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Direct evidences on bacterial growth pattern regulating pyrene degradation pathway and genotypic dioxygenase expression

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ABSTRACT

Pyrene degradation by *Mycobacterium* sp. strain A1-PYR was investigated in the presence of nutrient broth, phenanthrene and fluoranthene, respectively. Fast bacterial growth in the nutrient broth considerably enhanced pyrene degradation rate, whereas degradation efficiency *per* cell was substantially decreased. The addition of nutrient broth could not alter the transcription levels of all dioxygenase genotypes. In the PAH-only substrates, bacterial growth completely relied on biological conversion of PAHs into the effective carbon sources, which led to a higher degradation efficiency of pyrene *per* cell than the case of nutrient broth. Significant correlations were only observed between *nid*A-related dioxygenase expression and pyrene degradation or bacterial growth. The highest pyrene degradation rate in the presence of phenanthrene was consistent with the highest transcription level of *nid*A and 4,5-pyrenediol as the sole initial metabolite. This study reveals that bacterial growth requirement can invigorate degradation of PAHs by regulating metabolic pathway and genotypic enzyme expression.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the environment. This group of compounds are toxic to human beings due to high carcinogenicity, mutagenicity and teratogenicity (Mastral and Callén, 2000). In the past decades, a large variety of bacterial strains that are able to degrade PAHs have been successfully isolated from highly PAH-contaminated environments (Bacosa and Inoue, 2014). Physiological properties and genomic information of these microbial degraders, including catabolic pathways, and key genes and enzymes related to PAH degradation, have been well characterized (Chauhan et al., 2008; Di Gennaro et al., 2011; Peng et al., 2008; Seo et al., 2009).

Microbes are generally posed at a more complex situation in the real environments than in the degradation assays, such as the concurrence of biodegradable and recalcitrant PAHs (Chen et al., 2006; Zhou et al., 2012). Cometabolism is considered as one of important strategies for the microbe to cope with recalcitrant pollutants (Juhasz and Naidu, 2000; Luo et al., 2014; Sahinkaya and Dilek, 2006). It refers to the phenomenon that microorganisms remove the non-growth and intractable pollutants in the presence of easily-assimilating growth substrates as the energy and carbon supplier (Nzila, 2013). In recent years, Tobajas et al. reported that 4-chlorophenol degradation by *Comamonas testosteroni* was enhanced by phenol or glucose (Tobajas et al., 2012). Cometabolism is frequently found for microbial removal of PAHs, e.g., pyrene and anthracene could improve fluoranthene degradation by two strains (*Mycobacterium flavescens* and *Rhodococcus* sp.) isolated from contaminated sediments (Dean-Ross et al., 2002). Nevertheless, it was also exhibited that the supplementation of nutrient broth substantially suppressed microbial mineralization of phenanthrene and pyrene (Carmichael and Pfaender, 1997). Contradictory phenomena highlight the needs to elucidate the exact molecular mechanisms responsible for the cometabolism of PAHs in the microbe.

Microbial transformation of PAHs was initiated by a dioxygenation process, in which the ring hydroxylating dioxygenase (RHD) is key for oxidizing PAHs *via* the addition of two hydroxyl groups (Cerniglia, 1992; Kauppi et al., 1998). The α subunit of RHD contains a conserved region with the function of electron transport. Different genotypes of RHD α subunit have been found in a large variety of microorganisms, such as *nahAc* in *Pseudomonas putida* G7, *phdAc* in *Comamonas testosteroni* GZ39, and *phnAc* in *Burkholderia* sp. RP007 (Dunn and Gunsalus, 1973; Goyal and Zylstra, 1996; Laurie and Lloyd-Jones, 1999). With respect to *Mycobacterium* genus, *nid*A is the most common RHD α subunit genotype, and its enzyme product is important to pyrene degradation by some strains of *Mycobacterium* (Khan et al., 2001). Another two genotypes of RHD α subunit, *pdo*A2 and *nid*A3, are often carried by *Mycobacterium* genus (Krivobok et al., 2003; Kweon et al., 2010). However, it has yet to be known how genotypic dioxygenases are transcribed consistently or differentially in response to the addition of extra substrates, which could be in particular helpful to fully understanding of cometabolic mechanism of PAHs in the microbe.

