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Triphenyltin degradation and proteomic response by an engineered *Escherichia coli* expressing cytochrome P450 enzyme

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ABSTRACT

Although triphenyltin (TPT) degradation pathway has been determined, information about the enzyme and protein networks involved was severely limited. To this end, a cytochrome P450 hydroxylase (CYP450) gene from *Bacillus thuringiensis* was cloned and expressed in *Escherichia coli* BL21 (DE3), namely *E. coli* pET32a-CYP450, whose dosage at 1 g L^{-1} could degrade 54.6% TPT at 1 mg L^{-1} within 6 d through attacking the carbon-tin bonds of TPT by CYP450. Sequence analysis verified that the CYP450 gene had a 1214 bp open reading frame, encoding a protein with 404 amino acids. Proteomic analysis determined that 60 proteins were significantly differentially regulated expression in *E. coli* pET32a-CYP450 after TPT degradation. The up-regulated proteins enriched in a network related to transport, cell division, biosynthesis of amino acids and secondary metabolites, and microbial metabolism in diverse environments. The current findings demonstrated for the first time that P450 received electrons transferring from NADH could effectively cleave carbon-metal bonds.

1. Introduction

Bacterial cytochrome P450s belong to the superfamily of proteins with a conserved heme-iron center, catalyzing various molecules as substrates in enzymatic reactions, which made them attractive as potential catalysts for chemical reactions in industries (Grogan, 2011) and environmental remediation. Regarding pollutant degradation, those approved reactions included hydroxylation, oxidation (Ballesteros-Gomez et al., 2015; Frank et al., 2014; Whitehouse et al., 2012), denitrification (Shinkai et al., 2016), sulfoxidation (Renard et al., 2014), N-dealkylation (Roberts et al., 2016), cyclopropanation (Coelho et al., 2013) and intramolecular sp^3 C-H amination (Singh et al., 2014). The metabolism pathways of xenobiotics by cytochrome P450, including benzopyrene, naphthalene, aflatoxin, trichloroethylene, dimethylbenzanthracene, bromobenzene, nitronaphthalene and 1,1-dichloroethylene, have been summarized been summarized in the kegg database (http://www.kegg.jp/dbget-bin/www_bget?map00980). However, the transformation of organometallic compounds with carbon-metal bonds that have characters in between ionic and covalent, and the cleavage of these bonds by P450 are still not clear. The elucidation of these reactions will extend the earlier findings about P450 catalysis, and exhibit insights into the biodegradation of compounds with carbon-metal bonds.

As an organometallic compound, triphenyltin (TPT) has been extensively used as an active component of herbicides, disinfectants, biocides, antifouling paints and plastic catalysts (Antes et al., 2011; Renard et al., 2014). However, its high toxicity to various invertebrates and vertebrates has been approved, seriously disturbing the endocrine system, preventing enzyme expression and causing reproductive problem (Graceli et al., 2013; Harada et al., 2015; Zuo et al., 2014). Except for its toxicity, the biodegradation of TPT has also been investigated. The results certified that TPT dephenylation was related to the cellular metabolism of ions, carbohydrates and organic acids (Gao et al., 2014). To further reveal the mechanism of TPT degradation by the effective microbes, it is vital to study the response of cellular proteome rather than only focus on the expression of a single effective enzyme because the cellular metabolic pathways associated with TPT degradation are regulated by the entire set of proteins and their networks.

Among the effective microbes selected for TPT degradation, *Bacillus thuringiensis* is a Gram-positive bacterium, commonly used as a biopesticide, which has been proved without negative impact on human, wildlife, pollinators and most other beneficial insects (Lu et al., 2012; Melo et al., 2016). Apart from insect control, *B. thuringiensis* was also used to degrade pollutants, such as dimethyl phthalate (Brar et al.,

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2009), fipronil (Mandal et al., 2013) and TPT (Huang et al., 2014). Metabolite analysis confirmed that TPT was degraded through the cleavage of the Sn-C bonds producing diphenyltin, monophenyltin and tin, respectively (Huang et al., 2014). Based on these degradation products, and the reactions catalyzed by P450 (Arnold, 2015; Ren, et al., 2016) and genomic analysis of *B. thuringiensis*, P450 expressed in this strain was speculated to be the enzyme for TPT degradation.

