STABLE-ISOTOPE PROBING-BASED INVESTIGATIONS OF POLYCYCLIC
AROMATIC HYDROCARBON-DEGRADING BACTERIA IN CONTAMINATED SOIL

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Abstract

Maiysha D'ora Jones: Stable-Isotope Probing-based Investigations of Polycyclic Aromatic Hydrocarbon-Degrading Bacteria in Contaminated Soil

(Under the direction of Michael D. Aitken)

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic contaminants that are a global environmental problem. These compounds become more recalcitrant to remediation and increase in carcinogenic potential with increasing molecular weight. Engine exhaust and industrial process waste, like that from the sites of former manufactured-gas plants, contain high concentrations of PAHs and are major sources of benzo[*a*]pyrene contamination in the environment. Bioremediation, the use of microorganisms to remove PAH contamination, is the dominant strategy for removing PAH contamination from soil because many microorganisms can grow on PAHs. Stable-isotope probing (SIP) is a cultivation-independent technique used to identify microorganism able to grow on specific chemicals, such as PAHs. SIP was used to identify bacteria in soil from the site of a former manufactured-gas plant that are capable of degrading naphthalene, phenanthrene, anthracene, pyrene, fluoranthene, or benz[*a*]anthracene. Group-specific quantitative PCR primers were developed to determine whether the bacteria identified were capable of growth

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on the respective PAH. SIP with naphthalene, phenanthrene, and fluoranthene selected

bacteria previously associated with the degradation of those compounds, and *Pigmentiphaga*

was newly associated with naphthalene, phenanthrene, and anthracene degradation. A group

of uncultivated Gammaproteobacteria known as Pyrene Group 2 was newly associated with

fluoranthene and benz[*a*]anthracene degradation, and it was the only group of bacteria

associated with pyrene degradation. A group of uncultivated Alphaproteobacteria was the

primary anthracene-degrading group and was designated Anthracene Group 1;

Herminiimonas was also newly associated with anthracene degradation. In experiments to

evaluate the biases associated with using a commercial DNA extraction kit, performing

multiple DNA extractions on the same anthracene-enriched soil sample did not affect

qualitative results; however, shifts in the relative abundances of anthracene-degrading

bacteria were observed between extracts. Since no microorganisms are known to grow on

benzo[*a*]pyrene, a carcinogenic PAH, mineralization experiments and the results of the SIP

investigations were used to obtain indirect evidence suggesting that bacteria capable of

growth on other PAHs might participate in benzo[*a*]pyrene metabolism. None of the major

SIP-identified bacteria were associated with benzo[*a*]pyrene mineralization, but members of

the genera *Cupriavidus*, *Luteimonas*, and *Rhizobium* may be associated with benzo[*a*]pyrene

mineralization.

To my mom, whose fortitude made me the woman I am

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1. Introduction

Polycyclic aromatic hydrocarbon (PAH) contamination of environmental systems is a global problem (1-5). PAHs are chemically stable organic compounds, and most of them are poorly soluble in water. Both of these characteristics contribute to the environmental persistence of PAH contamination. Of the sixteen US EPA priority pollutant PAHs, seven are probable human carcinogens (6), including benzo[*a*]pyrene (BaP).

Soil is a receptor for PAH contamination transferred from air, surface runoff and industrial sources, but bioremediation, the use of microorganisms to remove chemical contamination, has proven useful toward reducing PAH contamination in soils. The foundation for understanding the biological removal of PAHs from contaminated soil is the identification of specific bacterial groups capable of degrading individual PAHs and PAH mixtures and the characterization of the genes directing PAH metabolism. Although the genetic elements responsible for lower molecular weight (LMW) PAH metabolism, particularly those genes encoding ring-hydroxylating dioxygenase (RHD) systems, have been identified in several genera (7-10), the characteristics of bacterial RHD enzymes and their associated genes required for the biodegradation of BaP are not yet known. Therefore, identifying bacteria associated with BaP metabolism is a first step toward this end.

In the last 7 years, several studies have focused on the identification of PAH-degrading bacterial groups using the cultivation-independent technique stable-isotope probing (SIP), which was first used as a tool in microbial ecology by Radajewski and

colleagues (11). With DNA-based SIP, isotopically dense cellular material is produced by feeding an environmental sample, and therefore its native microbial community, a ^{13}C-labeled growth substrate. The result is a pool of ^{13}C-enriched nucleic acids that can be isolated by density-gradient ultracentrifugation and further investigated to asses various endpoints. Our group has previously had success with SIP investigations of two geographically distinct PAH-contaminated soils using uniformly ^{13}C-labeled substrates (12-16), with isolating phenanthrene- (7) and pyrene-degrading (unpublished) organisms from these soils, and with characterizing a phenanthrene-degradation gene cluster from an isolated *Acidovorax* strain that was identified in an SIP experiment (7).

Using a third soil, the most comprehensive SIP investigation of a single soil to date was performed with the LMW growth substrates naphthalene, phenanthrene, and anthracene, and the higher molecular weight (HMW) growth substrates pyrene, fluoranthene, and benz[*a*]anthracene, to better understand the microbial ecology of PAH-contaminated soil. These are the first SIP experiments in which [U-^{13}C] anthracene, fluoranthene, or benz[*a*]anthracene has been used as a growth substrate. Identifying organisms capable of growth on specific PAH substrates will facilitate their isolation and direct future investigations into the genetic elements responsible for specific PAH metabolism, including that of HMW PAHs such as BaP.

1.1. Specific Research Objectives and Rationale

1.   Determine the effect of multiple DNA extractions (performed on the same soil aliquot) on the identification and quantification of anthracene-degrading bacteria native to PAH-contaminated soil and identified by DNA-based stable-isotope probing.

The effectiveness of molecular methods to describe microbial diversity depends on our ability to efficiently extract and purify macromolecules from microbial cells native to an environmental sample (17). Commercially available kits are commonly used to extract nucleic acids from environmental samples using a single-extraction approach. Recently, this approach was shown to underestimate genomic DNA mass yield and small-subunit ribosomal gene copy number and to bias the diversity of bacterial groups identified using molecular methods (18). In each DNA-based SIP study published to date in which soil communities were investigated, a single DNA extraction was performed on the soil sample using a commercial DNA extraction kit prior to recovering the ^{13}C-labeled (heavy) DNA by density-gradient ultracentrifugation. The effect of multiple DNA extractions on the bacteria identified by DNA-based SIP has not been examined. Objective 1 considers the hypothesis that beyond what is recovered in the first extract, additional genomic DNA and small-subunit ribosomal gene copies will be recovered, and additional bacterial groups will be associated with anthracene degradation, as a result of multiple DNA extractions.

2.   Use DNA-based SIP to identify 2-, 3-, and 4-ring PAH-degrading bacteria indigenous to PAH-contaminated soil, and design and validate quantitative PCR primers and standard curves to quantify SIP-identified groups.

Traditional microbiological techniques, including culture-based isolation methods, are biased toward selecting organisms that can grow under static conditions on defined media. This indicates that cultivation efforts alone may be insufficient for characterizing microbial life. Cultivation-independent molecular methods, like DNA-based SIP, eliminate the need to isolate microorganisms from their natural habitat. Instead, molecular methods are used to detect and quantify microorganisms in environmental samples by extracting and

measuring their cellular material. With DNA-based SIP, microorganisms capable of growth on a stable-isotope-labeled (^{13}C) substrate assimilate the labeled carbon atoms into newly synthesized DNA; this heavy DNA is then analyzed by additional molecular methods. As a result, microorganisms are identified based on their ability to perform a specific metabolic function, and they can be quantified based on their specific DNA sequence. Objective 2 considers the hypotheses that DNA-based SIP will be useful in the cultivation-independent identification of naphthalene-, phenanthrene-, pyrene-, fluoranthene-, and benz[*a*]anthracene-degrading bacteria and that specific PAH-degrading bacteria can be quantified based on differences in their 16S rRNA gene sequences.

3.   *Compare the effects of pre-enrichment and co-incubation of 2-, 3-, and 4-ring PAH with benzo[a]pyrene on benzo[a]pyrene mineralization, and determine whether SIP-identified bacteria are associated with benzo[a]pyrene mineralization.*

Although benzo[*a*]pyrene is not a bacterial growth substrate, it can be metabolized by bacteria pre-grown on other substrates (pre-enrichment) or grown in the presence of other substrates (co-incubation). Various LMW and HMW PAHs, as well as non-PAH substrates, have served as growth substrates in studies investigating benzo[*a*]pyrene metabolism, but the effect of pre-enrichment versus co-incubation has not been compared in the same soil sample. In addition, the community dynamics associated with benzo[*a*]pyrene have not been investigated using pyrosequencing-based analyses. Objective 3 considers the hypotheses that naphthalene, phenanthrene, pyrene, fluoranthene, or benz[*a*]anthracene will influence benzo[*a*]pyrene mineralization under pre-enrichment or co-incubation conditions, and that naphthalene-, phenanthrene-, pyrene-, fluoranthene-, or benz[*a*]anthracene-degrading

bacteria will be associated with benzo[*a*]pyrene mineralization via pyrosequencing-based analyses.

1.2. Dissertation Organization

My dissertation consists of three manuscripts, each detailing the research performed to address the previously described research objectives. The manuscript associated with Chapter 3 has been modified to address comments that were received from reviewers after initial submission to *Applied and Environmental Microbiology*. The manuscript associated with the Chapter 4 has been submitted to *Environmental Microbiology*. The manuscript associated with the third objective (Chapter 5) is in draft form, but my intent is to submit this work for publication as well. Chapter 2 reviews the literature published to-date that is relevant to the experiments and analyses described in the three manuscripts, and Chapter 6 concludes the dissertation and makes recommendations for future research.

2. Literature Review

2.1. Global Distribution of PAH Contamination

PAH contamination of environmental systems is a global problem (1-5). PAH contamination usually occurs as a complex mixture of compounds and is a result of both the combustion of natural and anthropogenic organic materials and the accidental or improper disposal of industrial materials containing high concentrations of PAHs. Natural sources of PAH contamination include forest fires, volcanic eruptions, and natural oil seeps, but anthropogenic sources remain the primary concern (4). Anthropogenic sources of PAH contamination include residential fireplace use, high heat cooking practices like grilling with charcoal and wood stove use, cigarette smoke, engine exhaust, coal gasification and other industrial releases.

Airborne PAH contamination is suspected to be the cause for the global distribution of PAHs, with PAH concentrations being the greatest in urban areas (3, 4, 19). For example, atmospheric benzo[*a*]pyrene (BaP) emissions in the US have been estimated to be 3.6×10^6 kg/year (20). Most of this mass partitions onto suspended organic particulates, but the main receptor of atmospheric BaP is soil or sediment (20). Particle-associated PAHs can deposit on surfaces and be stripped from the air and from surfaces during rain events (5, 20, 21). Surface run-off can then lead to contamination of surface water, soil, and sediment (4). Ultimately, PAHs can bioaccumulate in the food chain and pose a significant risk to human health (20-22).

2.2. Former MGP Sites are a Source of Soil PAH Contamination in the United States

In the mid-1800s, the need for energy distribution in towns and cities grew in the United States. This need was met by manufacturing gas from coal. Each city had its own manufactured-gas plant (MGP) and some wealthy estates and large facilities like prisons, hospitals, and military installations did as well. In the US, MGPs have been estimated to have numbered 1,000 to 2,000 during their nearly 100 years of operation (23), but the US EPA estimates that up to 45,000 sites across the country require clean-up from former MGP processes (24).

Manufactured gas was generated by heating coal or oil in the absence of oxygen. The gas was cooled and then stored before distribution to the end user via underground pipes. In addition to manufactured gas, the coal gasification process produced hazardous by-products including coal tar (which contains high concentrations of PAHs), volatile organic compounds, inorganic compounds, and metals (23). Coal tar and other byproducts were sold for profit. Some byproducts were also dumped in sewers and surface waters or buried on-site. The need for MGPs declined as natural gas and electricity replaced manufactured gas as an energy source in the mid-1900s. Some plants were converted to natural gas or electricity producing facilities, but many were abandoned, leaving behind hazardous waste products including PAHs.

Since Percival Pott's association of scrotal cancer with soot exposure in chimney sweeps in 1775 and von Volkman's report of increased skin cancer incidence in coal tar workers 100 years later, there has been an awareness of the negative health effects of human exposure to combustion by-products (5, 21). To date thousands of research studies have provided evidence that some PAHs are carcinogenic (25).

2.3. Physical and Chemical Properties of PAHs

The United States Environmental Protection Agency (US EPA) regulates sixteen PAHs as priority pollutants, seven of which are probable human carcinogens (Figure 2.1). PAHs contaminate the environment as a result of the combustion of natural and anthropogenic organic material and the accidental or improper disposal of industrial materials containing high concentrations of PAHs. PAH molecules are composed of at least two fused benzene rings, are inherently stable due to the movement of resonant electrons, and autofluoresce when exposed to UV light (26). Except for certain substituted PAHs, most PAHs are nonpolar, hydrophobic, insoluble in water, and persist in the environment. The hydrophobicity, aqueous solubility, and environmental persistence of each PAH is dictated by the number and geometric orientation of benzene rings in its molecular structure (27). LMW PAHs are less hydrophobic and more water soluble, whereas HMW PAHs are more hydrophobic and less water soluble. Hydrophobicity can be measured by the tendency for a compound to remain in the organic phase versus the aqueous phase as in octanol-water partitioning, for example. Environmental persistence is associated with a tendency toward partitioning to the particle or soil phase, and it increases with increased number of aromatic rings and with increased molecular weight (28). These trends are listed in Table 2.1 for the sixteen US EPA priority pollutant PAHs.

