Pyrene Degradation by *Mycobacterium* sp. KMS:

Biochemical Pathway, Enzymatic Mechanisms, and Humic Acid Effect

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ABSTRACT

Pyrene Degradation by *Mycobacterium* sp. KMS: Biochemical Pathway, Enzymatic Mechanisms, and Humic Acid Effect

by

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Utah State University, 2006

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Department: Civil and Environmental Engineering

Pyrene, a four-ring polycyclic aromatic hydrocarbon (PAH), was identified as the chemical that requires the largest land area for soil bioremediation due to the slow rate of biodegradation at the Libby, Montana, Superfund site. Prepared bed land treatment is the specific bioremediation technology that is currently employed at this site. Although bioremediation has been widely accepted for treatment of contaminated soil due to its low cost, the effective application of bioremediation is often hindered by the lack of information related to: 1) biochemical pathways, 2) enzymatic mechanisms, and 3) effects of amendments.

Mycobacterium sp. KMS is a new strain isolated from the land treatment units of the Libby site and has been found to utilize pyrene as a carbon and energy source. The genome of *Mycobacterium* sp. KMS was sequenced by Joint Genome Institute (JGI) and is publically available in the NCBI database.

This dissertation is comprised of seven chapters. Chapter I provides information concerning PAH characteristics, the Libby Superfund site, accelerated bioremediation approaches, and the hypotheses for this dissertation. Chapter 2 addresses the pyrene degradation pathway used by *Mycobacterium* sp. KMS based on isolating and identifying pyrene degradation intermediates. Chapter 3 describes the enzymatic mechanism of pyrene degradation by *Mycobacterium* sp. KMS.

Chapter 4 presents the effect of Elliott soil HA (ESHA) amendment on pyrene solubility in soil slurry systems and pyrene mineralization in unsaturated soil microcosms. Chapter 5 describes the overall effect of ESHA amendment on pyrene distribution in a soil slurry system.

Chapter 6 addresses the engineering significance of this study and future research recommendations. Chapter 7 summarizes the dissertation. The main theme of this dissertation is to provide the basic scientific information and knowledge for better understanding, better control, and improvement of the bioremediation process at PAH-contaminated sites.

(182 pages)

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Yanna Liang

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CHAPTER 1

INTRODUCTION

SOURCES OF PAHs

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds composed of two or more fused benzene rings in linear, angular, and cluster arrangements. PAHs are major constituents of crude oil, creosote and coal tar. They have been detected in air, water, soil, and food and are ubiquitous contaminants in nature. Processes and sources that can produce or contain PAHs are listed in Table 1.

TOXICITY OF PAHs

Research on PAH carcinogensis began two centuries ago when physician John Hill reported the link between excessive use of tobacco snuff and nasal cancer in 1761 (2). In 1775, Percival Pott related chimney sweep's scrotal skin cancer with exposure to soot. In 1915, Yamigiwa and Ickikawa reported that tumors were formed after repeated application of coal tar on the ears of rabbits. From 1930 to 1955, Kennaway, Hieger, Cook, and Hewett established that the carcinogenic fraction of coal tar contained PAHs. In the 1970s, James and Elizabeth Miller (8) showed that many chemicals require metabolic activation to express toxicity. It is now well known that PAHs must be metabolically activated first to elicit their latent mutagenic, genotoxic, and carcinogenic properties (3).

PAH carcinogenesis has recently been reviewed and related to at least four mechanisms (4) (Fig. 1): 1) the dihydrodiol epoxide mechanism, which involves

TABLE 1.1. Processes and sources producing or containing PAHs (3)

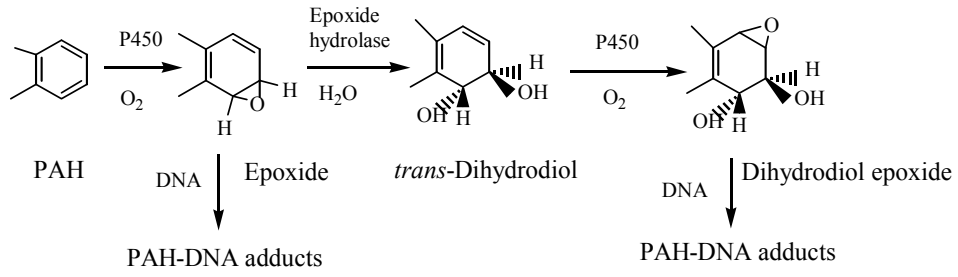
Natural oil seeps	Combustion of fossil fuels
Refinery and oil storage waste	Tobacco and cigarette smoke
Accidental spills from oil tankers and other ships	Forest and prairie fires
Municipal and urban wastewater discharge runoff	Rural and urban sewage sludge
River-borne pollution	Refuse and waste incineration
Atmospheric fallout of fly ash particulates	Coal gasification and liquefaction processes
Petrochemical industrial effluents	Creosote and other wood preservative wastes
Coal tar and other coal processing wastes	Chronic input associated with boating activitites
Automobile engine exhausts	

