fujikawa et al., 1993). The high-molecular-weight (HMW) PAHs with four or more fused benzene rings are more recalcitrant than low-molecular-weight (LMW) ones, persisting in the environment for a very long time (Shuttleworth and Cerniglia, 1995).

Microbial degradation and transformation are mainly responsible for natural attenuation of PAHs. In bacteria, extensive studies showed that LMW PAHs can be effectively mineralized to CO_2 and the catabolic pathways for the mineralization have been described in detail (Peng et al., 2008; Lu et al., 2011). By contrast, bacterial degradation to HMW PAHs is less understood. The relevant work on HMW PAHs was initially performed in 1988, in which *Mycobacterium vanbaalenii* PYR-1 was found to degrade 4-ring pyrene (Heitkamp and Cerniglia, 1988). The particular cell wall layer such as mycolic acids plays a vital role in mycobacterial degradation on hydrophobic PAHs (Abbasnezhad et al., 2011). Pyrene-degrading mycobacteria are capable of utilizing a broad range of PAHs, including naphthalene, phenanthrene, and fluoranthene as sole carbon source, and even five-ring benzo[*a*]pyrene via cometabolic

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biodegradation (Kweon et al., 2011). The catabolic versatility is attributed to the broad substrate specificity of the degradative enzyme they possessed (Kweon et al., 2014; Jouanneau et al., 2015). PAH ring-hydroxylating dioxygenases (PAH-RHDs) are crucial for PAHs degradation by aerobic bacteria, catalyzing the initial oxidation step by introduction of atoms of O_2 to formation of a *cis*-dihydrodiol and controlling the degradation rate (Kim et al., 2007; Chemerys et al., 2014). Numerous works showed that abundance and diversity of PAH-RHDs genes-carrying populations were usually related to PAHs contamination under natural conditions (Li et al., 2015; Yang et al., 2015).

Increasing attentions have been recently devoted to identification of RHDs for oxidizing HMW PAHs (Kweon et al., 2010; Wu et al., 2014; Li et al., 2015), allowing a closer look at pathways potentially useful in bioremediation of HMW PAHs. Furthermore, HMW PAH-RHDs will be valuable in construction of transgenic plants for phytoremediation. It was shown that the transgenic plants expressing PAH-RHDs exhibit enhanced PAHs tolerance and thus degrade PAHs more effectively (Peng et al., 2014). In this study, a gene encoding RHD capable of oxidizing HMW PAHs was cloned from a pyrene-degrading mycobacterium, and the catalytic function of this oxygenase toward 3 to 5 rings PAHs was examined. Despite that the enzyme highly related to a reported dioxygenase PdoAB from *Mycobacterium* sp. S65 (Sho et al., 2004), the substrate preference of this dioxygenase has not been studied yet. Besides, *in silico* molecular modeling analysis was performed to investigate structural characteristics of the RHD and its region-specificity toward various PAHs.

2. Materials and methods

2.1. Chemicals and media

Anthracene, phenanthrene, fluoranthene, pyrene, benzo[*a*]anthracene and benzo[*a*]pyrene were purchased from Sigma-Aldrich (Sigma, USA). 3-hydroxybenzo[*a*]pyrene is obtained from Toronto Research Chemicals, Inc. (Toronto, Canada). *N,O-bis* (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was obtained from Supelco (Bellefonte, USA). M9-glucose medium contained (per liter) 12.8 g of $Na_2HPO_4 \cdot 7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 0.4 g NH_4Cl, 0.24 g $MgSO_4$, 4 g glucose.

2.2. Bacterial strains and plasmids

Mycobacterium sp. NJS-P capable of utilizing pyrene as sole carbon and energy source was previously isolated from a PAH contaminated soil (Zeng et al., 2010). The strain was deposited in the China Center for Type Culture Collection with No. CCTCC M 2011011 as a reference. *Escherichia coli* JM109 and BL21(DE3) were used for gene cloning and expression experiments, respectively. Plasmid pBRCD for coexpressing electron carriers from *Nocardioides* sp. strain KP7 was kindly provided by Dr. Yves Jouanneau (Krivobok et al., 2003).