2.4. Health Effects Resulting from Exposure to PAHs

PAHs are readily metabolized in the human body, with PAH metabolites being detected in adipose tissue, expired air, breast milk, blood, and urine (31, 32). Human exposure to PAHs can result from occupational, domestic, and recreational activities. The magnitude of human exposure to PAHs depends both on the source and the receptor's
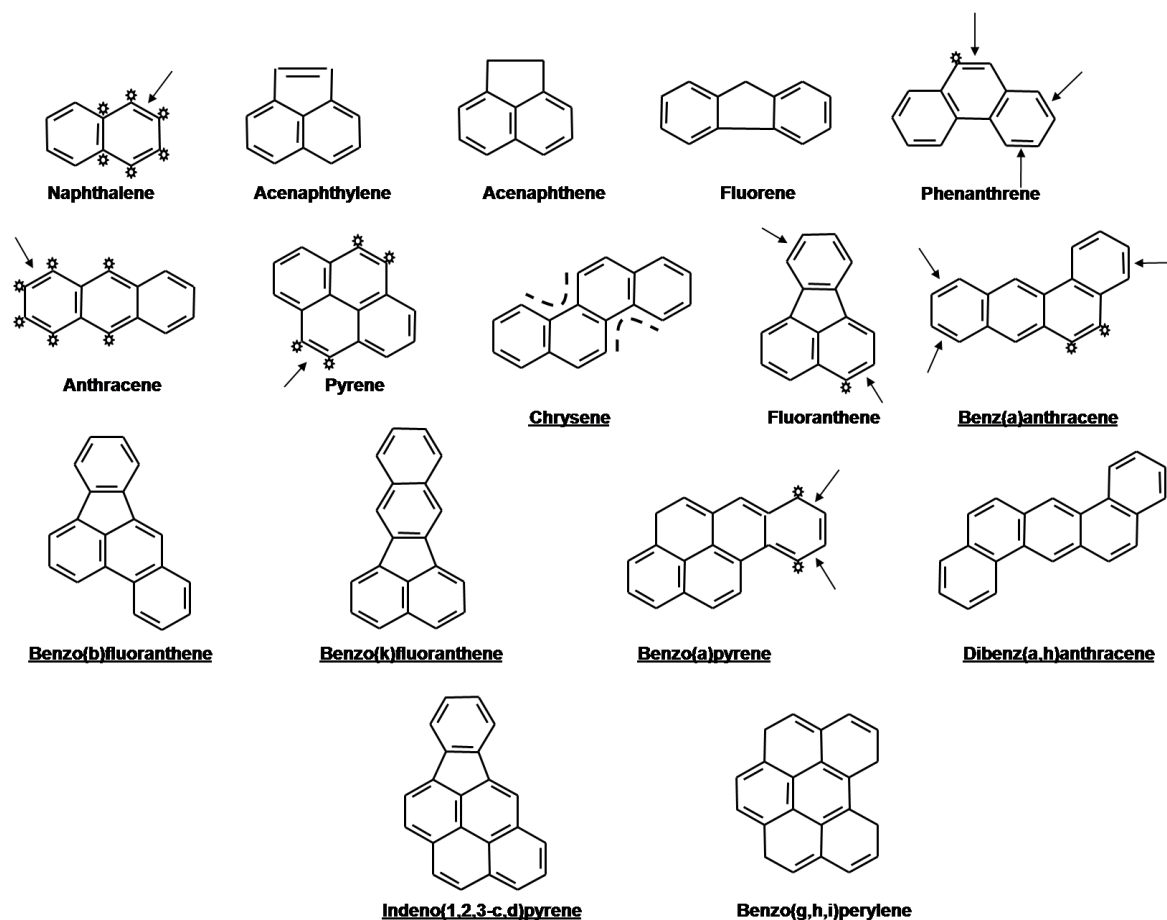
Figure 2.1 Names and structures of the sixteen US EPA priority pollutant PAHs. Names of US EPA Group B2 probable human carcinogens are underlined. Locations of ^{14}C atoms of radiolabeled PAHs used in this work are indicated with a star (�). Bay regions are shown with dotted lines for chrysene. Arrows indicate predominant sites of dioxygenase attack during aerobic biodegradation (22).

geographic location, climate, occupation, and lifestyle. Human exposure to PAHs can result

in mutagenic, cytotoxic, genotoxic or carcinogenic effects. Exposure to PAHs can also result

in other adverse health effects, ranging in severity from general malaise to hemolysis to

neurological, developmental, or reproductive impairment (31). The severity of the adverse

effects depends on the PAH, the dose, the method of exposure, and the receptor's genetic

status and health status at the time of exposure.

Table 2.1. Select properties of the sixteen US EPA priority pollutant PAHs.

Compound	Number of Rings[1]	Molecular Weight (g/mol)	Log(K$_{ow}$)[2]	Aqueous Solubility (mg/L)[2]	Carcinogenic Potency[3]
Naphthalene (NAP)	2	128.17	3.37	31.0	
Acenaphthylene	3	152.19	4.0	16.1	
Acenaphthene	3	154.21	3.92	3.80	
Flourene	3	166.22	4.18	1.90	
Phenanthrene (PHE)	3	178.23	4.57	1.10	
Anthracene (ANT)	3	178.23	4.54	0.045	
Pyrene (PYR)	4	202.25	5.18	0.132	
Chrysene	4	228.29	5.65	0.002	0.001
Fluoranthene (FLA)	4	202.25	5.22	0.26	
Benz[a]anthracene (BaA)	4	228.29	5.91	0.011	0.1
Benzo[b]fluoranthene	5	252.31	5.80	0.0015	0.1
Benzo[k]fluoranthene	5	252.31	6.0	0.0008	0.01
Benzo[a]pyrene (BaP)	5	252.31	6.04	0.0038	1.0
Benzo[g,h,i,]perylene	5	278.35	6.5	0.00026	
Dibenza[a,h]anthracene	6	276.33	6.75	0.0006	1.0
Indeno[1,2,3-c,d]pyrene	6	276.33	7.66	0.062	0.1

[1] PAHs with 2 or 3 rings are LMW, 4 or more rings are HMW.
[2] As in reference (29).
[3] Estimated relative cancer risk from oral exposure to US EPA Group B2 probable human carcinogens (30).

Empirical evidence of genotoxicity and carcinogenicity in animal models defines compounds in the US EPA Group B2 as probable human carcinogens, but for some PAHs there is insufficient evidence to determine carcinogenic potential (31-34). Carcinogenic potential is correlated with the presence of four or five benzene rings in an angular arrangement that includes a bay region (21, 35). LMW PAHs and PAHs with a linear molecular structure are less likely to be carcinogenic. The potential for adverse health effects generally increases with increasing affinity for an organic phase, in this case lipids, which increases with PAH molecular weight. The different levels of toxicity exhibited by different

isomeric forms of PAH metabolites is additional evidence of the relationship between a compound's structure and its potential to cause adverse health effects (21, 22, 36).

Benzo[*a*]pyrene is one of the most carcinogenic, most widely studied PAHs and has been used as the model compound for setting regulatory standards. Human exposure to BaP has been estimated to be a combined 2.2 ug/day from inhalation and ingestion of food and water (20). A series of experiments in the early 1900s led to the discovery that BaP was one compound responsible for the carcinogenicity of the mixture of compounds constituting coal tar (21). It was later found that BaP exerts this carcinogenicity through the bay region dihydrodiol epoxide pathway (21). Benzo[*a*]pyrene (BaP) has recently been classified by the International Agency for Research on Cancer as a carcinogenic to humans based on evidence of genotoxicity and carcinogenicity in animal models (32), but no studies have provided evidence for carcinogenicity in humans as a direct result of exposure to BaP.

2.5. Microorganisms can Reduce PAH Concentrations in Soil

Bioremediation is the dominant mechanism for removal of PAH contamination from soil (37). Bioremediation takes advantage of an indigenous microbial community and its ability to degrade the contaminants present at a given site. In order for the contaminant of interest to be transformed or completely degraded, it must be accessible to the degrading community, an appropriate microbial community and its nutritional requirements must be present, and that community must possess the genetic potential to be metabolically active against the contaminant.

Many species of microorganisms utilizing different lower molecular weight (LMW) PAHs, those composed of 2 or 3 fused benzene rings, as a sole carbon and energy source have been identified and cultivated in the laboratory environment. Fewer microorganisms are

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known to use higher molecular weight (HMW) PAHs, those composed of 4 or more fused benzene rings, as a sole carbon and energy source. Bacterial growth on LMW PAH has been shown to enhance the metabolism of HMW PAHs (38). Because HMW PAHs have been associated with carcinogenicity in humans (39), more attention should be given to identifying and isolating bacteria capable of metabolizing HMW PAHs to non-harmful products.

Traditional microbiological techniques, including culture-based isolation methods, are biased toward selecting organisms that can grow under static conditions on defined media. This indicates that cultivation efforts alone may be insufficient for characterizing microbial life. Amann and colleagues suggest that traditional techniques hardly address the extant microbial diversity and suggest a molecular approach to understanding the uncultured microbial majority (40). Several recent studies have focused on the identification of PAH-degrading bacterial groups using the cultivation-independent technique stable-isotope probing, which was first used as a tool in microbial ecology by Radajewski and colleagues (11).

2.6. Ingredients for Successful Bioremediation in Contaminated Soil

Soil is a receptor for PAH contamination transferred from air, surface runoff and industrial sources. Bioremediation is the dominant mechanism for removal of PAH contamination from soil (41). There are several factors that must work in concert for bioremediation to be successful in PAH-contaminated soil. These include the physical and chemical state of the soil itself, the behavior of PAHs within the soil matrix, PAH bioavailability, and the condition of the soil's microbial community.

2.6.1. Behavior of PAHs in the Soil Matrix

Soil is a dynamic and heterogeneous mixture of minerals, metals, and organic and inorganic material. The soil matrix is composed of aggregates of particles of various sizes resulting in pore spaces that liquids, gases, and microorganisms can permeate. Various depictions of the soil matrix have been published (42-44). Soil texture, porosity, pH, oxygen status, moisture content, temperature, and organic carbon content can influence permeability, nutrient distribution, sorption of PAHs to nonaqueous compartments, and the rate of PAH sorption and desorption in the soil matrix. Because of their hydrophobicity, poor aqueous solubility, and low volatility, PAHs associate with nonaqueous-phase liquids (NAPLs) and mineral and organic surfaces of the soil matrix more than the aqueous or gas phase (42). This tendency for PAHs to compartmentalize within the soil matrix increases with PAH molecular weight, leads to PAH sequestration in the soil matrix (42, 45-47), and contributes to decreased bioavailability and biodegradation (42, 47-49), especially over time (42, 45, 47-49).

2.6.2. Bioavailability of PAHs in the Soil Matrix

Successful bioremediation of PAH-contaminated soil requires that PAHs be bioavailable, or physically and biologically accessible to the soil microorganisms responsible for their degradation. This means that the PAH and the microorganisms must be in close physical proximity, and the PAH must be in a chemical form the microorganism can metabolize. PAH bioavailability in soil is primarily controlled by the mass transfer of PAHs from the nonaqueous-phase to the bioavailable fraction of the soil matrix (42-44, 49-52). The bioavailable fraction has been defined as PAHs in the aqueous phase such that bacterial metabolism cannot take place unless the substrate is dissolved in water (53, 54). However,

bacteria are known to grow on PAH crystals (44, 55, 56) and at NAPL-water interfaces (57-59) indicating that the substrate in each case was bioavailable to the bacteria even if it was not dissolved in a bulk aqueous phase. One model used to quantify PAH bioavailability to microbial cells determined that reduced mass transfer rates can indicate reduced substrate bioavailability and can result in less biodegradation (44). However, increased mass transfer rates do not always result in increased biodegradation, especially for HMW PAHs (60).

2.6.3. Microbial Factors Affecting Bioremediation

The absence of a metabolically active microbial community, insufficient numbers of specific microorganisms or quantities of required nutrients, or lack of the necessary genetic pathways will limit biodegradation and could hinder targeted bioremediation efforts. In order for PAHs to be transformed or mineralized, they must be accessible to the degrading community, an appropriate microbial community and its nutritional requirements must be present and sustainable, and that community must possess the genetic potential to be metabolically active against the contaminants.

Identifying organisms capable of metabolizing specific PAH substrates is crucial to choosing the appropriate bioremediation strategy. For example, the application of fungal-bacterial cocultures to contaminated sites has been proposed as an effective strategy for BaP degradation (61). Though fungal-bacterial cocultures have been shown to degrade HMW PAHs in uncontaminated soil spiked with a mixture of PAHs (62), the effectiveness of fungal-bacterial cocultures has not been examined in field-contaminated soil. Increased numbers of microorganisms have been shown to correspond to increased PAH removal (63), but does not always lead to enhanced biodegradation (51). Nutrient addition is generally

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considered beneficial to microbial systems, but does not always result in enhanced

biodegradation (64-67).

PAH mixtures are environmentally relevant, but can be challenging for microbial

communities to address effectively. Competitive inhibition can lead to decreased metabolism

when more than one substrate is metabolized by the same enzyme system (68-70).

Metabolites produced from one PAH can result in the inhibition of the metabolism of another

PAH in the same system causing the second PAH to persist (71). Additionally, the presence

or absence of an appropriate co-substrate can affect the degradation of an otherwise

bioavailable compound (68, 72, 73). Knowing which organisms are responsible for the

degradation of which individual compounds may help better address the challenge of

remediating PAH mixtures.

2.7. Stable-Isotope Probing

In the last 7 years, several studies have used the cultivation-independent technique

stable-isotope probing (SIP) to identify PAH-degrading bacteria. With SIP, isotopically

dense cellular material is produced by feeding an environmental sample, and therefore its

native microbial community, a ^{13}C-labeled growth substrate. The result is a pool of ^{13}C-

enriched nucleic acids that can be isolated by density-gradient ultracentrifugation. Members

of the alpha, beta, and gamma subclasses of Proteobacteria have been identified by SIP as

being capable of degrading PAHs (12, 13, 15, 74, 75). SIP has also revealed several PAH-

degrading bacterial groups that have not previously been associated with PAH degradation

(12, 15, 75).

Several details should be considered when performing a DNA-based SIP experiment.

DNA-based SIP requires that organisms assimilate labeled carbon atoms from the supplied

labeled substrate into new cellular material. Assimilation of labeled carbon atoms occurs

during bacterial growth, cell replication, and DNA synthesis. A high level of DNA labeling is

essential for the increased density needed to more completely separate labeled DNA from

unlabeled DNA during ultracentrifugation. To achieve a high level of DNA labeling the

addition of uniformly labeled substrate is preferred for community enrichment, but care must

be taken that the added label is not diluted by unlabeled growth substrates present in the

original sample. The presence of mixtures of unlabeled potential growth substrates is

unavoidable when working with contaminated soil, but to minimize partial labeling of DNA,

the labeled substrate should be the most abundant and most bioavailable carbon source in the

system. Enrichment with partially labeled substrate will likely lead to poor separation

resulting from partially labeled DNA. Insufficient incubation time can also result in partially

labeled DNA especially for slower-growing organisms. Extended incubation time may result

in crossfeeding, or assimilation of label from metabolites of the labeled substrate by

secondary consumers (76, 77). Crossfeeding can be minimized by stopping the SIP

incubation once the labeled substrate is consumed (13).

2.8.   Molecular Methods for DNA Analysis

Molecular methods used for DNA analysis include PCR, DGGE, cloning, and

sequencing. These methods have inherent biases that begin with the extraction of DNA from

the environmental sample (17). The failure of a cell to lyse during the extraction procedure

precludes that cell's DNA from being detected in any downstream molecular application.