microsomal cytochrome P450 enzymes to activate PAHs to reactive epoxide and diol-epoxide intermediates that form covalent adducts with DNA, perhaps resulting in mutations that lead to tumorigenesis; 2) the radical-cation mechanism, which involves one-electron oxidation to generate radical-cation intermediates that may attack DNA, resulting in depurination; 3) the quinone mechanism, which involves enzymatic dehydrogenation of dihydrodiol metabolites to yield quinone intermediates that may either combine directly with DNA or enter into a redox cycle with oxygen to generate reactive oxygen species capable of attacking DNA; and 4) the benzylic oxidation mechanism, which entails formation of benzylic alcohols that are converted by sulfotransferase enzymes to reactive sulfate esters that may attack DNA.

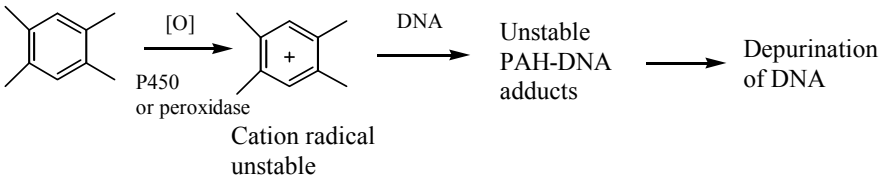
BIODEGRADATION

The environmental fate of PAHs is of great concern since they are the largest class of chemical carcinogens known today (1). Engineering of biodegradation, the biological

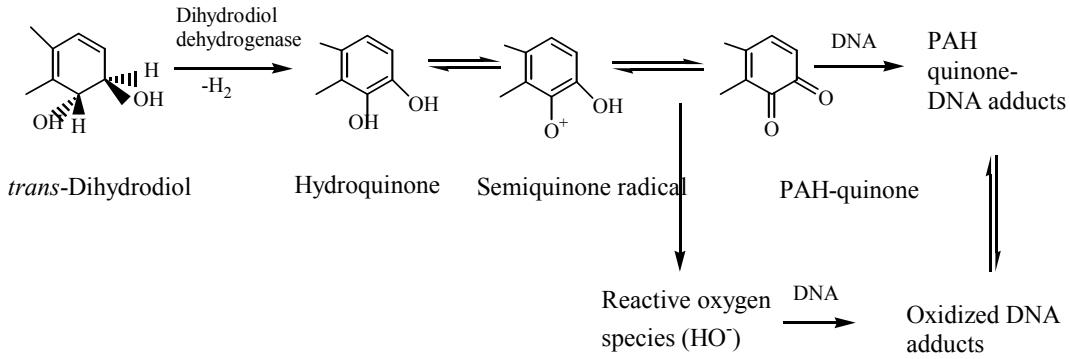
1. Diol-epoxide mechanism



2. Radical-cation mechanism



3. Quinone mechanism



4. Benzylic oxidation mechanism

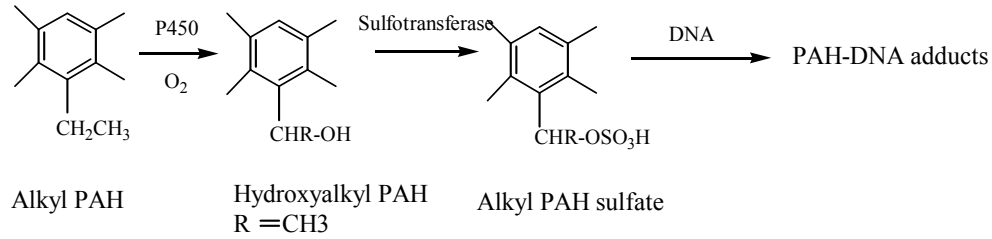


FIG. 1.1. Mechanisms of PAH carcinogenesis (4).

catalyzed reduction in complexity of chemicals (11), was developed in the 1970's and has been widely accepted as a cost effective tool for site decontamination.

One site currently utilizing biodegradation is the Champion International Superfund Site in Libby, MT. This site experienced heavy contamination of PAHs and pentachlorophenol (PCP) from wood treatment operations from 1946-1969. Prepared bed land treatment, a process of stimulating soil microorganisms to degrade contaminants, was initiated in 1989 in two 1-acre, lined, land treatment units and subsequently expanded to 12 acres of unlined treatment area (10).