To prove the above hypothesis, CYP450 gene was cloned and expressed in *Escherichia coli* BL21 (DE3), the recombinant host for CYP450 expression (Biggs et al., 2016; Colthart et al., 2016). The insight into the interaction among P450, cellular proteome and TPT degradation was investigated through an iTRAQ based quantitative proteomic technology.

2. Materials and methods

2.1. Strains and chemicals

B. thuringiensis GIMCC1.817 was an effective strain for TPT degradation (Tang et al., 2016) and was stored at the Microbiology Culture Center of Guangdong Province, China. PUCm-T and pET32a vectors were used for gene cloning and expression, respectively. PET32a-CYP450 was an expression vector containing CYP450. *E. coli* pET32a-CYP450 and *E. coli* pET32a were the expression strains containing CYP450 and an empty plasmid without CYP450, separately. Triphenyltin chloride (purity = 98.8%) was obtained from Sigma Aldrich (St. Louis, MO, USA). Lysogeny broth (LB) medium consisted of (in g L^{-1}) 10 tryptone, 5 yeast extract and 5 NaCl was used to culture *B. thuringiensis* and *E. coli*. The mineral salt medium (MSM) for TPT degradation contained (in mg L^{-1}) 150 $Na_2HPO_4{\cdot}12H_2O$, 50 KH_2PO_4, 30 NH_4Cl, 5 $Zn(NO_3)_2$ and 5 $MgSO_4$ (Huang et al., 2014).

2.2. Cloning of cytochrome P450 hydroxylase gene

The genomic DNA of *B. thuringiensis* was used as the template for PCR amplification. The coding region of CYP450 gene was amplified by PCR with the specific primers, CYP450F (5′-GGGGGATCCATGGCTTCACCTGAAAAT-3′) and CYP450R (5′-CCCCTCGAGTTATTTAGCTTTCAATCGAATAGG-3′), which were designed according to the complete genome of *B. thuringiensis*. PCR was performed at 94 °C for 4 min, followed by 30 cycles at 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min, and 72 °C for 10 min as a final extension step by using a thermal cycler. The PCR product was purified, linked to the pUCm-T vector and sequenced. Homology searches of nucleotide sequences were finished by BLAST at NCBI.

2.3. Expression of CYP450 gene in E. coli

The above PCR product was purified by gel extraction kit, digested with restriction enzymes BamHI and XhoI, and then ligated into pET32a(+) vector which was digested with the same enzymes to form the recombinant expression plasmid pET32a-CYP450. The recombinant vector pET32a-CYP450 was transferred into *E. coli* BL21 (DE3) for protein expression. Meanwhile empty plasmid without CYP450 was also transferred into *E. coli* BL21 (DE3) competent cells. *E. coli* pET32a-CYP450 and *E. coli* pET32a were separately grown at 37 °C in 200 mL LB medium containing 100 mg L^{-1} ampicillin at 180 r min^{-1}. When the optical density at 600 nm of the medium was about 0.6–0.8, isopropyl-β-D-thiogalactoside was added at a final concentration of 1 mM, and the mixture was further cultured at 37 °C for 4 h. SDS-PAGE was performed using 12.5% separation gel and 4.5% stacking gel. Protein bands were stained with coomassie brilliant blue R-250. Zymogram analysis was performed according to the method used by Lee et al. (2007).