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PYR degradation by *Mycobacterium* sp. has been extensively described (Kim et al., 2007; Krivobok et al., 2003; Laurie and Lloyd-Jones, 1999; Sho et al., 2004; Su et al., 2012; Zhong et al., 2006), whereas PYR cometabolism with other substrates (e.g., degradable PAHs) that more truly reflected their concurrence in the real environments than single PAH degradation is far beyond our knowledge. The interactions between easily and poorly degradable PAHs, including cometabolism, inhibition and cross-acclimation, could be reflected by the profiles of PYR metabolites and genotypic enzymes that pertain to certain degradation pathways (Carmichael and Pfaender, 1997; DeBruyn et al., 2012; Kim et al., 2007; Kweon et al., 2014; Zhong et al., 2006). The objective of this study is to uncover inherent links among bacterial growth patterns, degradation pathway and genotypic enzyme expression, which might be insightful into the molecular mechanism of PYR cometabolism with other growth substrates.

2. Materials and methods

2.1. Chemicals and materials

Phenanthrene (PHE), fluoranthene (FLA), pyrene (PYR), *m*-terphenyl, 1,2-naphthalenediol, 1-hydroxy-2-naphthoic acid, 1-pyrenol, 9,10-phenanthroquinone and [bis(trimethylsilyl)trifluoroacetamide (BSTFA), the derivatization reagent] were purchased from Sigma-Aldrich (St. Louis, MO). Four phenanthrols (2-,3-,4-phenanthrols and 3-phenanthrol-D_9) were purchased from Dr. Ehrenstorfer (Augsburg).

The mineral salts medium (MSM) was composed of $(NH_4)_2SO_4$ (1000 mg L^{-1}), $MgSO_4 \cdot 7H_2O$ (200 mg L^{-1}), $FeSO_4 \cdot 7H_2O$ (12 mg L^{-1}), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (1 mg L^{-1}), $ZnSO_4 \cdot 7H_2O$ (3 mg L^{-1}), $MnSO_4 \cdot H_2O$ (3 mg L^{-1}), $CoSO_4 \cdot 7H_2O$ (1 mg L^{-1}), $CaCl_2$ (86 mg L^{-1}), and NaCl (20 g L^{-1}). The salinity of MSM was approximately 20‰, and was consistent with that in natural mangrove wetland. All chemicals were prepared in 0.05 M Phosphate buffered saline (PBS) (pH = 7.2). A 25-mL aliquot of MSM was added in a 100-mL conical flask and sterilized at 121 °C for 20 min.

2.2. Microorganism and culture conditions

Mycobacterium sp. strain A1-PYR was isolated from surface mangrove sediments in the Futian National Nature Reserve, Shenzhen, China using pyrene as the sole carbon source (Zhong et al., 2006). This strain is capable of utilizing phenanthrene, fluoranthene or pyrene as the sole carbon and energy sources (Zhong et al., 2011, 2006). The bacteria were incubated at 30 °C and 150 rpm in nutrient broth (NB, which contains 10 g of peptone, 3 g of yeast extract and 5 g of NaCl in 1000 mL of distilled water) until the late exponential phase. Bacterial culture was centrifuged at 7070*g* for 10 min, and washed twice with sterilized NaCl (0.85%). Cell pellet was resuspended in NaCl solution with a final optical density of 1.0 at the wavelength of 600 nm.

A batch of assays were carried out to study the cometabolism of PYR by *M.* sp. strain A1-PYR in the presence of PHE, FLA or NB as growth substrates. Stock solutions of PYR, PHE and FLA (1000 mg L^{-1}) were prepared in the acetone. Stock solution of PYR (250 μL) was mixed with 25 mL of MSM, and the final concentration of PYR was 10 mg L^{-1}. Thereafter, PHE (250 μL, 1000 mg L^{-1}), FLA (250 μL, 1000 mg L^{-1}) or NB (0.45 g) was added as the growth substrates, respectively. The acetone was evaporated by shaking the flasks on a rotary shaker at 150 rpm for about 12 h. The effect of acetone residue on bacterial growth was considered to be negligible as reported in our previous study (Zhong et al., 2006). Cell suspension (500 μL, 1.04×10^9 CFU mL^{-1}) was then inoculated to each of flasks. All flasks were incubated on the rotary shaker (150 rpm) at 30 °C for 7 days in the dark. Triplicate samples (200 μL) were collected respectively from each flask (containing 25 mL medium) at days 0, 1, 3, 5, and 7 for counting cell number and the remainder for analyzing residual PAH concentrations. Bacterial growth rate was determined by counting colony forming units (CFU) on Luria-Bertani agar plates using a series of 10-fold dilution with 0.85% NaCl. The samples were also analyzed for the concentrations of various PAH metabolites and the relative transcription levels of dioxygenase-related genes.