Molecular microbial ecologists design primers, short oligonucleotide sequences, to detect,

quantify, and monitor specific bacterial groups in environmental samples without cultivation

and with minimal time and expense. These primer pairs are designed to flank a segment of

nucleic acid specific to a particular organism's 16S rRNA gene or functional genes such that the gene can be amplified by PCR.

Beyond DNA extraction, PCR is the first step in most molecular techniques. Though the imperfections of PCR have been reported (17, 78, 79), both conventional and quantitative PCR are used routinely. Conventional PCR and DGGE provide qualitative evidence of the presence or absence of a particular target in a sample, and DGGE is used to resolve mixtures of PCR-amplified targets based on sequence differences. Quantitative PCR relies on amplicon fluorescence beyond a defined threshold value to quantify the absolute or relative abundance of gene targets in a sample using a standard curve. The specificity of a primer pair to a gene target must be evaluated and standard curves must be validated for the primer pair before it can be used for quantitative purposes. Constructing an *in vivo* clone library (usually using *E. coli* as a host) is another way to resolve a mixture of PCR-amplified targets. By Sanger-based capillary sequencing and determining the taxonomy of the gene fragment within each host cell, one can obtain a semi-quantitative analysis of the composition of the targets in a sample. However, clone libraries can be biased because they tend to detect the most abundant, and possibly less diverse, fraction of the PCR-amplified sample. Though Sanger-based sequencing technologies are useful, the low-throughput (96- or 384-well plate format) method limits the amount of sequence information that can be obtained in a reasonable timeframe.

Pyrosequencing is a high-throughput technology (delivering thousands of sequences using picotiter plates) that is faster and less expensive than Sanger sequencing (80). This metagenomic, or whole community genome, deep-sequencing approach facilitates the recovery of large amounts of sequence data from a single sample or from multiple pooled

samples, and its applications include genotyping, detecting single-nucleotide polymorphisms, and identifying microorganisms (81). Pyrosequencing libraries are less labor-intensive to prepare than *in vivo* clone libraries and can detect low abundance, possibly more diverse, members of a microbial community that might be masked in an *in vivo* clone library (82). By barcoding the sequences within each unique sample in the pool, members of multiple microbial communities can be simultaneously resolved. Various computer programs are able to filter sequences into libraries based on the unique barcode such that each library can be further analyzed.

Like the previously mentioned molecular analyses, preparing a sample (or set of samples) for pyrosequencing begins with the PCR-amplification of a target gene or gene fragment. Researchers commonly sequence segments of the 9 variable regions (as opposed to the conserved regions) of the 16S rRNA gene to classify bacteria. The outcome of microbial classification (83-85) and diversity estimates (86) resulting from PCR-based analyses can be influenced by the variable region amplified. Phylum to family level classification based on pyrosequencing the V1+V2 variable region has been shown to best match that obtained from whole-genome sequencing of the same sample (85), and V1+V2 has been shown to be the ideal region to use for microbial community analyses (84). The V1+V2 region has also been shown to overestimate species richness compared to near full-length 16S rRNA gene fragments and compared to other variable regions (86). Nevertheless, short DNA sequences (100-250 bp) are adequate for analyzing microbial communities (84).

2.9. Co-metabolism of Benzo[*a*]pyrene by Bacteria

There have been no reports of bacteria capable of utilizing BaP as a sole carbon and energy source, though several bacteria can oxidize BaP when a growth substrate is provided.

Poglazova and colleagues (87) described the ability of several bacteria, including two

Bacillus species, isolated from the grounds of an oil refinery to remove endogenous BaP

from sterilized soil slurried with meat peptone broth. Khesina and colleagues (19) showed

that microorganisms indigenous to PAH contaminated soil are also capable of oxidizing BaP.

No bacteria were reportedly isolated in this work and there is no mention of the total or

LWM PAH concentration in the soil, only the concentration of BaP was reported. Further,

the authors did not address the effect that the presence of meat peptone or other PAHs may

have had on the removal of BaP. Gibson and colleagues (88) described a mutant strain of

Beijerinckia, later identified as *Sphingomonas yanoikuyae* (89), that could not grow on

biphenyl but could oxidize BaP to a *cis*-dihydrodiol after growth on succinate in the presence

of biphenyl. The authors failed to address the effect of biphenyl on the ability of the mutant

strain to oxidize BaP. It is likely that the meat peptone and LMW PAHs served as growth

substrates for Poglazova's isolates and Khesina's indigenous soil community, respectively,

while BaP was oxidized as a result of the production of enzymes used to oxidize the non-BaP

carbon sources. Similarly, the presence of biphenyl likely induced the enzymes necessary for

BaP oxidation by the *Beijerinckia* mutant. The concept of co-metabolism, the metabolism of

one substrate at the expense of another, seems not to have been established by the time these

studies were conducted, but, barring the unlikely growth on BaP itself, it is clear that the

oxidation of BaP observed by these research groups was the result of the presence of the

primary substrates.

The structural similarity between LMW and HMW PAHs is likely responsible for the

co-metabolism of HWM PAHs as a result of microbial growth on LWM PAHS (62, 90, 91).

Though the specific PAH responsible for BaP oxidation was not uncovered, incubating

Burkholderia cepacia at high cell numbers in basal salts medium with a mixture of three- to

seven-ring PAHs, including phenanthrene and fluorene, resulted in greater degradation of

BaP than when BaP was supplied as a single substrate (90). In another study, phenanthrene

stimulated BaP degradation by *B. cepacia* in basal salts medium, but the effect of fluorene

was not tested (91). The presence of naphthalene vapor or phenanthrene in silicone oil

stimulated the removal of BaP from slurried soil (59). Phenanthrene and anthracene supplied

together to cells separated from soil enhanced BaP mineralization compared to controls (92).

In each of these studies BaP and the LMW PAH were incubated together, but BaP

metabolism has also been induced by pre-incubation with the LMW PAH growth substrate.

Juhasz et al. (90) observed that *B. cepacia* removed 20-22% of BaP after growth on pyrene at

high cell numbers in basal salts medium. In another study, Chen and Aitken (93) induced

BaP mineralization in *Pseudomonas saccharophila* P15 by pre-incubating a culture with

phenanthrene. The effect of pre-incubation versus co-incubation on BaP removal or

mineralization has not been compared in the same soil sample. Some primary substrates used

to influence the removal of BaP by bacteria are listed in Table 2.2.

Table 2.2. Primary substrates used to influence BaP removal.

Substrate(s)[1]	Innoculum[2]	Matrix	Effect on BaP[3]	Ref.
Co-incubation Experiments				
Meat peptone	Native soil microorganisms	Soil in meat peptone broth	20-40% removal	(87)
Meat peptone	*Bacillus sphaericus, B. megaterium mutilate, or Pseudomonas* sp. 146	Sterile soil in meat peptone broth	48-86% removal	(87)
Presumably LMW PAHs	Native soil microorganisms	Soil in pots in a heated greenhouse	34-70% removal	(19)
PHE+ANT	Native bacteria separated from soil and enriched on 3-ring PAHs in the presence of BaP	Minimal salts medium	37% transformation; 6% mineralization[4]	(92)
PYR+FLT	Native bacteria separated from soil and enriched on 4-ring PAHs in the presence of BaP	Minimal salts medium	32% transformation	(92)
NAP or PHE	Microbial consortium enriched in a two-liquid-phase bioreactor	Bushnell-Haas mineral salt medium	> 80% removal	(59)
PYR	*Stenotrophomonas maltophilia* VUN 10,009	Basal salts medium	23% removal	(62)
PYR	Bacterial consortium VUN 10,010	Basal salts medium	32% removal	(62)
Mixture of 3- to 7-ring PAHs	*Burkholderia cepacia* VUN 10,001	Basal salts medium	78% removal	(90)
Pre-incubation Experiments				
Succinate, biphenyl	*Sphingomonas yanoikuyae*	Potassium phosphate buffer	Oxidation to *cis*-dihydrodiol	(88)
PYR	*Burkholderia cepacia* VUN 10,001	Basal salts medium	20-22% removal	(90)
PHE	*Pseudomonas saccharophila* P15		30% mineralization[5]	(93)
Salicylate	*Pseudomonas saccharophila* P15		20% mineralization[5]	(93)

[1]PHE, ANT, NAP, and PYR are as in Table 2.1 FLT, fluoranthene.

[2]All soils were naturally contaminated with PAHs and were assumed to have contained a PAH degrading microbial community.

[3]All reported values are significantly different from their respective controls.

[4][7,10-^{14}C]BaP.

[5][7-^{14}C]BaP.

3. Multiple DNA extractions coupled to stable-isotope probing of anthracene-degrading bacteria in contaminated soil[1]

Maiysha D. Jones[2], Wei Sun[3], and Michael D. Aitken

3.1. Abstract

In many of the DNA-based stable-isotope probing (SIP) studies published to date in which soil communities were investigated, a single DNA extraction was performed on the soil sample, usually using a commercial DNA extraction kit, prior to recovering the ^{13}C-labeled (heavy) DNA by density-gradient ultracentrifugation. Recent evidence suggests, however, that a single extraction of a soil sample may not lead to representative recovery of DNA from all of the organisms in the sample. To determine whether multiple DNA extractions would affect DNA yield, eubacterial 16S rRNA gene copy number, or the identification of anthracene-degrading bacteria, we performed seven successive DNA extractions on the same aliquot of contaminated soil either untreated or enriched with [U-^{13}C] anthracene. Multiple extractions were necessary to maximize DNA yield and 16S rRNA gene copy number from both untreated and anthracene-enriched soil samples. Sequences within the order Sphingomonadales, but unrelated to any previously described genus, dominated the

16S rRNA gene clone libraries derived from ^{13}C-enriched DNA and were designated "Anthracene Group 1". Sequences clustering with the *Variovorax* and *Sphingobium* genera were also highly represented, and sequences related to the genera *Herminiimonas* and *Pigmentiphaga* were newly associated with anthracene degradation. The bacterial groups collectively identified across all seven extracts were all recovered in the first extract, although quantitative PCR analysis of SIP-identified groups revealed quantitative differences in extraction patterns. These results suggest that performing multiple DNA extractions on soil samples improves extractable DNA yield and quantifiable eubacterial 16S rRNA gene copies, but has little qualitative effect on the identification of the bacterial groups associated with the degradation of a given carbon source by SIP.

3.2.   Introduction

Molecular methods are increasingly being used to explore the microbial diversity of environmental systems without needing to isolate microorganisms from their natural environment, especially because many relevant organisms have proven difficult to isolate from their environmental sources (94-96). The effectiveness of molecular methods to describe microbial diversity depends on our ability to efficiently extract and purify macromolecules from microbial cells native to an environmental sample (17). Commercially available kits are commonly used to extract nucleic acids from environmental samples by physical and/or chemical lysis of microbial cells followed by purification of the nucleic acids from cell debris and other organic material. Feinstein, et al. (18) recently demonstrated that extracting a soil aliquot only once with a commercial kit can lead to incomplete DNA extraction, thus biasing estimates of genomic DNA mass yield, small-subunit ribosomal gene copy number, and the bacterial groups identified; multiple extractions led to broader recovery of organisms in the soil community.

Bioremediation is the primary method of removing polycyclic aromatic hydrocarbons (PAHs) from PAH-contaminated environments (6), but our understanding of the roles of specific organisms within PAH-degrading microbial communities and the metabolic mechanisms responsible for PAH degradation is still developing. Stable-isotope probing (SIP) is one cultivation-independent molecular technique that can link the identity of a microorganism with its metabolic function without isolating that organism from its natural environment (97). DNA-based SIP has been used to identify bacteria capable of degrading aromatic hydrocarbons in PAH-contaminated environments, and in some cases it has revealed novel bacterial groups (12, 13, 15, 75, 98). Earlier SIP studies on pyrene-degrading bacteria conducted in our lab revealed members of previously uncultivated β- and γ-Proteobacterial groups, neither of which is related to any cultivated genus (12, 15). SIP investigations have also facilitated the isolation of ecologically relevant organisms (7, 75, 99) and have been used to reduce the complexity of community DNA slated for metagenomic analysis (100). To date, SIP of anthracene-degrading bacteria has not been reported.

In many of the DNA-based SIP studies published to date in which soil communities were investigated, a single DNA extraction was performed on the soil sample, usually using a commercial DNA extraction kit, prior to recovering the ^{13}C-labeled (heavy) DNA by density-gradient ultracentrifugation. In the present study, we performed successive DNA extractions on the same aliquot of PAH-contaminated soil either untreated or enriched with uniformly ^{13}C-labeled anthracene to determine whether multiple DNA extractions would affect DNA yield, eubacterial 16S rRNA gene recovery, or the identification of anthracene-degrading bacteria. In addition, we tested the effects of soil loading and multiple extractions on the efficiency of the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, OH).

3.3. Materials and Methods

3.3.1. Soil processing

PAH-contaminated soil was collected from a former manufactured-gas plant site in Salisbury, Rowan County, NC. The total PAH concentration was approximately 890 mg/kg, and the anthracene concentration was 32 mg/kg. Large objects were removed by hand. The soil was then sieved through a 10-mm wire screen, blended, and sieved again prior to storage in the dark at 4°C. The processed soil (64% sand, 30% silt, 6% clay, 15% moisture, pH=7.6) was further prepared by manually removing any remaining small stones and other debris immediately before use in experiments.

3.3.2. Chemicals

Natural abundance isotopomer (unlabeled) anthracene (scintillation grade) was obtained from Eastman Kodak (Rochester, NY). [U-^{13}C] Anthracene was synthesized according to methods to be described elsewhere (Z. Zhang, L.M. Ball, A. Gold, personal communication). [1,2,3,4,4a,9a-^{14}C]Anthracene (17.3 mCi/mmol) was obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were the highest purity available. All solvents were molecular biology or high-pressure liquid chromatography (HPLC) grade.

3.3.3. Enrichment with anthracene

Soil slurries were prepared in 125 mL flasks containing 1 g of soil (wet wt) and 30 mL of simulated groundwater amended with 0.37 mM NH_4NO_3 and 0.08 mM K_2HPO_4. The groundwater was prepared to reproduce the major ion concentrations in the groundwater of Rowan County, NC (1) (0.7 mM $CaCl_2·H_2O$, 0.2 mM $MgSO_4·7H_2O$, 1.0 mM $NaHCO_3$, 0.06 mM KCl, 1 N H_2SO_4; pH=7.5) and was filter-sterilized through a 0.1 μm pore-size flow-through, hollow-fiber membrane water filter (Sawyer Products, Safety Harbor, FL). Four sets of flasks

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containing the soil slurry were incubated in parallel. For each set, after two days of shaking (225

rpm) in the dark at room temperature to reduce the concentrations of native PAHs, the aqueous

phase was replaced with fresh nitrogen- and phosphorus-amended groundwater, duplicate flasks

were spiked with 625 µg of either unlabeled (set 1) or [U-^{13}C]anthracene (set 2) to enrich

anthracene-degrading microorganisms, and the flasks were returned to the shaker (t=0). In

addition, triplicate flasks containing unlabeled anthracene (set 3) were prepared to monitor

anthracene disappearance by HPLC and to archive community DNA over time. Another set of

triplicate flasks (set 4) containing a mixture of unlabeled and radiolabeled anthracene (20,000

dpm) was prepared to monitor anthracene mineralization by liquid scintillation counting of $^{14}CO_2$

trapped in KOH-soaked filter paper (101). Inhibited controls were prepared by acidifying

incubations to pH < 2 using 200 µl of 85% phosphoric acid.

