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Phenanthrene degradation in *Arthrobacter* sp. P1-1: Initial 1,2-, 3,4- and 9,10-dioxygenation, and *meta*- and *ortho*-cleavages of naphthalene-1,2-diol after its formation from naphthalene-1,2-dicarboxylic acid and hydroxyl naphthoic acids

Jong-Su Seo, Young-Soo Keum, Yuting Hu, Sung-Eun Lee, Qing X. Li *

Department of Molecular Biosciences and Bioengineering, University of Hawaii, 1955 East–West Road, Honolulu, HI 96822, USA

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Abstract

Arthrobacter sp. P1-1, isolated from a polycyclic aromatic hydrocarbon (PAH)-contaminated site in Hilo, HI, USA, can decompose phenanthrene (40 mg l^{-1}) completely within 7 days. A detailed phenanthrene metabolism map was constructed based on metabolite analysis and replacement cultures. Initial dioxygenation occurs on 1,2-, 3,4-, and 9,10-C of phenanthrene, dominantly on 3,4-C positions. Rapid accumulation of 5,6- and 7,8-benzocoumarin suggests that phenanthrene-1,2- and -3,4-diols mainly undergo *meta*-cleavage. However, a trace amount of *o*-carboxyvinylnaphthoates and diphenic acid indicates a limited extent of *ortho*-cleavage of the diols. Naphthalene-1,2-diol, as a common and converged metabolite, was formed from 1-[(*E*)-2-carboxyvinyl]-2-naphthoic acid, naphthalene-1,2-dicarboxylic acid, and 1-hydroxy-2-naphthoic acid in separate culture tests. Naphthalene-1,2-diol is then degraded in a dominant phthalic acid pathway and a minor salicylic acid pathway. Several metabolites of phthalic acid were found, while no salicylic acid metabolites were detected. The strain P1-1 likely has a very diverse set of PAH-degrading enzymes or the enzymes having relaxed substrate-specificity.
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Keywords: *Arthrobacter*; Biodegradation; Dioxygenation; Carboxyvinylnaphthoate; *ortho*-Cleavage; *meta*-Cleavage; Phenanthrene

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are global pollutants, of which the sources are highly related with industrial activities. Because of their toxicological potency and resistance to degradation, PAHs pose a serious risk to the environment and human health (Atsushi et al., 1998). Research efforts on the remediation of PAHs include various types of chemical, physical, and biological methods (Guieysse et al., 2004; N'Guessan et al., 2004). It is well known that microorganisms play an important role in the degradation of persistent organic pollutants in the environment. A large number of bacterial species are isolated and characterized as efficient PAH degraders (Pinyakong et al., 2000; Kim et al., 2005; Keum et al., 2006; Seo et al., 2006). Among such bacteria, several *Arthrobacter* species can decompose a variety of contaminants including PAHs, pesticides, phenols, and polychlorinated biphenyls (PCBs) (Furukawa and Chakrabarty, 1982; Kohler et al., 1988; Havel and Reineke, 1993; Hayatsu et al., 1999; Daane et al., 2001; Negrete-Raymond et al., 2003). However, detailed catabolic researches of PAHs are still limited within this genus (Samanta et al., 1999).

This study was focused on the isolation and identification of metabolites of phenanthrene. In addition to the

* Corresponding author. Tel.: +1 808 956 2011; fax: +1 808 956 3542.
E-mail address: qingl@hawaii.edu (Q.X. Li).

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metabolic pathways reported in the literature, new catabolic pathways of phenanthrene by strain P1-1 were found in this study. Catabolic pathways were proposed based on metabolites identified using replacement culture and metabolite standards.

2. Materials and methods

2.1. Chemicals

Phenanthrene (>98% purity), diphenic acid, 1-hydroxy-2-naphthoic acid, 2-hydroxy-1-naphthoic acid, 2-carboxycinnamic acid, phthalic acid, 2-formylbenzoic acid, and protocatechuic acid were purchased from Sigma–Aldrich (Milwaukee, WI). Salicylic acid, gentisic acid, and catechol were obtained from Fisher Scientific (Morris Plains, NJ). 1-Hydroxy-2-naphthaldehyde, 2-hydroxy-1-naphthaldehyde, naphthalene-1,2-dicarboxylic acid anhydride, and naphthalene-1,2-diol were purchased from TCI America (Portland, OR). 5,6- and 7,8-Benzocoumarin, 2-[(*E*)-2-carboxyvinyl]-1-naphthoic acid, 1-[(*E*)-2-carboxyvinyl]-2-naphthoic acid, *trans*-4-(2-hydroxy-1-naphthyl)-2-oxobut-3-enoic acid, and *trans*-4-(1-hydroxy-2-naphthyl)-2-oxobut-3-enoic acid that were not commercially available were previously synthesized (Keum et al., 2005). *cis*-Phenanthrene-9,10-dihydrodiol was synthesized according to the method of Shing et al. (1994). Naphthalene-1,2-dicarboxylic acid was prepared by basic hydrolysis of naphthalene-1,2-dicarboxylic acid anhydride. Ethyl acetate and the other solvents were purchased from Fisher Scientific (Morris Plains, NJ). The carboxyl and phenolic metabolite standards were methylated into corresponding methyl esters or ethers with diazomethane that was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide in a diazomethane generator (Aldrich).

