Degradation of Pyrene by *Mycobacterium aromativorans* Strain JS19b1

Jong-Su Seo1,2, Young-Soo Keum2,3, Kyun Kim1, and Qing X. Li*1

1Analytical Research Center, Korea Institute of Toxicology, Daejeon 305-343, Republic of Korea
2Department of Molecular Biosciences and Bioengineering, University of Hawaii, Honolulu, HI 96822, USA
3Department of Molecular Biotechnology, KonKuk University, Seoul 143-701, Republic of Korea

Received February 23, 2010; Accepted April 7, 2010

*Mycobacterium aromativorans* JS19b1T, isolated from a polycyclic aromatic hydrocarbon-contaminated soil, can grow with pyrene as a sole source of carbon and energy. A detailed pyrene metabolic map was constructed based on metabolite analysis by GC-MS and replacement cultures supplemented with the two intermediate metabolites phenanthrene-4,5-dicarboxylic acid and naphthalene-1,2-dicarboxylic acid. Strain JS19b1T can degrade pyrene through branched metabolic pathways, including its initial 4,5-dioxygenation and subsequent *ortho*- and *meta*-cleavages of 3,4-dihydroxyphenanthrene and naphthalene-1,2-diol. Dioxygenation of pyrene occurred on K-region to produce phenanthrene-4,5-dicarboxylic acid. 2,2'-Dicarboxy-6,6'-dihydroxybiphenyl and cis-3,4-dihydroxy-3,4-dihydrophenanthrene were also detected in phenanthrene-4,5-dicarboxylic acid-supplemented replacement cultures, which suggests branching of pyrene catabolism to a typical pathway of 3,4-dihydroxyphenanthrene.

**Key words:** biodegradation, bioremediation, *Mycobacterium aromativorans* JS19b1, pyrene

Polycyclic aromatic hydrocarbons (PAHs) are global pollutants and are produced from petroleum industry, fossil fuel combustion, and waste incineration. PAHs and some of their degradation products pose a toxic risk to the environment and human, since they have mutagenic and carcinogenic effects [Atsushi et al., 1998]. The U.S. Environmental Protection Agency listed 16 PAHs as priority pollutants. Many factors influence microbial degradation of pollutants. To monitor and improve microbial degradation of PAHs in the environment, it is necessary to understand their catabolic pathways. Microbial degradation is associated with plants in the environment and various bioremediation methods of PAHs have been studied [Daane et al., 2001; Parrish et al., 2004]. Pyrene, anthracene, and phenanthrene have been often used as model PAHs to study various aspects of their degradation by microorganisms, including bioavailability, metabolism, genomics, and compatibility with other remediation methods [Friedrich et al., 2000; Pinyakong et al., 2000; Dean-Ross et al., 2001; Stingley et al., 2004].

A variety of bacterial species are able to utilize two or three-ring PAHs as a sole source of carbon and energy [Springfellow and Aitken, 1995; Johnsen et al., 1996]. Recently, *Cycloclasticus*, *Mycobacterium*, *Rhodococcus*, *Sphingomonas*, and *Staphylococcus* species have been reported to degrade three- or five-ring PAHs [Walter et al., 1991; Geiselbrecht et al., 1998; Pinyakong et al., 2000; Kim et al., 2005; 2007; Mallick et al., 2007]. The initial degradation reactions are catalyzed by multi-component dioxygenases to produce dihydrodiols [Moody et al., 2004; Kim et al., 2005; Mallick et al., 2007]. Desaturation, hydroxylation, and monooxygenation also occur in *Pseudomonas* [Gibson et al., 1995].

Pyrene degradation by *Mycobacterium* species is initiated with incorporation of mono- or dioxygen at different positions [Vila et al., 2001; Kim et al., 2005; 2007]. *M. vanbaalenii* can metabolize pyrene to cis-4,5-dihydrodiol and cis-1,2-dihydrodiol [Kim et al., 2007]. 4,5-Dihydroxyphenanthrene is cleaved to phenanthrene-4,5-dicarboxylic acid, which is further decomposed either through 3,4-dihydroxyphenanthrene [Rehmann et al., 1998; Kim et al., 2007] or 2,2'-dicarboxy-6,6'-dihydroxybiphenyl [Vila et al., 2001; Kim et al., 2005; López et al., 2008].

A previous study with *Mycobacterium aromativorans* JS19b1T revealed that this species was able to utilize various PAHs, specifically, could degrade 100% of...
phenanthrene and pyrene (each 40 mg/L) in 7 and 14 days, respectively [Seo et al., 2007]. In the present study, metabolites of pyrene were identified and the catabolic pathways were constructed.