3.3.4. Monitoring anthracene disappearance

Soil slurry from each triplicate flask in set 3 (1 mL) was mixed with 1 mL of ethyl acetate

in each of triplicate 15-mL conical-bottom glass centrifuge tubes. The tubes were vortexed at

maximum speed for 1 min and centrifuged for 5 min at 3,500 rpm. The organic layer of each

resulting supernatant was filtered through a 0.45 µm pore-size nylon filter and stored in a gas-

chromatography vial at -20 °C prior to HPLC analysis. The extracts were diluted with

acetonitrile as needed immediately before HPLC analysis. The HPLC system included a Waters

(Milford, MA) 600E system controller, a Waters 717 Plus autosampler, and a Perkin Elmer

(Beaconsfield, UK) LS40 fluorescence detector. Analyte standards were prepared from an EPA

610 Polynuclear Aromatic Hydrocarbons Mixture stock (Sigma-Aldrich, St. Louis, MO) and

used to create a four-point calibration curve for sample quantification. Samples were injected

through a 3-µm particle-size Supelcosil™ LC-PAH column (Sigma-Aldrich, St. Louis, MO)

using a gradient mobile phase of acetonitrile and water and analyzed as previously described (101).

3.3.5. DNA extraction

DNA was extracted from the soil in each flask from sets 1 and 2 in two 500-mg aliquots using the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, OH) according to the accompanying instructions with the following exceptions. Samples were secured horizontally to a bench-top vortexer set at maximum speed for homogenization. After each extraction, fresh sodium phosphate and MT buffers were added to the Lysing Matrix E tube containing the original soil aliquot and the extraction procedure was repeated. DNA was eluted from each successive extraction with Tris-EDTA buffer (TE, pH=8.0) into a clean catch tube until seven extractions had been performed. The equivalent extracts of each 500-mg aliquot from a given incubation flask were pooled prior to further analysis; because there were duplicate incubation flasks, there were duplicate series of seven DNA extracts. For the flasks from set 3, the FastDNA® Spin Kit for Soil was used to perform a single DNA extraction on a soil pellet resulting from 1 mL of soil slurry containing approximately 33 mg of soil (wet wt). In a subsequent experiment, DNA was extracted from untreated soil in duplicate aliquots of 33, 100, 250, or 500 mg (wet wt) using the same multiple-extraction procedure described above, except that six successive extractions were performed.

3.3.6. DNA and 16S rRNA gene quantification

The DNA mass yield was quantified with a NanoDrop 3300 fluorospectrometer (NanoDrop Products, Wilmington, DE) using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Eugene, OR). The 16S rRNA gene copy number of a targeted sequence was determined by quantitative PCR (qPCR) using 1μl of DNA as template, primers (final

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concentration 600 mM) as identified in Table 3.1, and QuantiTect™ SYBR® Green PCR Master

Mix (Qiagen, Valencia, CA) with the SmartCycler platform (Cepheid, Sunnyvale, CA) in a 25 μl

reaction. Primer sets for targeted quantification of several SIP-identified groups were designed

and validated as described elsewhere (12) except that sequences were aligned within the myRDP

personalized workspace (102). The qPCR temperature program included 15 min at 95°C

followed by 45 cycles of 15 sec at 95°C, 30 sec at the annealing temperature (Table 3.1), and 30

sec at 72°C. Data were collected during primer extension, and reaction products were analyzed

by melt curve analysis between 65 and 95°C. The r^2 value for each qPCR standard curve (cycle

threshold vs. log gene copy number) was ≥0.995, and the amplification efficiencies of curves

from eubacterial and group-specific primer sets were close to 2.0 (Table 3.1). To compare the

abundance of the SIP-identified bacteria in the heavy DNA to their abundance in the light DNA,

each group-specific primer set was used to quantify the corresponding sequences in each fraction

from one ultracentrifuge tube.

3.3.7. DNA separation and recovery

DNA extracted from anthracene-enriched samples was mixed with 20 μl of SYBR safe™

(Invitrogen, Carlsbad, CA), and the mixture was added to 6-mL polyallomer ultracrimp tubes

(Kendro Laboratory Products, Newtown, CT). SYBR safe™ is an alternative to ethidium

bromide in CsCl density gradients used in DNA-based SIP assays (106) that simplifies the

cleanup of fractions collected from ultracentrifuge tubes because ethidium bromide does not

have to be extracted. Including a fluorescent dye in the CsCl solution allowed us to visualize

bands of DNA post-separation, thus locating approximate positions of fractions containing DNA

of interest. The tubes were filled with a cesium chloride solution (ρ=1.72 g/mL), crimp-sealed,

and ultracentrifuged (RC70 ultracentrifuge, Sorvall, Newtown, CT) at 175,800 x g and 20°C for

Table 3.1. Quantitative PCR primers used in this study.

Target Group	Primer Name	Primer Sequence (5'→3')	T_M (°C)[1]	qPCR Standard[2]	Amplicon Length	Amp. Eff.[4] (Bac; Group)	RDP II Hits[5]	Reference
Bacteria	341F	CCTACGGGAGGCAGCAG	60	--	177	--	--	(103)
	517R	ATTACCGCGGCTGCTGG						
Anthracene Group 1	AG1F	TTCGGAATAACTCCTC	50	sbant93	102	1.97; 2.03	15	This study
	AG1R	TCACCAACTAGCTAATCC						
Variovorax	VARIO.2F	AGCTGTGCTAATACCGCATA	55	sbant158	67	2.11; 1.94	634	This study
	VARIO.2R	TCCATTCGCGCAAGGTCTTG						
Sphingobium	SGB.5F	ACAGTACCGGGAGAATAAGCTC	56	sbant43	158	1.98; 1.92	128	This study
	SBG.5R	CAAGCAATCCAGTCTCAAAGGCTA						
Herminiimonas	HERM.1F	TATCGGAACGTACCCTAG	52	sbant22	116	1.95; 1.97	380	This study
	HERM.1R	TATCGGCCGCTCCATG						
Pigmentiphaga	PIGMF	CAGGCGGTTCGGAAAG	56	sbnap45[3]	63	1.91; 2.03	17	This study
	PIGMR	TGACATACTCTAGTTCGGGA						

[1] PCR annealing temperature.

[2] Clone name for plasmid DNA used to generate standard curves, linearized with NcoI.

[3] *Pigmentiphaga*-related sequences were identical to those recovered from an earlier SIP experiment with naphthalene (results to be published elsewhere), thus the difference in the sequence name relative to the names of the other sequence standards.

[4] Amp. Eff., Amplification efficiency (104) with eubacterial (Bac) and group-specific (Group) primers.

[5] Number of sequences returned by the Ribosomal Database Project II release 10.18 (105) (excluding sequences from this study) with no mismatches to primer pairs.

40 h in a TV-1665 vertical rotor (Sorvall). A tube containing 1 µg each of *Escherichia coli* K12 DNA from a culture grown in LB broth and a *Pseudomonas putida* G7 culture grown on uniformly labeled [^{13}C]glucose (Cambridge Isotope Laboratories, Inc.) was included as a control to verify separation of unlabeled DNA from ^{13}C-labeled DNA. DNA bands were visualized with the Safe Imager™ blue light transilluminator (Invitrogen, Carlsbad, CA) before collecting 24 fractions of 250 µl each from the bottom of each ultracentrifuge tube as described by Singleton, et al (13). Separation of unlabeled and ^{13}C-labeled DNA achieved in the control tube is illustrated in Appendix A (Figure A1). DNA in each fraction was recovered by ethanol precipitation (107) and resuspended in 100 µl of 0.2 µm filter-sterilized TE (pH=8.0).

3.3.8. Identification of heavy and light DNA fractions

The DNA concentration and eubacterial 16S rRNA gene copy number in each DNA fraction recovered were quantified, and the eubacterial community profile in each fraction was visualized by denaturing-gradient gel electrophoresis (DGGE). PCR for DGGE targeted the V1-V3 hypervariable region of the 16S rRNA gene and was performed with 5Prime Mastermix (Gaithersburg, MD) using 1µl of DNA as template and primers 63F-GC and 517R (final concentration 200 nM each) in a 20 µl reaction as previously described (108). The temperature program was modified such that 10 cycles of touchdown PCR was followed by 15 cycles of conventional PCR. PCR products were loaded onto a 6.5% polyacrylamide gel without denaturant stacked on top of a 6.5% polyacrylamide gel with a urea-formamide denaturing gradient between 30% and 60% and run for 16 h at 60 V on a DCode system (Bio-Rad Laboratories, Hercules, CA). DGGE gels were post-stained with ethidium bromide, and bands were visualized under UV transillumination.

Consecutive fractions in the ultracentrifuge tube with similar DNA concentrations, 16S rRNA gene copy numbers, and community profiles were pooled and identified as the composite heavy or light DNA fraction, depending on the section of the tube from which the fractions were removed. Effort was made not to interrupt a peak when deciding which individual fractions to combine to create composite heavy and light fractions. DNA from the composite heavy fraction of each anthracene-enriched replicate was screened for archaeal rRNA gene sequences using primers 25F (109) and 1492R (110) and for fungal rRNA gene sequences using primers ITS1F (111) and ITS4 (112) before being used as template to generate a eubacterial 16S rRNA gene clone library.

3.3.9. Clone library preparation and analysis

After identifying the fractions corresponding to heavy DNA in extracts from each [U-^{13}C]anthracene-enriched replicate, PCR was performed with 1 µl of heavy DNA as template, primers 8F (113) and 1492R (110) (final concentration 200 nM), and 5Prime Mastermix in a 50 µl reaction. The PCR temperature program included 10 min at 94°C followed by 25 cycles of 1 min at 94°C, 1 min at 50°C, and 3 min at 72°C, and ended with one 15 min cycle at 72°C. PCR products were cloned using a TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) according to the accompanying instructions. Plasmid DNA from a random subset of clones was subjected to restriction analysis prior to sequencing to ensure successful ligation of the insert to the plasmid vector. Inserts were partially sequenced with primer 8F by Functional Biosciences, Inc (Madison, WI).

Sequences were analyzed by VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen) to remove vector contamination, Bellerophon (114) was used to screen for chimeric sequences, and RDP Classifier (115) was used to determine the closest cultivated genus to each

sequence. Each sequence was compared to public sequence databases using BLASTN (116) and RDP release 10.17 (105) to identify closely related sequences. Multiple sequence alignments and guide trees were produced using myRDP (102) and ClustalX (117), respectively, which facilitated grouping of sequences most similar to one another and to those in GenBank. Rarefaction curves were generated at 3% sequence distance to test whether clone libraries of adequate size had been generated. The UniFrac significance test (118) was used to determine whether the clone libraries were significantly different from one another. Clones were named to indicate the source soil (SB: Salisbury) and growth substrate (ANT: anthracene) and were numbered.

3.3.10. Nucleotide sequence accession numbers

Sequences recovered from this study were deposited in GenBank with accession numbers HM596084-HM596270.

3.4. Results

3.4.1. Anthracene removal and mineralization

Soil slurry was incubated in triplicate with unlabeled anthracene (to follow anthracene removal) or a mixture of unlabeled and radiolabeled anthracene (to follow mineralization) for 20 d. After 3 d, less than 10% of the added anthracene remained in the flasks containing unlabeled anthracene. However, the mineralization experiment continued until the rate appeared to decline at day 20 (Figure 3.1), which was then selected as the time to terminate SIP incubations with [U-^{13}C]anthracene.
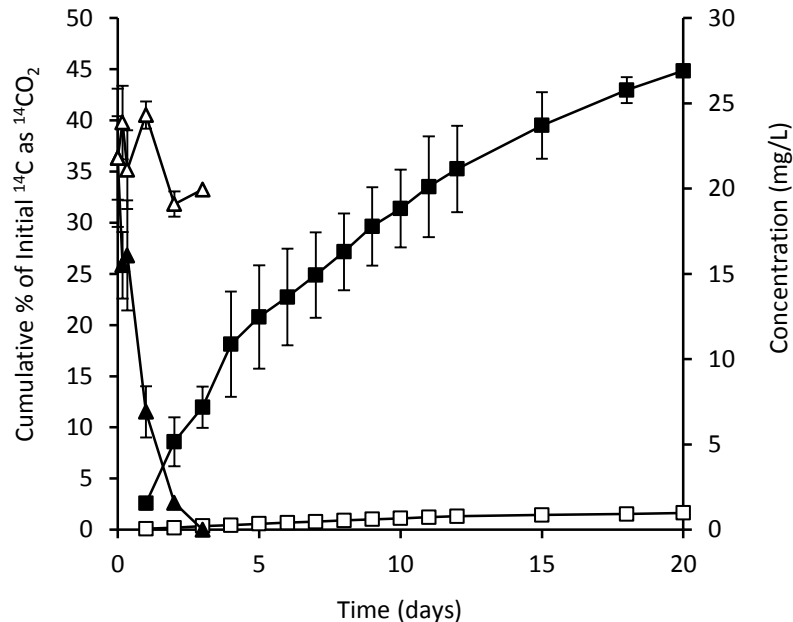
Figure 3.1. Cumulative $^{14}CO_2$ recovered from incubations with ^{14}C-anthracene (squares) and anthracene removal from incubations with unlabeled anthracene (triangles) by the indigenous Salisbury soil microbial community. Filled and open symbols represent live and inhibited incubations, respectively. Values are the mean and range of duplicate incubations for mineralization or the mean and standard deviation (n = 3) for anthracene removal. Some error bars are smaller than the symbol.