2.4. TPT degradation experiments

E. coli pET32a-CYP450 and *E. coli* pET32a were inoculated into the culture medium at 37 °C on a rotary shaker at 130 r min^{-1}, respectively. After then, the cells were separated from the medium by centrifugation at 3500 *g* for 10 min and washed three times with sterile distilled water before use in the further experiments. Biodegradation of TPT at 1 mg L^{-1} by 1 g L^{-1} *E. coli* pET32a-CYP450 or *E. coli* pET32a was performed in 20 mL MSM at 30 °C on a rotary shaker at 130 r min^{-1} for 1–6 d. Three samples for each experiment were taken and the mean values were used in calculations. After degradation, 10 mL hexane was added into the MSM. The mixture was sonicated for 20 min in an ultrasonic bath. After the organic phase was collected, 10 mL hexane was added into the aqueous phase to extract TPT again. The collected organic part was concentrated using a rotary evaporator at 30 °C. Subsequently, the residues were dissolved by 5 mL methanol and derivatized in pH 4.5 acetate buffer with 2 mL of 2% sodium diethyl dithiocarbonate. TPT was analyzed according to previously published methods (Ye et al., 2013) by gas chromatography-mass spectrometry (GC-MS, 7890/5975 C, Agilent Technologies, Santa Clara, CA, USA) equipped with an Rxi-5MS GC column (30 m × 0.25 mm × 0.25 μm). Briefly, helium at 1.1 mL min^{-1} was used as the carrier gas. The column temperature program started at 50 °C for 1.5 min. Subsequently, the oven was heated to 300 °C at an efficiency of 10 °C min^{-1} for 4 min. The solvent cut time was set to 2.6 min. Mass spectra were recorded at 1 scan s^{-1} under electronic impact with electron energy of 70 eV, and mass ranged 55–650 atoms to mass unit. The detection limit of TPT was 250 ng L^{-1}.

2.5. Protein preparation and digestion

The cells of *E. coli* pET32a-CYP450 before and after degradation were suspended in 1 mL lysis buffer added with 0.2 g L^{-1} phenylmethylsulfonyl fluoride, 2% v/v IPG buffer and 0.6 g L^{-1} DTT (Ou, et al., 2017). The samples were frozen in liquid nitrogen thrice for 15 min per time, and subsequently treated by ultrasonication for 20 min. Nuclease was added to the lysate at a final concentration of 1% v/v. After the mixture was incubated at 4 °C for 30 min, the cell debris was removed at 4 °C by centrifugation at 13500 *g* for 1 h. The resultant proteins from each sample were reduced by 10 mM DTT for 1 h at 37 °C, and blocked with 1 μL cysteine blocking reagent for 10 min at room temperature. Subsequently, the samples were added in 10 KD Amicon Ultra-0.5 centrifugal filter devices, and centrifuged at 12000 *g* for 20 min. The proteins in filter devices were digested by 50 μL trypsin (Promega, V5280, USA) at 4% w/w overnight at 37 °C. After centrifugation, 1 μg trypsin was added to each filter for 2 h. The concentration of tryptic peptides in the liquid collection tube was measured using the Bradford method.

2.6. ITRAQ labeling and desalination

Tryptic peptides were labeled with an iTRAQ reagent multiplex kit (Sigma, PN 4352135, USA) according to the manufacturer's instructions. Briefly, the contents of an iTRAQ® Reagent vial was transferred to a sample tube, and incubated at room temperature for 1 h. Subsequently, 100 μL water was added to each sample to stop the reaction. After determination of the labeling efficiency by ABI 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA), the iTRAQ-labeled samples were mixed, centrifuged, desalinated with Strata-X (Phenomenex, USA), separated by strong cation exchange chromatography (SCX), and dried in a vacuum concentrator, respectively. The samples were then resolved with solution (2% v/v acetonitrile, 0.1% v/v formic acid), centrifuged at 12000 r min^{-1} for 20 min and detected by an AB SCIEX Triple TOF 5600 mass spectrometer (AB SCIEX, Framingham, MA, USA) equipped with a Nanospray III source (AB SCIEX) using the following parameter settings (Cui et al., 2015): spray voltage, 2.3 kV; sheath gas (nitrogen) pressure, 30 psi; collision