2.3. Gene cloning and construction of the plasmid for RHD overexpression

RHD gene in strain NJS-P was first detected by PCR with primers Nid-for and Nid-rev1 designing for amplification of *nidA*-like genes (Zhou et al., 2006). The PCR product was then purified and sent to be sequenced. The sequence was aligned with published sequences with NCBI BLASTN, showing high identity (99%) to *pdoA* of *Mycobacterium* sp. S65 (Fig. S1) (Brezna et al., 2003). The full length of *pdoAB* gene was obtained from the genomic DNA of *Mycobacterium* sp. NJS-P with primer pairs 5′-GTAT*CCATGG*GCAACGCGGTCGCGGTGGAC-3′/5′-ACG-*GATCC*TCATCGAGCACCGCCGCGGAACTG-3′, using high-fidelity DNA polymerase PrimerSTAR HS (Takara). The primers contained unique *Nco*I and *Bam*HI restriction site (italics) in the forward and backward primers, respectively. The amplified fragment was cloned into the p*EASY*-T1 simple vector (Transgen), and then excised with *Nco*I and *Bam*HI and re-ligated into pET15b (Novagen) by treating with the same restriction enzymes to generate *pdo*AB-15b. The final construct was sequenced to ensure that no mutation was incorporated during PCR.

2.4. Dioxygenase overexpression and in vivo assays

E. coli BL21 (DE3) co-transformed with *pdo*AB-15b and pBRCD was incubated at 37 °C by inoculating a colony in LB medium supplemented with ampicillin and gentamicin. When the culture reached an optical density of ~0.6 at 600 nm, it was induced by adding 0.5 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and further incubated overnight at 30 °C. The cells were then harvested by centrifugation, washed and resuspended in 100 mL M9-glucose medium supplemented with ampicillin and gentamicin. Cells (25 mL) were then incubated for 48 h at 30 °C with 5 mL silicone oil which contained single PAHs. The tested PAHs include 100 mg L^{-1} of anthracene, phenanthrene, pyrene, fluoranthene and benzo[*a*]anthracene, and 50 mg L^{-1} of benzo[*a*]pyrene.

After incubation, hydrosoluble products were analyzed for each sample of aqueous phase which were centrifuged and passed through solid-phase extraction column. The columns were washed with 10 mL water and eluted with 1 mL ethyl acetate; then the solvents were dried over sodium sulfate and evaporated under nitrogen gas. The dried extracts were dissolved in 100 μL hexane before derivatization with BSTFA containing 1% TMCS, and then trimethylsilyl (TMS) derivatives were analyzed by gas chromatography - mass spectrometry (GC/MS) analysis. The GC peak areas of parent PAHs and the hydroxylation metabolites in each extract were recorded for calculating relative conversion rate, represented as the ratio of the metabolite to its parent compound.

2.5. Analytical methods

A Varian CP3800 gas chromatograph coupled to a Saturn 2200 ion-trap mass spectrometer (Agilent, Paolo Alto, CA, USA) equipped with a Varian CP-8 column (Agilent, Paolo Alto, CA, USA) (30 m × 0.25 mm, 0.25 μm), was used for analysis the products in the extracts of PAHs excluding of benzo[*a*]pyrene. It was programmed from 80 to 260 °C at 5 °C min^{-1}, with a final hold time of 27 min. Due to that both of benzo[*a*]pyrene and TMS derivative of 3-hydroxybenzo[*a*]pyrene were hardly detected using Varian CP3800/Saturn 2200 system, the products from benzo[*a*]pyrene culture was analyzed on the Agilent 7890A system coupled with a 5975c quadrupole mass spectrometry (Agilent, Paolo Alto, CA, USA) which was equipped with J&W HP-5 ms capillary GC column (Agilent, Paolo Alto, CA, USA) (30 m × 0.25 mm, 0.25 μm). It was programmed from 50 to 200 °C at 25 °C min^{-1} and from 200 to 283 °C at 8 °C min^{-1}, and then increased to 290 °C at a rate of 2 °C min^{-1} with a final hold time of 2 min. The mass spectrometer was operated in the selected ion-monitoring mode, selecting *m/z* values corresponding to the expected masses (M^+) of the derivatives of hydroxylation products.

2.6. In silico analysis

The Swiss-Model server (Schwede, 2003) was used to generate homology model for the α-subunit of mycobacterial PAH-RHDs, including PdoAB, PodA1B1, NidAB, NidA3B3 and PdoA2B2, using the naphthalene dioxygenase (PDB accession code 2B24) from *Rhodococcus* sp. strain NCIMB 12038 as a template (Gakhar et al.,