2.3. Synthesis of orthopyrenediol

The standards of 1,2-pyrenediol and 4,5-pyrenediol were synthesized in the form of catecholdiacetate pyrene according to the modified procedures reported in the literatures (Venkataramana et al., 2011; Wu et al., 2010). Prior to use, pyrene catecholdiacetates were saponificated into pyrenediols. The details were provided in the SI.

2.4. Analysis of PAHs

PAHs were extracted from the samples using ethyl acetate following the procedure described in the literature (Tam et al., 2002). A 250-μL aliquot of *m*-terphenyl (1000 mg L^{-1}) was added in each of samples as the internal standard prior to extraction, and its final concentration was 10 mg L^{-1}. Gas chromatography coupled with mass spectrometry (GC–MS) (7890A and 5975C, Agilent Technologies, Palo Alto, CA, USA) was used to determine the concentrations of PAHs. Chromatographic separation of PAHs and their metabolites was achieved on an HP-5 fused silica capillary column (length, 30 m; i.d., 0.25 mm; film thickness, 0.25 μm). The oven temperature program was as follows: 80 °C for 2 min, increasing to 160 °C at the rate of 15 °C min^{-1}, then to 220 °C at the rate of 4 °C min^{-1}, finally to 300 °C at the rate of 20 °C min^{-1} and holding at 300 °C for 10 min. The temperature of injector inlet and mass detector was 280 °C. Helium was used as carrier gas, and its flow rate was 1.0 mL min^{-1} (Zhong et al., 2006). Recovery rates of PAHs were obtained by spiking PAHs into the pure MSM with a final concentration of 10 mg L^{-1}. Recovery rates of PYR and its metabolites were greater than 90%.

2.5. Analysis of PAH metabolites

The medium was centrifuged at 11,780*g* at 4 °C for 10 min. An 8-mL aliquot of supernatant was transferred to a 10-mL brown screw vial with iron cap. The rest of the supernatant was discarded, and cell pellet was collected to measure the transcription levels of dioxygenase-encoded genes. The 3-Phenanthrol-D_9 was used as internal standard to calculate the recovery rates of PAH metabolites. PAH metabolites were analyzed using GC–MS and identified following the reported method (Luan et al., 2007). An 85-μm polyacrylate fiber (Supelco, Bellefonte, PA, USA) with the length of 2 cm was directly immersed in the solution for the extraction and enrichment of PAH metabolites. On-fiber silylation solid-phase microextraction (SPME) was automatically performed using a Multipurpose Autosampler MPS2 (Gerstel, München, Germany).

2.6. Identification of dioxygenase-encoded genes

Bacteria cells in the medium were harvested by centrifugation at 9425*g* for 10 min. DNA was then extracted using the E.Z.N.A. Bacteria DNA Kit (Omega, Norcross, GA, USA) according to the manuals. Primers specific to different genotypes of ring hydroxylating dioxygenase α subunit (*nid*A, *pdo*A2 and *nid*A3 genes) were synthesized by Invitrogen (Shanghai, China) according to reference sequences in the previous studies (Table S1, Supplementary Information) (DeBruyn et al., 2012; Miller et al., 2004; Pagnout et al., 2007).

Traditional PCR reactions were conducted in a 25-μL solution containing 1 μL of dNTP mixture (2.5 mM), 2.5 μL of a 10× PCR buffer (Mg^{2+} Plus), 1 μL of each primer (20 μM), 0.25 μL of Taq polymerase (5 U/μL) (TaKaRa Biotechnology, China), and 1.25 μL of DNA template. Thermal cycling conditions for PCR amplification were as follows: initially denaturing at 94 °C for 5 min, followed by 40 cycles of denaturing