3.4.2. SIP with anthracene and identification of heavy DNA

After 20 d, seven successive DNA extractions were performed on duplicate aliquots

of 500 mg (wet wt) of soil from each of duplicate flasks containing unlabeled or [U-

^{13}C]anthracene. For comparison, six successive extractions were performed on replicate 500-

mg aliquots of the untreated original soil sample. Quantifiable amounts of DNA were

obtained through the six extractions of the untreated soil (total over 6 extractions = 0.98 ±

0.01µg/g dry soil), although the number of 16S rRNA genes in the sixth extract was

negligible (Figure 3.2; total over 6 extractions = $2.24 \times 10^8 \pm 1.99 \times 10^7$ gene copies/g dry

soil).The pairwise Wilcoxon signed rank test determined that the amount of DNA (p = 0.85)
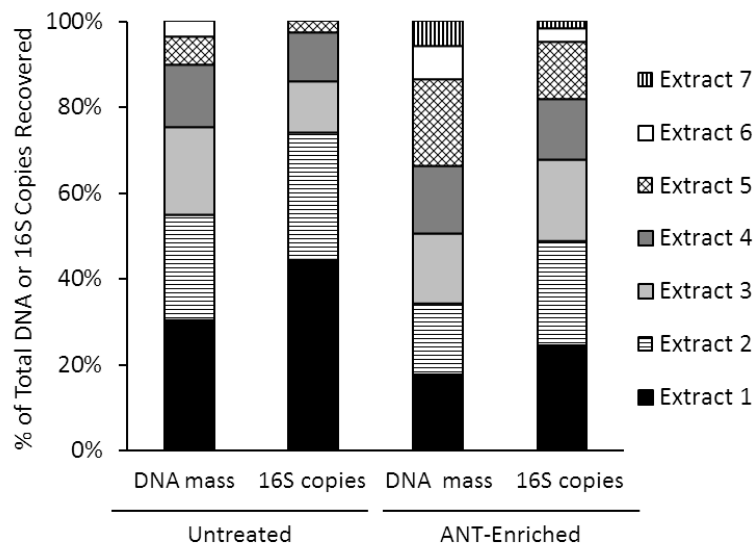
Figure 3.2. Recovery of DNA mass and eubacterial 16S rRNA genes from successive DNA extracts of the original (untreated) or anthracene-enriched soil. DNA mass values are the means of triplicate measurements from each of duplicate soil aliquots. Values for 16S rRNA gene copy number are means of a single analysis of each of two soil aliquots. ANT, anthracene.

and 16S rRNA genes (p = 0.36) recovered at each extraction step was similar in each of the duplicate extraction series. Both total DNA (39.0 ± 2.65 µg/g dry soil) and 16S rRNA genes ($2.37 \times 10^{11} \pm 2.60 \times 10^9$ gene copies/g dry soil) were quantifiable through the seventh extraction of anthracene-enriched soil (Figure 3.2). The amount of DNA (p = 0.30) and 16S rRNA genes (p = 0.81) recovered at each extraction step was similar in each of the duplicate extraction series.

DGGE analysis of anthracene-enriched samples revealed the same banding patterns for extracts 1-4, but additional bands were present in lanes containing amplicon from extracts 5-7 (not shown). Based on this analysis, for each incubation flask, the DNA from extracts 2-4 (19 µg) was pooled and the DNA from fractions 5-7 (13 µg) was pooled prior to ultracentrifugation. The pooled extracts from each duplicate flask were loaded into separate ultracentrifuge tubes and centrifuged in parallel to duplicate tubes containing DNA from

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extract 1 (7 µg).

Fractions collected from each of the ultracentrifuge tubes were analyzed by measuring DNA concentration and eubacterial 16S rRNA gene abundance, as well as by DGGE. In the tubes containing DNA from incubations with unlabeled anthracene, there was a single peak over a range of fractions corresponding to where unlabeled ("light") DNA was observed in the control tube (Figure A2A in Appendix A). No measurable DNA was observed in lower fractions, and 16S rRNA gene abundance was at a background level (2.0 to 2.5 log gene copies) in the range of fractions corresponding to the location of heavy DNA in the control tube. DGGE bands were visible in these lower fractions, but the banding patterns were identical to those from fractions containing unlabeled DNA (Figure A3A). In the tubes containing DNA from incubations with ^{13}C-labeled anthracene, the fractions containing measurable DNA also had quantifiable eubacterial 16S rRNA genes up to three orders of magnitude above the background level (Figure A2B-D). Differences in DGGE banding patterns (Figure A3B-D) between fractions were used to select the fractions corresponding to heavy DNA in each tube. No archaeal or fungal rRNA genes were detected in any heavy DNA fraction.

3.4.3. Analysis of sequences recovered from 16S rRNA gene clone libraries

A 16S rRNA gene clone library was generated from the heavy DNA recovered from each replicate of extract 1, pooled extracts 2-4 and pooled extracts 5-7. For each replicate, 32 clones were partially sequenced (192 total), of which one containing a vector sequence and four containing chimeras were excluded from further analyses. Phylogenetic analysis of the recovered sequences is illustrated in Figure A4, and major groups corresponding to these sequences are summarized in Table 3.2. Rarefaction analysis indicated that an adequate

number of clones was sequenced (Figure A5). The pairwise UniFrac significance test (weighted and normalized to account for sequence abundance and branch lengths, respectively) determined that libraries from extract 1 and extracts 5-7 were significantly different from one another ($p \leq 0.002$); however, all groups identified in extracts 2-4 and 5-7 were also identified in extract 1. Other library pairs (extract 1 vs extracts 2-4 and extracts 2-4 vs extract 5-7) were not significantly different from one another ($p > 0.1$). Sequences clustering with members of the order Sphingomonadales, but that are unrelated to any previously described genus, dominated each clone library (14 of 61 for extract 1, 25 of 63 for pooled extracts 2-4, and 42 of 63 for pooled extracts 5-7) and were designated "Anthracene Group 1" (AG1). Other well-represented sequences were similar to sequences representing the genera *Variovorax* (93% similar) and *Sphingobium* (93%); sequences related to *Herminiimonas* (99%) and *Pigmentiphaga* (99%) were found less frequently (Table 3.2).

Table 3.2. Bacterial groups associated with anthracene degradation by stable-isotope probing.

Genus or Group	Number of Clones			
	Extract #1	Extract #2-4	Extract #5-7	Total
Variovorax	17	20	8	45
Anthracene Group 1	14	25	42	81
Sphingobium	9	12	5	26
Pigmentiphaga	3	3	1	7
Herminiimonas	7	0	1	8
Other (# of different groups)	11 (5)	3 (3)	6 (4)	20 (11)
Total	61	63	63	187

3.4.4. Quantification of SIP-identified bacteria

Primers for quantitative PCR that targeted the 16S rRNA gene were developed to measure the abundances of the major bacterial groups associated with anthracene degradation

by SIP (Table 3.1). Quantitative PCR analyses of unseparated DNA reserved from each of the ^{13}C-anthracene enrichments revealed that there was differential recovery of each SIP-identified group compared to the eubacterial community with successive extractions (Figure 3.3); the most disparate recovery pattern occurred with AG1 sequences. To test the hypothesis that the extraction patterns of 16S rRNA genes from the different groups are not correlated over the seven extractions, we calculated the correlation between each pair of groups, permuted the data 2000 times, and calculated correlations using the permuted data (Table A1). We could not reject the hypothesis for comparisons between AG1 and any other group ($p = 0.19$ to 0.57), which suggests that the pattern of 16S rRNA gene extraction for AG1 was different from the extraction pattern of each other group. The tests between *Sphingobium* and *Herminiimonas* ($p = 0.08$) and between *Variovorax* and *Herminiimonas* ($p = 0.10$) were marginally significant, suggesting a relatively weak correlation. Tests between
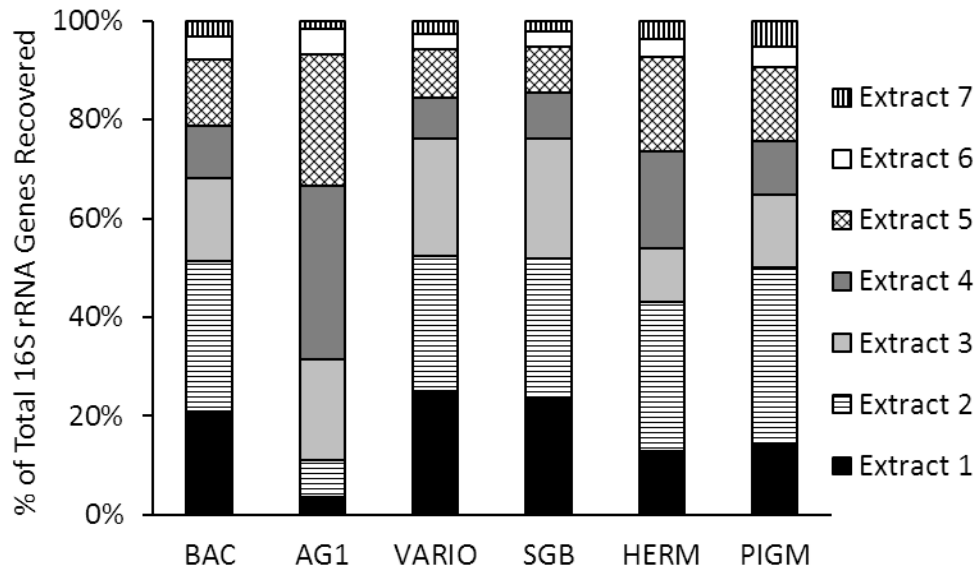
Figure 3.3. Differential recovery of eubacterial (BAC) and group-specific 16S rRNA genes in unseparated DNA from each successive extract of soil enriched with ^{13}C-anthracene. Values are means of triplicate qPCR analyses. AG1, Anthracene Group 1; VARIO, *Variovorax*; SGB, *Sphingobium*; HERM, *Herminiimonas*; PIGM, *Pigmentiphaga*.

all other pairs were significant ($p < 0.02$), suggesting similar extraction patterns.

During incubations in the presence of unlabeled anthracene, there was up to a 3-log increase in 16S rRNA genes of SIP-identified groups after three days (Figure 3.4), corresponding to when the majority of the added anthracene had been removed. Of the sequences associated with anthracene degradation by SIP, only those related to *Sphingobium* were above the quantification limit in the original soil and after two days of pre-incubation before anthracene addition (Figure 3.4). The abundance of *Pigmentiphaga*-related sequences did not change significantly after day 3. Sequences representing AG1 also did not increase in abundance after day 3, but decreased by over an order of magnitude between day 15 and day 20, when the SIP experiment was terminated. *Variovorax*-, *Herminiimonas*-, and *Sphingobium*-related sequences continued to increase in abundance between day 3 and day 7. There was little change after day 7 for *Variovorax* and *Sphingobium*-related sequences, but *Herminiimonas*-related sequences decreased by an order of magnitude between day 15 and day 20.

Each group-specific primer set was used to quantify the corresponding sequences in each DNA fraction recovered from extract 2-4 of the SIP incubation (Figure 3.5). Although the peaks for each group were in lower fractions than the peak of unlabeled DNA (Figure A2A), the peaks were relatively broad and in some cases (HERM, SGB, and VARIO) were shifted to higher fractions than the other groups (AG1 and PIGM), suggesting that the DNA may have been only partially enriched in ^{13}C (16).

3.4.5. Effects of soil loading and multiple extractions on extraction efficiency

Using the FastDNA Spin Kit for Soil®, DNA was extracted six successive times from untreated soil in duplicate aliquots of 33, 100, 250, or 500 mg to determine the optimum soil
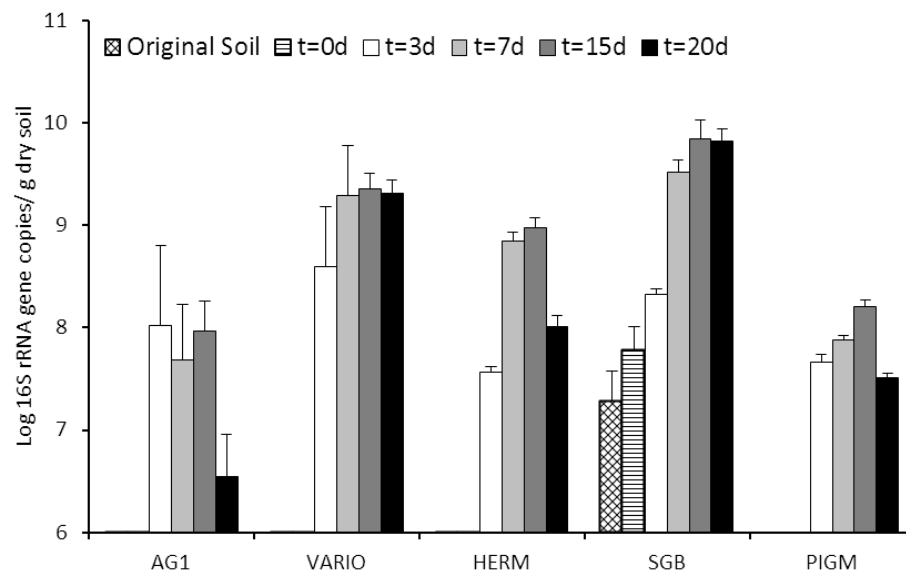
Figure 3.4. Abundances of group-specific 16S rRNA genes over time (days) in response to enrichment with unlabeled anthracene; t = 0 is when anthracene was first added to the incubation flask after two days of incubating the soil slurry without anthracene. Values are the mean and standard deviation (n=3). Eubacterial values are the mean and standard deviation of the combined triplicate qPCR reactions calculated for each of the group-specific templates. Absent bars indicate that the value was below the quantification limit of the assay (AG1: 1.29×10^6; VARIO: 8.10×10^7; HERM: 1.04×10^6; PIGM: 8.86×10^6 gene copies). Abbreviations are as in Figure 3.3.

load and number of extractions needed to maximize DNA mass yield and 16S rRNA gene recovery from the same aliquot of PAH-contaminated soil (Figure A6). With a single extraction, DNA mass yield decreased with increasing soil load ($r^2 = -0.94$), but soil loads less than 500 mg did not affect the yield of quantifiable 16S rRNA genes recovered in the first extraction (500 mg vs 33, 100, and 250 mg; p = 0.07). Subsequent extractions resulted in further recovery of DNA and 16S rRNA genes, but there was relatively little recovery beyond the second extraction for soil loads up to 250 mg.