Laboratory treatability studies indicated that prepared bed land treatment at the Libby site was an effective method for reducing contaminant levels and residual toxicity (6, 10). However, the treatment process affected by the environment was slow. For pyrene, a four-ring PAH, the half life was beyond one year. In order to increase the degradation rate, accelerated bioremediation, a bioremediation process accelerated beyond the normal actions of the naturally occurring microbial community and chemical and geological conditions, usually by the addition of amendments or specialized microbes, has been proposed.

ACCELERATED BIOREMEDIAITON

Accelerated biodegradation is achieved by adding either amendments or specialized microbes. In the case of adding supplemental carbon sources or other nutrients to stimulate the activity of indigenous or inoculated PAH degrading microbial strains, the process is categorized as biostimulation (5). In the case of adding microbial strains with desired degradative capacities, the process is defined as bioaugmentation (5).

Mycobacterium sp. KMS was isolated from the vadose zone soil of the Libby prepared bed land treatment system. It was found to be a *Mycobacterium* species based on Gram staining, electron microscopy, and 16S rDNA-sequencing. It has the potential to degrade pyrene, benzo[a]pyrene, and other PAHs (7). Humic acid (HA), which is polyfunctional, can act as a binding agent and detoxicant, sobent, and flushing agent, redox mediator of abiotic and biotic reactions, nutrient carrier, and growth-stimulator (9). The possibilities of applying *Mycobacterium* sp. KMS for bioaugmentation and HA for biostimulation will be discussed in this dissertation. This work adds to the current body of knowledge involving *Mycobacterium* species for PAH biodegradation and HA addition for faster and more complete bioremediation.

HYPOTHESES

The following hypotheses were tested in this research:

Hypothesis 1: The pyrene degradation pathway by *Mycobacterium* sp. KMS is different to the pathway used by other bacteria.

Hypothesis 2. *Mycobacterium* sp. KMS degradation of pyrene and pyrene-4,5-dione are initiated by certain enzymes induced by the two chemicals.

Hypothesis 3: Standard Elliot soil humic acid (ESHA) increases pyrene's solubility and mineralization rate.

Hypothesis 4: Addition of standard ESHA to the Libby soil increases the formation of bound residues.

Hypotheses 1, 2, 3, and 4 are the subjects of Chapter 2, 3, 4, and 5, respectively. Specific objectives, methods, and results of each study are presented in the individual

chapters. These studies provide a better understanding of the chemical and biological aspects of PAH-degrading *Mycobacterium* sp. KMS.

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CHAPTER 2

IDENTIFICATION OF PYRENE-4,5-DIONE AS A NOVEL PYRENE DEGRADATION METABOLITE BY *MYCOBACTERIUM* SP. KMS[1]

ABSTRACT

Mycobacterium sp. KMS, isolated from vadose zone soil at the Champion International Superfund Site in Libby, Montana, has demonstrated the ability to degrade pyrene and other PAHs including benzo[a]pyrene. Pyrene degradation pathway experiments revealed intermediates including: pyrene-4,5-dione, *cis*-4,5-pyrene-dihydrodiol, phenanthrene-4,5-dicarboxylic acid, and 4-phenanthoic acid. This is the first study to report pyrene-4,5-dione as a pyrene degradation intermediate in a Gram-positive bacterium. Pyrene-4,5-dione, which accumulates as an end product in some Gram-negative bacterial cultures, can be further utilized and degraded by *Mycobacterium* sp. KMS. This study provides new information on pyrene-4,5-dione formation and degradation by *Mycobacterium* KMS, which occurs at PAH-contaminated sites and is involved in soil bioremediation processes.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) have been detected in air, water, soil, and food and are ubiquitous contaminants in nature (24). High-molecular-weight (HMW) PAHs are hydrophobic, very stable, and relatively inert molecules in the environment (34). However, their transformation products are bioactive and have been shown to be both acutely and chronically toxic (1, 5, 7, 10, 11, 17, 31, 39). The mechanisms of PAH

[1]Coauthored by Yanna Liang, Dale R.Gardner, Charles D. Miller, Anne J. Anderson, and Ronald C. Sims.

carcinogenesis have recently been reviewed and include, 1) the dihydrodiol epoxide mechanism, 2) the radical-cation mechanism, 3) the quinone mechanism, and 4) the benzylic oxidation mechanism (15, 16). Therefore, it is important to understand how PAHs are degraded, what kinds of products are produced, and whether the metabolites are toxic.