Materials and Methods

Chemicals. Pyrene (98%), 1-hydroxy-2-naphthoic acid, and phthalic acid were purchased from Sigma-Aldrich (Milwaukee, WI). Catechol was obtained from Fisher Scientific (Morris Plains, NJ). Naphthalene-1,2-diol and naphthalene-1,2-dicarboxylic acid anhydride were purchased from TCI America (Portland, OR). 4-[(1-Hydroxynaphthalene-2-yl)-2-oxo-but-3-enoic acid, 2-(2-carboxy-vinyl)naphthalene-1-carboxylic acid, and 7,8-benzocoumarine were previously synthesized [Keum et al., 2009]. Naphthalene-1,2-dicarboxylic acid was prepared by basic hydrolysis of naphthalene-1,2-dicarboxylic acid anhydride. Phenanthrene-4,5-dicarboxylic acid, 2,2'-dicarboxy-6,6'-dihydroxybiphenyl, and 4-oxa-pyrene-5-one were prepared according to the methods of Young and Funk [1998], Lefler and Graybill [1959], and Gillis and Porter [1989], respectively. Ethyl acetate and other solvents were purchased from Fisher Scientific (Morris Plains, NJ).

Bacterial strain and growth conditions. Strain JS19b' was previously isolated and characterized [Seo et al., 2007; Hennessee et al., 2009]. This strain was deposited in the American Type Culture Collection (ATCC BAA-1378) (Manassas, VA, USA). It was cultured in minimum medium [Bastaens et al., 2000] supplemented with pyrene (500 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). Cultures were also done with phenanthrene-4,5-dicarboxylic acid and naphthalene-1,2-dicarboxylic acid through separate cultures, referred to as replacement cultures. All cultivations were done in triplicate.

Extraction and derivatization of metabolites. After incubation for 14 days, the cultures were filtered through glass wool followed by centrifugation (6,000×g, 10 min) to remove the unreacted solid pyrene. The supernatant was acidified to pH 2.3 with 6N HCl and extracted with ethyl acetate (3×500 mL). The combined organic layer was acidified to pH 2.3 and extracted with ethyl acetate (3×500 mL, acidic fraction). The metabolites in replacement cultures were extracted according to the same procedure. Autoclaved bacterial cells were used as controls.

Metabolites in the neutral fraction were either derivatized or not derivatized prior to gas chromatography-mass spectrometry (GC-MS) analysis. For diols or cis-dihydrodiols, ethyl acetate was removed and the residue was dissolved in acetone (10 mL) containing n-butyliboronic acid (50 mg). After refluxing for 30 min, the mixture was concentrated to 1 mL and analyzed by GC-MS. The residue was further derivatized with methyl iodide and re-analyzed with GC-MS [Seo et al., 2006]. Metabolites in the acidic fraction were derivatized with diazomethane that was prepared from N-methyl-N-nitroso-p-toluene sulfonamide in a diazomethane generator (Aldrich).

Analytical methods. GC-MS analysis was performed on a Varian QP5000 gas chromatograph-Saturn2000 mass spectrometer (Varian, Palo Alto, CA), equipped with ZB-1 column (60 m, 0.25 mm film thickness, Phenomenex, Torrance, CA) and helium as carrier gas set at a rate of 2 mL/min. The column temperature was held at 120°C (2 min), programmed to 280°C at a rate of 2°C/min, and held 280°C for 10 min. Injector and analyzer temperatures were set at 270 and 280°C, respectively. The mass spectrometer was operated in electron impact mode (70 eV).

Results and Discussion

Strain JS19b' can utilize pyrene as a sole source of carbon and energy and degrade it via K-region (4,5-positions) dihydrodiol intermediates as previously reported [Dean-Ross and Cerniglia, 1996; Rehmann et al., 1998; Vila et al., 2001; Kim et al., 2005; Liang et al., 2006]. A total of 16 metabolites were detected in acidic and neutral fractions and identified with the appropriate standards by GC-MS according to their retention times and mass fragmentations (Table 1).

Metabolite P1 (as dimethyl ester; Rt=57.35 min) was identified to be phenanthrene-4,5-dicarboxylic acid (Fig 1). The mass fragments, [m/z 294 (M+) 263 (M'-OCH, 235 (M'-COOCH,), 220 (M'-COOCH,CH,), are identical with those previously reported [Dean-Ross and Cerniglia, 1996; Vila et al., 2001]. Phenanthrene-4,5-dicarboxylic acid (P1) was transformed by intradiol cleavage of pyrene-4,5-diol and was decarboxylated to produce phenanthrene-4-carboxylic acid (P2) (Fig 4), which is one of the two pathways described by Kim et al. [2005]. 2,2'-Dicarboxy-6,6'-dihydroxybiphenyl (P13) and 4-oxa-pyrene-5-one (P14) were detected and confirmed by replacement culture treated with P1 as an initial substrate (Fig. 2). P13 may be derived from ring opening, decarboxylation, and mono-hydroxylation. The decarboxylation and mono-hydroxylation were also