3.5. Discussion

In DNA-based SIP experiments with soil, it is important to recover as much of the ^{13}C-labeled DNA as possible. Feinstein, et al. (18) recently demonstrated that multiple
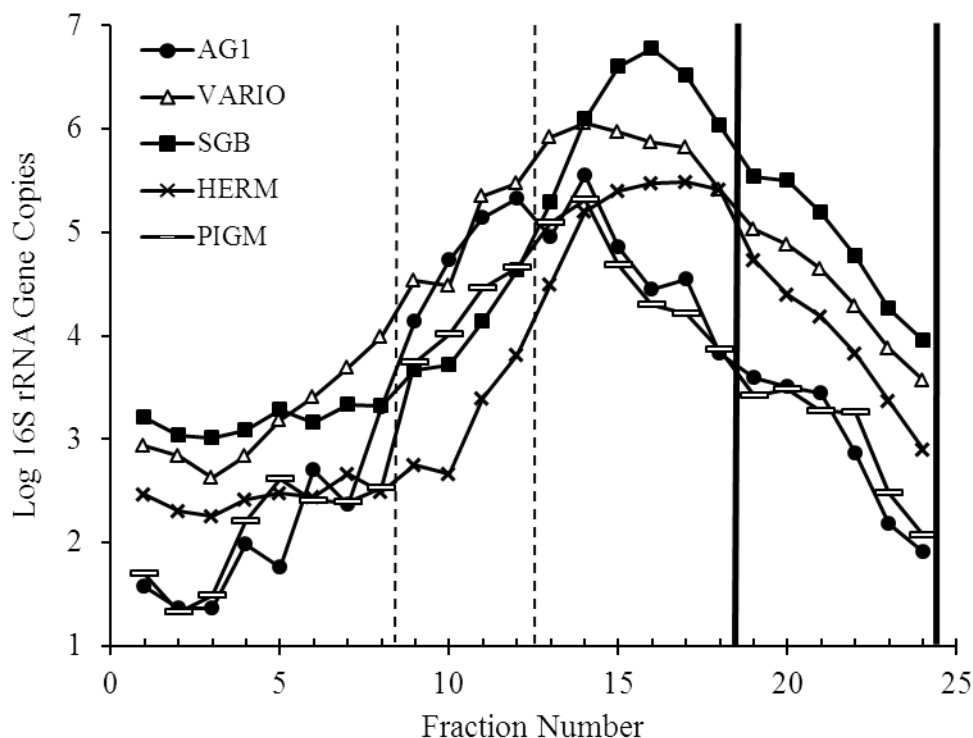
Figure 3.5. Absolute abundance of each SIP-identified group in each fraction of the ultracentrifuge tube from one replicate of extract #2-4. Each value is from a single reaction performed on each fraction using the group-specific primer sets listed in Table 3.1. The dashed or bold lines bracket the range of fractions identified as containing primarily heavy or light DNA on the basis of DGGE analysis (Figure A3C). Abbreviations are as in Figure 3.4.

extractions of an uncontaminated forest soil led to shifts in the relative abundance of various

phyla and in OTU composition in pyrosequence libraries between the first and sixth

successive DNA extract. The efficiency of DNA extraction kits used to recover DNA from

SIP experiments has not been determined. We performed multiple, successive DNA

extractions of 500-mg aliquots of PAH-contaminated soil that was enriched with anthracene

to determine whether multiple extractions would affect DNA yield, eubacterial 16S rRNA

gene recovery, or the identification of bacteria associated with anthracene degradation by

SIP. Compared to a single DNA extraction, multiple DNA extractions maximized DNA yield

and the recovery of 16S rRNA genes. Although an initial DGGE profile of each extract

suggested that DNA from additional bacteria may have been extracted in later extractions, clone libraries with adequate sequence coverage (Figure A5) did not support this observation (Table 3.2). Successive DNA extractions did not result in the identification of additional anthracene-degrading bacterial groups beyond those identified in the first DNA extraction (Table 3.2), but there was a significant shift in the relative abundance of the identified genera across successive extracts, specifically from extract 1 to extract 5-7 ($p \leq 0.002$). Overall, a group of bacteria within the Sphingomonadales, but not similar to any known genus (designated "Anthracene Group 1"), and members of the *Herminiimonas* and *Pigmentiphaga* genera were newly associated with anthracene degradation as a result of this study.

3.5.1. Assessment of anthracene-degrading microbial community activity

The activity of the anthracene-degrading microbial community native to the PAH-contaminated soil sample was assessed by measuring parent anthracene removal and radiolabeled anthracene mineralization. Because [1,2,3,4,4a,9a-^{14}C]anthracene is labeled on only one of the two end rings of the symmetrical anthracene molecule, the actual amount of mineralization should be at least twice the accumulation of $^{14}CO_2$ measured; thus, at least 25% of the anthracene was mineralized over the first three days, when the majority of anthracene was removed (Figure 3.1). However, mineralization continued through day 20 (Figure 3.1), which might be attributed to the transformation of at least some of the anthracene to one or more intermediates over the first three days, followed by slower mineralization of such intermediates and turnover of intracellular carbon. Nevertheless, substantial growth occurred over the first three days for all of the SIP-identified bacteria other than those related to *Sphingobium* (Figure 3.4), suggesting that these organisms grew on anthracene itself. Increases in abundance continued to occur after day 3 for sequences

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related to *Variovorax*, *Herminiimonas*, *Sphingobium*, and *Pigmentiphaga*, which may have resulted from growth on any anthracene metabolite(s) that had accumulated over the first three days or from slow growth on substrates unrelated to anthracene. This in turn may have led to the dilution of ^{13}C-labeled DNA and a decrease in the density and quantity of the heavy DNA belonging to these groups relative to AG1 (noticeable shifts of the peaks to the right in Figure 3.5). Any such dilution of ^{13}C-labeled DNA would have influenced the relative abundance of the SIP-identified groups in the clone libraries (Table 3.2). We note, however, that the absolute abundance of AG1, which dominated the heavy DNA clone libraries, was much higher at earlier time points than at the time the SIP incubation was terminated (Figure 3.4). Terminating the experiment at an earlier time point would have reduced the chances for label dilution, but allowing enough incubation time for adequate initial labeling of the DNA is also important to consider. In our experience, terminating the SIP incubation once reduced microbial activity is observed (as indicated by reduced mineralization rate) is a reasonable compromise.

Of the organisms associated with anthracene degradation by SIP, organisms related to AG1 and members of the genus *Herminiimonas* have previously been associated with hydrocarbon-contaminated environments, but not specifically with anthracene degradation. Sequences representing AG1 are 98.6 % similar to sequences previously recovered from the Rancho La Brea Tar Pits in Los Angeles, CA (119). Sequences 99.4% similar to the *Herminiimonas*-related sequences identified in this study were associated with a phenanthrene-degrading isolate recovered from PAH-contaminated soil from a former coal gasification plant in Iowa City, IA (120). *Variovorax* spp. have previously been associated with the degradation of biphenyl (121) and naphthalene (74, 75), but not with anthracene

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degradation. In the only previous study to associate the genus *Pigmentiphaga* with PAH contamination, 16S rRNA gene sequences > 97% identical to *Pigmentiphaga kullae* were recovered from creosote-contaminated soil, but an isolate was not recovered to be tested for specific PAH utilization (74, 122).

Sphingobium-related sequences were the only sequences recovered from SIP incubations that were present at an abundance $> 10^6$ gene copies/g dry soil in the original soil (Figure 3.4). These sequences increased in abundance during the two-day incubation period preceding anthracene addition, indicating that these organisms were capable of growing on the contaminants already in the soil. Although these sequences increased in abundance slightly after three days of incubation with anthracene, the greatest increase occurred after the added anthracene had been consumed. This observation suggests that *Sphingobium*-related bacteria grew on something other than anthracene itself. *Sphingobium* spp. have previously been associated with the degradation of PAHs composed of up to 5 rings (122-124), but only co-oxidation of anthracene by a *Sphingobium* sp. has been reported (124).

3.5.2. Effect of multiple DNA extractions

Similar to the findings of Feinstein, et al. (18), multiple DNA extractions did not result in the identification of additional bacterial groups compared to those identified from a single extraction. However, a single extraction might have resulted in much lower representation of sequences belonging to AG1 than we observed from the pooled extracts (23% vs. 43%, respectively, in Table 3.2; see also Figure 3.3), but there were shifts in relative abundance across the range of extracts. The DNA recovered from single extractions of multiple aliquots of soil from the same site is often pooled to reduce the potential effects of extraction bias with such heterogeneous material, but pooling DNA will also increase the

amount of total DNA recovered. Testing the efficiency of the FastDNA Spin Kit for Soil®

(Figure A6) revealed that a single extraction of the maximum 500-mg soil load

recommended by the manufacturer was not optimum for the recovery of genomic DNA or

16S rRNA genes from a PAH-contaminated soil. It is likely that at this soil load, natural

organic matter or contaminants in the soil co-extracted with DNA resulted in competition for

binding sites on the solid-phase sorbent and may also have interfered with PCR of the eluted

material (125). We cannot extrapolate our observations to other soils, but we recommend that

DNA extraction efficiency as a function of soil load be determined before extensive

experimentation with any given soil and DNA extraction kit.

The benefits of performing multiple DNA extractions on a sample will depend on the

downstream applications of the DNA. In an SIP investigation, it is important to maximize

DNA recovery, particularly if the heavy DNA in the metagenome is only partially labeled.

Partial labeling leads to weaker separation from unlabeled DNA during ultracentrifugation

than if the DNA were nearly 100% enriched in ^{13}C. Overall, this study demonstrated that

optimizing the recovery of ^{13}C-enriched DNA from an SIP experiment may represent a

compromise between the length of the incubations, the number of successive DNA

extractions, and the number of soil aliquots for a given soil load.

3.6.   Acknowledgements

4. Comprehensive stable-isotope probing of the polycyclic aromatic hydrocarbon-degrading bacterial guild in a contaminated soil [1]

Maiysha D. Jones[2], Douglas W. Crandell[3], David R. Singleton[4], Michael D. Aitken

4.1. Abstract

The bacteria responsible for the degradation of naphthalene, phenanthrene, pyrene, fluoranthene, or benz[*a*]anthracene in a polycyclic aromatic hydrocarbon (PAH)-contaminated soil were investigated by DNA-based stable-isotope probing (SIP). Clone libraries of 16S rRNA genes were generated from the ^{13}C-enriched ("heavy") DNA recovered from each SIP experiment, and quantitative PCR primers targeting the 16S rRNA gene were developed to measure the abundances of many of the SIP-identified sequences. Clone libraries from the SIP experiments with naphthalene, phenanthrene, and fluoranthene primarily contained sequences related to bacteria previously associated with the degradation of those compounds. However, *Pigmentiphaga*-related sequences were newly associated with naphthalene and phenanthrene degradation, and sequences from a group of uncultivated γ-Proteobacteria known as Pyrene Group 2 were newly associated with fluoranthene and benz[*a*]anthracene degradation. Pyrene Group 2-related sequences were the only sequences

recovered from the clone library generated from SIP with pyrene, and they were 82% of the sequences recovered from the clone library generated from SIP with benz[*a*]anthracene. In time-course experiments with each substrate in unlabeled form, the abundance of each of the measured groups increased in response to the corresponding substrate. These results provide a comprehensive description of the microbial ecology of a PAH-contaminated soil as it relates to the biodegradation of PAHs from two to four rings, and they underscore that bacteria in Pyrene Group 2 are well-suited for the degradation of four-ring PAHs.

4.2. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of hazardous organic compounds regulated by the U.S. Environmental Protection Agency and are listed among the top ten contaminants found at Superfund sites in the United States (6). PAHs are a natural component of coal, petroleum, and other fossil fuels, and PAH contamination can result from the incomplete combustion of these and other organic materials. PAHs can also enter environmental systems when industrial products or wastes containing high concentrations of these compounds are accidentally released to the environment or otherwise disposed of improperly.

Bioremediation is a viable option for reducing PAH contamination in soil (37), but in order to develop the most appropriate and cost-effective approaches to the bioremediation of a contaminated site, the microbial ecology of that site, as it relates to the contaminants of interest, should be understood to the fullest extent possible. Cultivation-based (126-129) and cultivation-independent (12, 13, 15, 16, 74, 75, 130) techniques have been used to evaluate the microbial ecology of PAH degradation. However, the traditional approach of isolating and culturing bacteria greatly underestimates the diversity of the prokaryotic world (131) and fails to account for the complex interactions of the members of microbial communities with each other and with

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their native environment. Cultivation-independent techniques can help us better estimate the prokaryotic diversity of complex systems (40, 94, 132), where it can be difficult to establish which organisms are responsible for the degradation of particular contaminants.

Stable-isotope probing (SIP) is a cultivation-independent technique that allows us to study the microbial ecology of specific-substrate degradation (11) . To date, SIP has been used to identify soil bacteria capable of degrading the PAHs naphthalene (13, 74, 75), phenanthrene (13, 16), pyrene (12, 15, 16) and anthracene (Chapter 3). As part of a larger project investigating strategies for the bioremediation of PAH-contaminated soil from a former manufactured-gas plant site, we performed DNA-based SIP with [U-^{13}C]naphthalene, phenanthrene, pyrene, fluoranthene, or benz[*a*]anthracene. The identification of fluoranthene- or benz[*a*]anthracene-degrading bacteria by SIP has not been previously reported. This study represents the most comprehensive SIP-based investigation of the bacterial guild responsible for the degradation of a range of related compounds in a contaminated soil.

4.3. Materials and Methods

4.3.1. Soil sample

PAH-contaminated soil from a former manufactured-gas plant site in Salisbury, NC was processed as previously described (Chapter 3) and stored in the dark at 4°C until use. The total concentration of PAHs regulated by the U.S. Environmental Protection Agency was determined by high-pressure liquid chromatography (HPLC) as previously described (101) and was approximately 890 mg/kg. The native concentrations of naphthalene, phenanthrene, pyrene, fluoranthene, and benz[*a*]anthracene were approximately 74, 362, 100, 34, and 65 mg/kg, respectively.

4.3.2. Substrates and chemical reagents

The natural abundance isotopomers (unlabeled versions) of naphthalene, phenanthrene, pyrene, and fluoranthene were obtained from Sigma-Aldrich (St. Louis, MO), and benz[*a*]anthracene was obtained from Acros Organics (NJ). [U-^{13}C] versions of each compound were synthesized by methods to be described elsewhere (Z. Zhang, L.M. Ball, and A. Gold, personal communication). [U-^{14}C]Naphthalene (17.8 mCi/mmol), [9-^{14}C]phenanthrene (8.3 mCi/mmol), [4,5,9,10-^{14}C]pyrene (61 mCi/mmol), and [3-^{14}C]fluoranthene (45 mCi/mmol) were obtained from Sigma-Aldrich (St. Louis, MO). [5,6-^{14}C]Benz[*a*]anthracene (54.6 mCi/mmol) was obtained from Chemsyn Science Laboratories (Lenexa, KS). All other reagents were the highest purity available. All solvents were molecular biology or HPLC grade.