Pyrene degradation pathways of *Mycobacterium* species including *vanbaalenii* PYR-1, *flavescens*, RJGII-135, KR2, and AP1 have been studied and are proposed to be similar (4, 6, 23, 32, 35, 38). Pyrene is first oxidized in the K-region by a dioxygenase to form *cis*-4,5-pyrene-dihydrodiol, which is rearomatized to form 4,5-dihydroxy-pyrene by dihydrodiol dehydrogenase. 4,5-Dihydroxy-pyrene is subsequently cleaved to yield phenanthrene-4,5-dicarboxylic acid. Following loss of a carboxyl group, 4-phenanthroic acid is formed and enters into the phenanthrene degradation pathway. Two additional pathways have been proposed. One proposes that pyrene hydroxylation takes place at the 1, 2 positions, leading to the formation of 4-hydroxy-perinaphthenone, which is a dead-end product and so far has been only found in *Mycobacterium vanbaalenii* PYR-1 cultures (4). Another pathway involves the accumulation of 6,6'-dihydroxy-2,2'-biphenyl-dicarboxylic acid in *Mycobacterium* sp. AP1 (38).

Pyrene-4,5-dione was identified to be a pyrene degradation intermediate of several bacteria. First, it was observed as a pyrene metabolite by *Sphingomonas yanoikuyae* strain R1 by Kazunga and Aitken (20). Formation of pyrene-4,5-dione probably proceeds by metabolism of c*is*-4,5-pyrenedihydrodiol, as strains R1 converted *cis*-4,5-pyrene-dihydrodiol to pyrene-4,5-dione essentially stoichiometrically and resulted in pyrene-4,5-dione accumulation in the culture. Second, *Mycobacterium vanbaalenii* strain PYR-1

formed significant amounts of pyrene-4,5-dione when it was incubated with a high concentration of *cis*-4,5-pyrene-dihydrodiol, although it was not reported as an intermediate when *Mycobacterium vanbaalenii* strain PYR-1 grew on pyrene (20). Third, pyrene-4,5-dione was identified to be a pyrene metabolite in the phagemid clone My6-pBK-CMV containing a dioxygenase gene when it was incubated with pyrene (22).

Mycobacterium sp. KMS was isolated from vadose zone soil of the Champion International Superfund Site in Libby, Montana and has the ability to degrade pyrene and other PAHs (29). It was found to have the dioxygenase gene for PAH degradation (13, 29). Due to the toxicity of pyrene-4,5-dione (30) and its possible presence during pyene degradation in *Mycobacterium* cultures, it is important to identify this metabolite and determine its fate during pyrene degradation, as it was observed as an end product in some Gram-negative cultures and may result in toxicity increase during *in-situ* soil bioremediation (20). The objectives of this work reported here were to: 1) determine the pyrene degradation pathway used by *Mycobacterium* sp. KMS by isolating and identifying the metabolites, and 2) determine the capability of *Mycobacterium* sp. KMS to degrade pyrene-4,5-dione.

MATERIALS AND METHODS

Chemicals

Pyrene (99%) was purchased from Fluka, Switzerland. C*is*-4,5-pyrenedihydrodiol was kindly provided from Dr. Michael Aitken at the University of North Carolina at Chapel Hill. Pyrenol (1-hydroxypyrene, 99%), phthalic acid (99%) were purchased from Aldrich Chemical Company, Inc. Radio-labeled [4,5,9,10-^{14}C] pyrene (95% purity,

specific activity = 56 mCi/mmol) was purchased from Amersham International (Burkinghamshire, England). All solvents (methanol, acetonitrile, ethyl acetate) used were HPLC grade or the equivalent and were purchased from Sigma-Aldrich, St. Louis, MO. Basal Salt Medium (BSM) and Luria Broth (LB) are the same as described by Miller (29). Deuterated solvents, methanol-D_4 (99.8%) was purchased from Sigma-Aldrich, St. Louis, MO and dimethyl sulfoxide–D_6 (DMSO) was purchased from Acros Organics, Morris Plains, NJ.

Pyrene-4,5-dione and phenanthrene-4,5-dicarboxylic acid were synthesized based on Yong and Funk's procedure (40). Briefly, pyrene was regioselectively oxidized to 4,5-phenanthrene-dicarboxylic acid by using hydrogen peroxide and tungstic acid. The carboxylated ion generated with sodium bicarbonate is alkylated with iodomethane in dimethyl formamide to produce dimethyl 4,5-phenanthrenedicarboxylate. Treatment of the diester with excess sodium in refluxing tetrahydrofuran resulted in a good yield of pyrene-4,5-dione. The purity and authenticity of synthesized pyrene-4,5-dione and phenanthrene-4,5-dicarboxylic acid were determined after analysis by high pressure liquid chromatography (HPLC) and ^{1}H nuclear magnetic resonance (NMR) spectroscopy.

Bacteria and growth condition

Mycobacterium sp. KMS cells were grown in BSM+ (a 9:1 mixture of BSM and LB) for five days to stationary phase, pelleted and washed twice with sterile distilled water. The suspension was used as an inoculum for the following experiments.

Pyrene mineralization in liquid culture