2.2. Isolation and identification of the phenanthrene-degrading strain

A PAH-contaminated soil collected from Hilo, HI, USA was incubated in minimal medium (MM) (1 g soil per 25 ml MM) for 3 months after addition of solid phenanthrene (1 mg ml^{-1}). The MM formula was adapted from Bastiaens et al. (2000) in this study. After 3 months, strain P1-1 was isolated via a classical shaken liquid medium enrichment method (Bastiaens et al., 2000). Strain P1-1 was identified by 16 S rDNA sequence analysis as follows. Genomic DNA was extracted with a mixture of phenol and chloroform (Marmur, 1961). The 16S rRNA gene was amplified from the genomic DNA (100–200 ng l^{-1}) by polymerase chain reaction (PCR) with *Taq* DNA polymerase (Takara Mirus Bio, Inc.) and primers 27F and 1492R (Lane, 1991). Amplification was performed with a Techne thermal cycler (Techne, Inc., Burlington, NJ) at 95 °C for 4 min followed by 30 cycles of 95 °C for 1 min, 55 °C for 50 s and 72 °C for 1.5 min, and a final elongation step at 72 °C for 7 min. The PCR product was purified with the Ultraclean PCR purification kit (Mo Bio Lab, Inc., Carlsbad, CA), and sequenced in both directions on an Applied Biosystems 377XL DNA sequencer. 16 S rDNA sequences were manually edited and assembled in Sequencher and Seqman (DNASTAR). Assembled sequence (1437 bases) was compared with that in the public domain through a BLASTn search (Altschul et al., 1997). Strain P1-1 (GenBank accession no. AY943390) was identified as *Arthrobacter* sp. through the comparison of 16S rRNA gene sequence. The closest neighbor was *Arthrobacter* sp. BS20 (AY452081) with 99% identity.

2.3. Biodegradation kinetics of phenanthrene

The bacterial cells were pre-grown in phenanthrene-supplied MM to an optical density of 0.2 at 540 nm. A 200-μl of phenanthrene stock solution (1000 mg l^{-1}) was placed into a sterilized culture tube. After the solvent was evaporated with nitrogen gas, the MM (4 ml) and pre-grown cells (1 ml) were added. The culture tubes were incubated (28 °C, 150 rpm in the dark), and extracted, at various time intervals, with ethyl acetate (5 ml) for GC-FID determination of concentration of remaining phenanthrene after degradation. The controls containing phenanthrene were cultures inoculated with boiled dead cells. All experiments were carried out in triplicates.

2.4. Growth of bacterium and extraction of metabolites

Strain P1-1 was grown in MM (Bastiaens et al., 2000) supplemented with phenanthrene (250 mg/1.5 l) as a sole source of carbon and energy at 28 °C and 150 rpm (C24 Rotary shaker, New Brunswick Scientific, NJ). After incubation for 3, 7, and 14 days, the cultures were filtered through glass wool and centrifuged (6000 × g, 10 min) (Keum et al., 2006). Supernatant was acidified to pH 2.3 with 6 N hydrochloric acid and extracted with ethyl acetate (3 × 500 ml). The combined organic phase was extracted three times with aqueous sodium hydroxide (500 ml, 10 mM). The remaining organic phase was dried over anhydrous sodium sulfate and concentrated to 5 ml of ethyl acetate (neutral fraction). The aqueous phase was acidified to pH 2.3 and extracted with ethyl acetate (500 ml, 3 times, acidic fraction). Degradation experiments for the PAH metabolites 1-[(*E*)-2-carboxyvinyl]-2-naphthoic acid, 1-hydroxy-2-naphthoic acid and naphthalene-1,2-dicarboxylic acid by strain P1-1 were also done according to the same procedures. Control experiments were performed with autoclaved cells according to the same procedures.

Metabolites in the neutral fraction were analyzed with gas chromatography–mass spectrometry (GC–MS) directly or after derivatization with *n*-butylboronic acid and methyl iodide (Keum et al., 2006). For the detection of diols or *cis*-dihydrodiols, ethyl acetate was removed and the residue was dissolved in acetone (10 ml) with *n*-butylboronic acid (50 mg) (Seeger et al., 2001). After refluxing for 30 min,

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