4.3.3. Identification and quantification of PAH-degrading bacteria

Soil slurries were prepared and spiked with a [U-^{13}C]PAH as previously described (Chapter 3). Briefly, duplicate soil slurries were prepared in 125-mL Erlenmeyer flasks and consisted of 1 g of the original soil sample (wet weight) and 30 mL of simulated groundwater amended with nitrogen and phosphorus. After two days of agitation without any added substrate to allow native PAH concentrations to decline, the aqueous phase was replaced and each flask was spiked with 625 µg of a PAH (t=0). Flasks were then agitated on an orbital shaker in the dark at room temperature until the predetermined endpoint. Each incubation endpoint was determined by triplicate mineralization experiments in which soil slurry was incubated with 20,000 dpm of a ^{14}C-labeled version of each PAH. The following endpoints were selected based on the mineralization data shown in Figure B1 in Appendix B: naphthalene, 8 h; phenanthrene, 16 h; pyrene, 12 d; fluoranthene, 17 d; and benz[*a*]anthracene, 21 d. For SIP experiments with

naphthalene, phenanthrene, and pyrene, DNA was isolated via a single extraction of each of two 500 mg soil aliquots with a FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, OH) according to the instructions provided with the kit, except that DNA was eluted in Tris-EDTA (TE; 10 mM Tris-HCl, 1 mM EDTA; pH=8.0). DNA extracts from the same source flask were pooled prior to CsCl separation by ultracentrifugation. ^{13}C-enriched (heavy) DNA was separated from unlabeled DNA, 16S rRNA gene sequences representing PAH-degrading bacteria were identified, and qPCR primers were developed as previously described (16). For SIP experiments with fluoranthene and benz[*a*]anthracene, DNA was isolated from each of four 250 mg soil aliquots with the FastDNA® Spin Kit for Soil and eluted in TE. Two successive extractions of each soil aliquot were performed, and DNA extracts from the same source flask were pooled prior to CsCl separation (Chapter 3). ^{13}C-enriched DNA was separated from unlabeled DNA, PAH-degrading bacteria were identified, and qPCR primers were developed as previously described (Chapter 3). The 16S rRNA gene sequences were grouped into OTUs and a representative sequence was chosen for each OTU using the complete linkage clustering and dereplicate tools, respectively, each with a maximum cluster distance of 3%, within RDP's Pyrosequencing Pipeline (105). Parallel triplicate incubations with unlabeled growth substrate were used to measure the abundance of each SIP-identified group by qPCR and to follow the disappearance of each growth substrate by HPLC over time as previously described (Chapter 3).

4.3.4. Nucleotide sequence accession numbers

Sequences of 16S rRNA genes recovered from SIP incubations were deposited in GenBank with accession numbers GU266293-GU266537 (naphthalene, phenanthrene, and pyrene) and HM640025-HM640206 (fluoranthene and benz[*a*]anthracene).

4.4. Results

4.4.1. Mineralization and growth substrate disappearance

Samples of the original soil were incubated as a slurry and spiked with a 2-, 3-, or 4-ring PAH. After 8 h (naphthalene), 16 h (phenanthrene), 12 d (pyrene), or 21 d (benz[*a*]anthracene) of incubation, the rate of mineralization had declined, and the residual parent compound was ≤ 6% for each substrate except pyrene, which was 25%. The mineralization data for each PAH are shown in Figure B1. In general, mineralization occurred over the same time scale as the disappearance of the parent compound. For fluoranthene, mineralization declined after 17 d even though 95% of the added fluoranthene had been consumed by day 4. We observed a similar discrepancy between mineralization and parent compound disappearance for anthracene in a separate SIP study on the same soil (Chapter 3).

4.4.2. 16S rRNA gene clones libraries

A 16S rRNA gene clone library was generated from the heavy DNA recovered from each SIP experiment, and 96 clones were sequenced for each experiment. After excluding vector sequences, poor reads, and chimeras, the clone libraries generated from DNA associated with the degradation of naphthalene, phenanthrene, pyrene, fluoranthene, and benz[*a*]anthracene contained 65, 85, 96, 91, and 91 sequences, respectively. The singleton sequences in each library were not included in subsequent analyses, but are listed in the supporting information (Table B1). The remaining sequences, along with sequences from our previous anthracene SIP experiment (Chapter 3), were grouped into operational taxonomic units (OTUs) based on 97% sequence similarity (Table 4.1). Figure 4.1 shows how the representative for each OTU is related to selected reference sequences from GenBank.

Table 4.1. Percent representation of SIP-identified groups in each clone library.[1]

OTU No.	Classification[2]	NAP	ANT	PHE	PYR	FLA	BaA
1	*Sphingobium*	-[3]	14	-	-	56	-
2	Pyrene Group 2	-	-	-	100	13	82
3	*Rhodobacter*	-	1	-	-	-	3
4	*Variovorax*	31	24	-	-	-	5
5	*Rhizobium*	-	3	-	-	-	4
6	*Sphingomonas*	-	-	-	-	25	-
7	*Pigmentiphaga*	8	4	13	-	-	-
8	*Acidovorax*	9	-	74	-	-	-
9	*Sphingobium*	25	-	9	-	-	-
10	*Achromobacter*	3	-	-	-	-	-
11	*Pseudoxanthomonas*	3	2	-	-	-	-
12, 13	*Pseudomonas*	22	2	-	-	-	-
14	Anthracene Group 1	-	43	-	-	-	-
15	*Herminiimonas*	-	4	-	-	-	-
16	Unclassified Rhizobiales	-	1	-	-	-	-
17	*Skermanella*	-	1	-	-	-	-

[1] NAP, naphthalene; ANT, anthracene; PHE, phenanthrene; PYR, pyrene; FLA, fluoranthene; BaA, benz[*a*]anthracene.
[2] Assigned using RDP Classifier (115) with an 80% confidence threshold.
[3] -, either not found or was a singleton sequence in that clone library.

The most abundant sequences in the clone library generated from SIP with naphthalene were related to members of the *Variovorax* (20 of 65 clones), *Sphingobium* (16 clones), and *Pseudomonas* (14 clones) genera. Other sequences were related to *Acidovorax* (6 clones), *Pigmentiphaga* (5 clones), *Achromobacter* (2 clones), and *Pseudoxanthomonas* (2 clones). *Sphingobium*- and *Pigmentiphaga*-related sequences were also present in the clone library generated from SIP with phenanthrene (11 and 8 of 85 clones, respectively), but most of the sequences recovered were related to *Acidovorax* (63 clones). All of the sequences in the clone library generated from SIP with pyrene were related to members of an uncultivated group of γ-Proteobacteria previously designated "Pyrene Group 2" (PG2) (12). Sequences related to PG2 were also present in the clone library generated from SIP with fluoranthene (12 of 91 clones), but
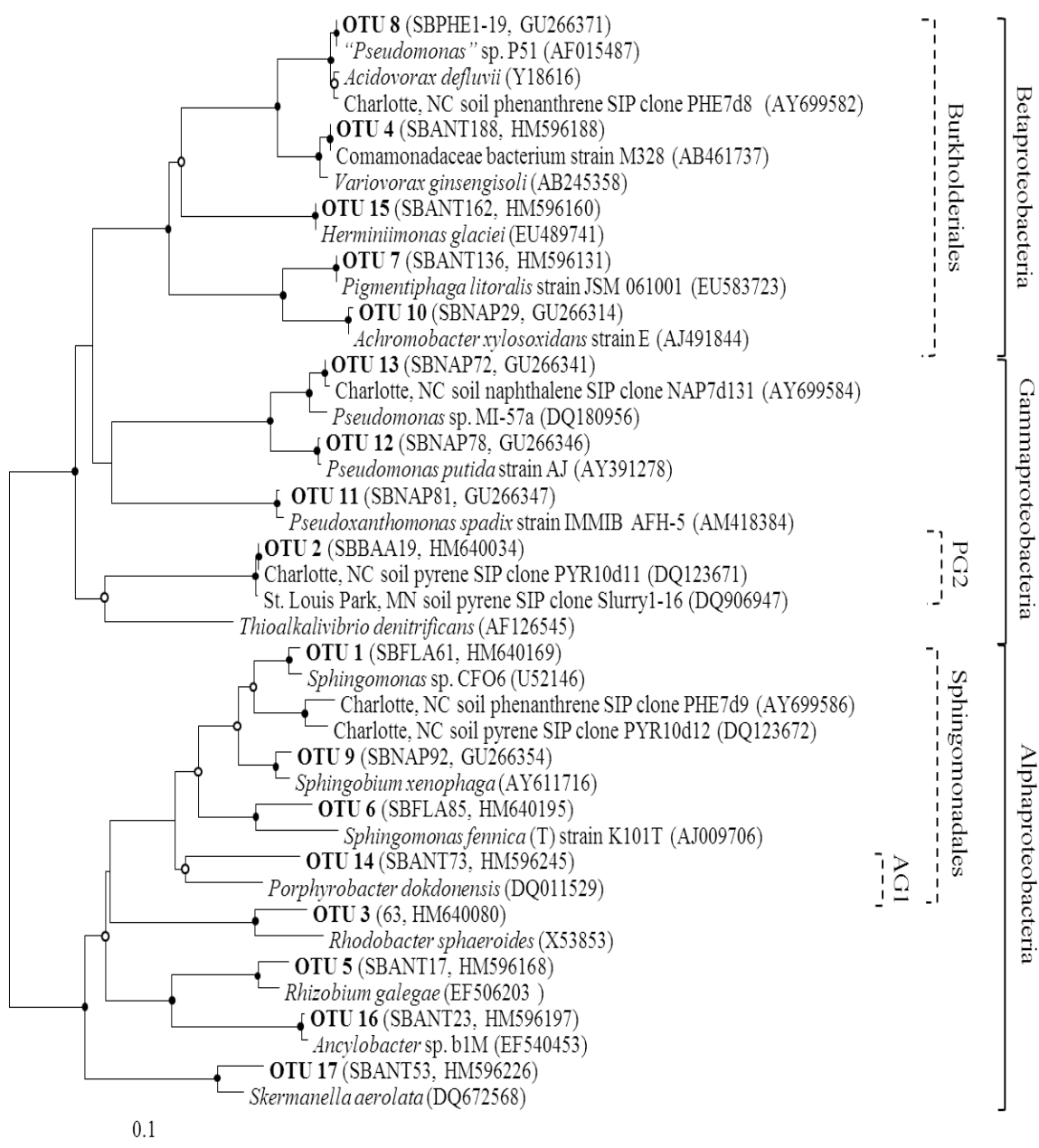
Figure 4.1. Phylogenetic tree of representative partial 16S rRNA gene sequences of bacteria from OTUs that contain sequences associated with the degradation of each of the five compounds investigated by SIP in this study and with anthracene degradation in the same soil (Chapter 3) and selected reference sequences. The tree was rooted with *Mycobacterium vanbaalenii* PYR100 (AY636002, not shown). Clones are named by the original soil sample (SB, Salisbury), the growth substrate (NAP, naphthalene; ANT, anthracene; PHE, phenanthrene; PYR, pyrene; FLA, fluoranthene; and BAA, benz[*a*]anthracene), and assigned an identifying number. The representative clone sequence and the GenBank accession numbers are in parentheses. OTUs are as in Table 4.1. Open and closed circles at nodes indicate ≥ 50% and ≥ 95% bootstrap support, respectively. PG2, Pyrene Group 2; AG1, Anthracene Group 1.

the majority of the sequences were related to *Sphingobium* (51 clones) and *Sphingomonas* (23 clones). PG2-related sequences also dominated the clone library generated from SIP with benz[*a*]anthracene (75 of 91 clones). Other sequences were related to *Variovorax* (5 clones), *Rhizobium* (4 clones), and *Rhodobacter* (3 clones).

4.4.3. Quantification of SIP-identified groups

Primers for quantitative PCR (qPCR) targeting the 16S rRNA genes of several SIP-identified groups were developed (Table 4.2) and used to determine the abundance of each group in response to the corresponding growth substrate. Except for the *Sphingobium-* and *Sphingomonas*-related bacteria associated with fluoranthene degradation, all of the targeted groups were below the respective quantification limit of each assay in the original soil sample (data not shown; see Table 4.2 for the quantification limits). Several of these groups increased to above the quantification limit during the two days of pre-incubation in the absence of the spiked PAH, but from the time the SIP incubation flasks were spiked with the ^{13}C-labeled PAH to the end of each SIP experiment, 16S rRNA gene copy numbers for each of these groups increased at least an order of magnitude in parallel flasks containing unlabeled substrate (Figure 4.2). The *Sphingobium-* and *Sphingomonas*-related bacteria associated with fluoranthene degradation were quantifiable in the original soil sample, and their 16S rRNA gene copy abundance increased by about 1.5 log by day 4 when the added fluoranthene had been consumed (Figure 4.3). PG2-related bacteria associated with fluoranthene degradation were below the quantification limit in the original soil sample, but their abundance also increased by about 1.5 log by day 4. None of the fluoranthene- associated groups increased in abundance between day 4 and day 17 when the SIP incubation was terminated.

Table 4.2. Quantitative PCR primers used in this study.

Target Group	Primer Name[1]	Primer Sequence (5'→3')	T_M (°C)[2]	qPCR Standard[3]	Amplicon Length	Amp. Eff.[4] (Bac; Group)	Quant. Limit[5]	RDP Hits[6]
Bacteria	341F	CCTACGGGAGGCAGCAG	60	--	--	--	--	--
	517R	ATTACCGCGGCTGCTGG						
Pigmentiphaga	PigmF	CAGGCGGTTCGGAAAG	56	SBNAP45	63	1.91; 2.03	8.86×10^6	17
	PigmR	TGACATACTCTAGTTCGGGA						
Sphingobium[7]	SGBF	ACGTAGGCGGCGATTT	59	SBNAP83	70	2.03; 2.03	1.44×10^7	329
	SGBR	CCTCTCCAAGATTCTAGCAA						
Sphingobium[8]	SGB.5F	ACAGTACCGGGAGAATAAGCTC	56	SBANT43	158	1.98; 1.92	2.32×10^7	128
	SGB.5R	CAAGCAATCCAGTCTCAAAGGCTA						
Variovorax	VarioF	AGCTGTGCTAATACCGCATAA	61	SBNAP02	279	2.05; 1.99	8.10×10^7	65
	VarioR	GAGACTTTTCGTTCCGTAC						
Acidovorax	AcidF	TAACGGAGCGAAAGCTT	55	SBPHE2-37	60	1.98; 2.01	2.08×10^7	331
	AcidR	GTCCGCGCAAGGCCTT						
Pyrene Group 2	PG2.4F	CCAAGCCGACGACGGGTAG	59	SBPYR03	94	2.02; 1.99	8.17×10^7	900
	PG2.4R	TTCCCCACTGCTGCCTC						
Sphingomonas	SPH.1F	CGGTACGGAATAACTCA	50	SBFLA15	202	1.98; 1.95	8.12×10^5	37
	Univ338R	GCTGCCTCCCGTAGGAGT						

[1] Bacterial primers are from Muyzer et al. (1993), SGB.5 primers are from Chapter 3, *Acidovorax* primers are from Singleton et al. (2007), and Univ338R are from Suzuki and Giovannoni (1996). All other primers were developed in this study.

[2] PCR annealing temperature.

[3] Clones from which plasmid DNA was used to generate standard curves. Each plasmid was linearized with NcoI. Clone names are as in Figure 4.1.

[4] Amp. Eff., Amplification efficiency (104) with eubacterial (Bac) and group-specific (Group) primers.

[5] Quantification limit (number of 16S rRNA gene copies) of each qPCR assay.

[6] Number of sequences returned by the Ribosomal Database Project II release 10.18 (105) (excluding sequences from this study) with no mismatches to primer pairs.

[7] Targets naphthalene- and phenanthrene-associated *Sphingobium* sequences.

[8] Targets fluoranthene-associated *Sphingobium* sequences which were similar to those recovered from an earlier SIP experiment with anthracene (Chapter 3).
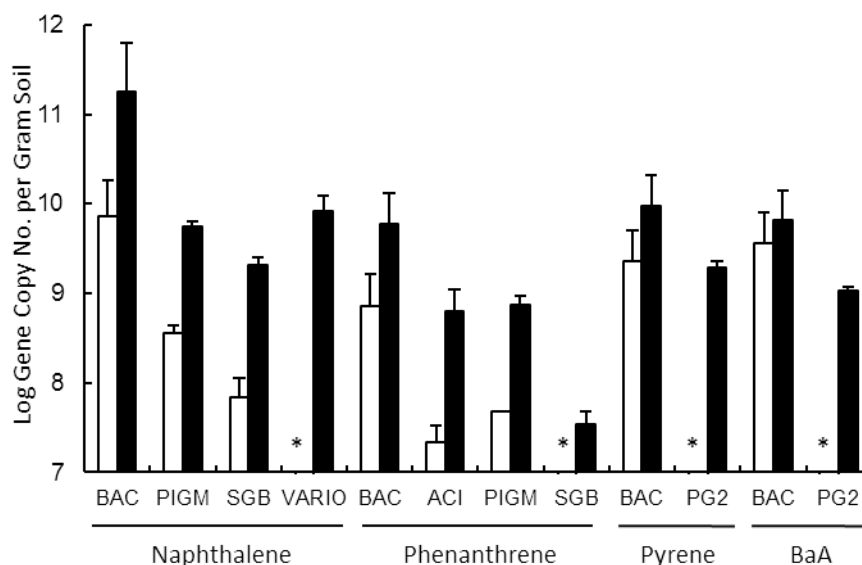
Figure 4.2. Abundances of eubacterial (BAC) and group-specific 16S rRNA genes at the beginning (open bars) and end (closed bars) of each SIP experiment in response to enrichment with the unlabeled substrate indicated. The beginning of the experiment (t = 0) is when each substrate was first added to the incubation flask after two days of pre-incubating the soil slurry without any added substrate. Group-specific values are the mean and range of duplicate reactions. Eubacterial values are the combined mean and standard deviation of the duplicate reactions calculated for each of the group-specific templates. Asterisks indicate that the value was below the quantification limit of the assay. PIGM, *Pigmentiphaga*; SGB, *Sphingobium*; VARIO, *Variovorax*; ACI, *Acidovorax*; BaA, benz[*a*]anthracene.

4.5. Discussion

Individual stable-isotope probing experiments were performed with five different uniformly ^{13}C-labeled PAHs to investigate the bacterial guild responsible for PAH degradation in a PAH-contaminated soil from the site of a former manufactured-gas plant. Coupled with SIP of anthracene-degrading bacteria in the same soil (Chapter 3), this work represents a comprehensive investigation of bacteria capable of degrading 2-ring (naphthalene), 3-ring (anthracene and phenanthrene), and 4-ring (benz[*a*]anthracene, fluoranthene, and pyrene) PAHs.
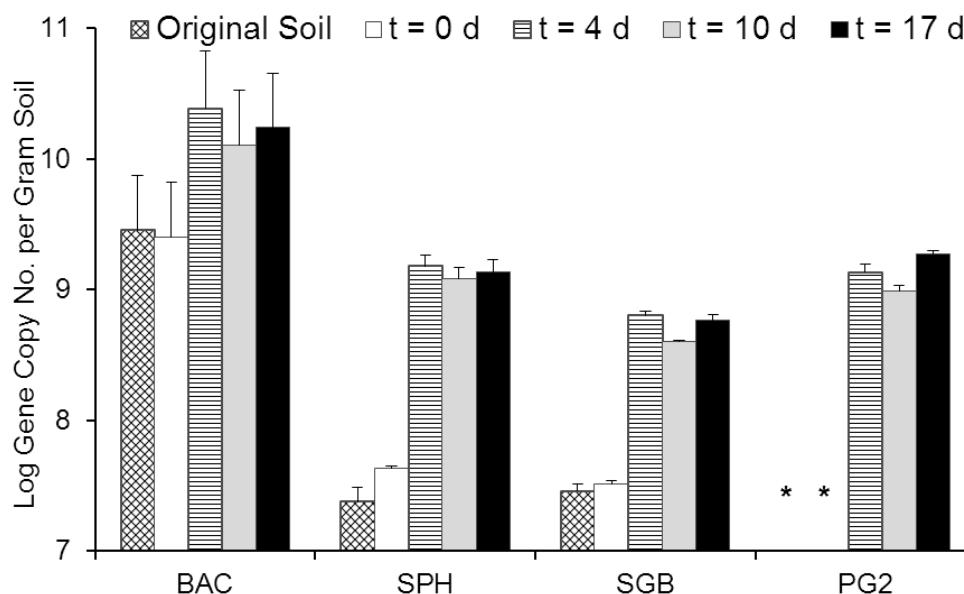
Figure 4.3. Abundances of eubacterial and group-specific 16S rRNA genes over time in response to enrichment with unlabeled fluoranthene; t = 0 is when fluoranthene was first added to the incubation flask after two days of pre-incubating the soil slurry without fluoranthene. Group-specific values are the mean and standard deviation of triplicate reactions. Eubacterial values are the combined mean and standard deviation of the triplicate reactions calculated for each of the group-specific templates. SPH, *Sphingomonas*. Other notes are as in Figure 4.2.

Collectively, a diverse range of bacteria spanning the α-, β-, and γ-Proteobacteria were found to grow on one or more of the six PAHs we evaluated (Figure 4.1). Of the 17 OTUs reported in Table 4.1, nine represented at least 10% of the clone library for at least one of the PAHs. However, only a few OTUs were well-represented in more than one clone library (Table 4.1), suggesting a degree of specialization for degrading a particular PAH. Two OTUs associated with growth on anthracene (*Rhodobacter* and *Rhizobium*) were also associated with growth on benz[*a*]anthracene, but they were not a major OTU in either clone library. None of the three OTUs associated with growth on phenanthrene grew on a four-ring PAH. All of the phenanthrene-degrading OTUs grew on naphthalene, but not *vice versa*.

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Sequences related to members of the orders Burkholderiales (*Variovorax*, *Acidovorax*, and *Pigmentiphaga*) and Sphingomonadales (*Sphingobium*, Anthracene Group 1) dominated the clone libraries generated from SIP with 2- and 3-ring PAHs. Most of the 16S rRNA gene sequences we recovered from SIP with naphthalene were similar to sequences from genera that have been associated with naphthalene degradation in previous DNA-based SIP studies, including *Pseudomonas* (74, 75, 133), *Acidovorax* (75, 133), and *Variovorax* (74, 75). Members of the genus *Sphingobium* have previously been associated with naphthalene degradation by other methods (122). *Pigmentiphaga*-related sequences have not previously been associated with naphthalene degradation, but were present in heavy DNA from incubations with naphthalene and increased in abundance in response to naphthalene addition (Figure 4.2). *Variovorax*- and *Sphingobium*-related sequences were also well-represented in the clone libraries generated from SIP with anthracene, but the most numerous sequences were related to an uncultivated and unclassified group within the order Sphingomonadales (Chapter 3). The *Variovorax*-, *Sphingobium*-, and *Pigmentiphaga*-related sequences associated with naphthalene were greater than 99% (over 815 bp of aligned sequence), 95% (908 bp), and 98.9% (817 bp) similar, respectively, to sequences recovered from SIP with anthracene (Chapter 3).

As with naphthalene, many of the 16S rRNA gene sequences we recovered from SIP with phenanthrene were similar to sequences from genera previously associated with phenanthrene degradation. *Acidovorax*-related sequences have previously been associated with phenanthrene degradation by SIP (13, 16), and *Sphingobium*-related sequences have been associated with phenanthrene degradation by other methods (122, 123). In addition, *Pigmentiphaga*-related bacteria were shown for the first time to be capable of growth on

phenanthrene as a result of this work. The *Sphingobium*- and *Pigmentiphaga*-related sequences associated with phenanthrene were greater than 99.1% (over 801 bp of aligned sequence) and 99.8% (816 bp) similar, respectively, to sequences recovered from SIP with naphthalene, and they were greater than 95.8% (801 bp) and 100% (816 bp) similar, respectively, to sequences recovered from SIP with anthracene (Chapter 3).

Sequences related to bacteria designated as PG2 were abundant in each clone library generated from SIP with a 4-ring PAH, but not in any clone library generated from SIP with a 2- or 3-ring PAH. PG2 was first identified in association with pyrene degradation via an SIP investigation of PAH-contaminated soil from a different manufactured-gas plant site (in Charlotte, NC) after the soil was treated in a laboratory bioreactor (12). PG2 organisms were also the primary pyrene degraders in an SIP investigation of PAH-contaminated soil from a former wood-treatment plant site in St. Louis Park, MN (15). The Salisbury, NC soil used in the present study is the third soil (of three tested) to be investigated by SIP with pyrene in which PG2 was the dominant group associated with pyrene-degradation. Organisms in PG2 did not respond to the addition of naphthalene or phenanthrene in the present study (data not shown), but PG2 organisms in the Charlotte, NC soil did grow on phenanthrene (16) .

In addition to PG2, *Sphingomonas*- and *Sphingobium*-related sequences were well-represented in the clone library generated from SIP with fluoranthene. These sequences did not increase in abundance between day 4, when the added fluoranthene had been consumed, and the end of the SIP incubation on day 17. This suggests that the PG2-, *Sphingomonas*-, and *Sphingobium*-related organisms in the soil grew primarily on fluoranthene itself, rather than a metabolite derived from fluoranthene. *Sphingomonas* and *Sphingobium* are genera that are known to include fluoranthene-degrading species (124, 134, 135). The *Sphingobium*-

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related sequences were greater than 96.3% (over 790 bp of aligned sequence), 95.9% (790

bp), and 99.5% (908 bp) similar to *Sphingobium*-related sequences from SIP with

naphthalene, phenanthrene, and anthracene, respectively, and they were the only sequences

recovered that increased in abundance in response to 2-ring, 3-ring, and 4-ring PAHs. This is

not surprising because sphingomonads are known to have an extensive substrate range that

includes both substituted and unsubstituted mono- and polyaromatic hydrocarbons up to 4

rings (124, 136-138). What little is known about the bacterial degradation of

benz[*a*]anthracene has resulted from studies of *Mycobacterium* isolates (139-142), but PG2

sequences dominated the clone library generated from SIP with benz[*a*]anthracene in the

present study.

Gram positive and Gram negative bacteria representing several different genera can

grow on both pyrene and fluoranthene and include *Alcaligenes*, *Pseudomonas*, *Stappia*,

Rhodococcus and *Microbacterium* (143), as well as *Stenotrophomonas* (144) , *Burkholderia*

(91), and *Mycobacterium* (145). However, there have been relatively few reports of bacteria

that can grow on multiple 4-ring PAHs. *Mycobacterium vanbaalenii* PYR-1 is the most

thoroughly studied bacterium capable of higher-molecular-weight PAH degradation.

Cultivation-based investigations of this bacterium (140, 146, 147) and other *Mycobacterium*

species (8, 129, 139, 148-154) have revealed that members of this genus can grow on the 4-

ring PAHs chrysene, pyrene, fluoranthene, and benz[*a*]anthracene. We have now shown that

PG2 bacteria can grow not only on pyrene, but also on fluoranthene and benz[*a*]anthracene

as well. This suggests that PG2 bacteria may be particularly well-suited for growth on 4-ring

PAHs.

The present work highlights the need for the continued use of cultivation-independent methods to gain further insights into the microbial groups responsible for PAH degradation. Of the six PAHs we have evaluated as growth substrates in this soil, three (anthracene, benz[*a*]anthracene, and pyrene) were primarily degraded by bacteria that are not closely related to any cultivated species. The results of such cultivation-independent approaches should be used to complement the discoveries made by studying bacteria isolated from environmental systems, and in fact can assist in targeting bacteria for isolation (7, 75, 99).

4.6. Acknowledgements

5. Association of polycyclic aromatic hydrocarbon-degrading bacteria in contaminated soil with benzo[*a*]pyrene mineralization

Maiysha D. Jones and Michael D. Aitken

5.1. Abstract

Benzo[*a*]pyrene (BaP) is a carcinogenic polycyclic aromatic hydrocarbon (PAH) that is not known to be a bacterial growth substrate but can be co-metabolized by PAH-degrading bacteria. However, the organisms capable of co-metabolizing BaP in complex, field-contaminated systems have not previously been identified. We evaluated the ability of various PAH growth substrates (naphthalene, anthracene, phenanthrene, pyrene, fluoranthene, or benz[*a*]anthracene) to influence the mineralization of BaP by a bacterial community from a bioreactor treating PAH-contaminated soil, both during co-incubation with or after pre-enrichment with each growth substrate. Pyrosequence libraries of 16S rRNA genes were used to identify the members of the bacterial community that were enriched on the added growth substrate as a means of associating specific organisms with BaP mineralization. Compared to conditions without an added growth substrate, co-incubating the bioreactor-treated soil with naphthalene, phenanthrene, or pyrene inhibited BaP mineralization over the 24-hour incubation period, while pre-enriching the soil on the same three PAHs for seven days enhanced BaP mineralization. Combined, these results suggest

that bacteria in the bioreactor community that are capable of growing on naphthalene, phenanthrene, or pyrene can metabolize BaP, with co-incubation competitively inhibiting BaP metabolism. Anthracene, fluoranthene, and benz[a]anthracene had little effect on BaP mineralization compared to incubations without an added growth substrate under either co-incubation or pre-enrichment conditions. Substantial increases in relative abundance after pre-enrichment with naphthalene, phenanthrene, or pyrene, but not the other PAHs, suggest that members of the genera *Cupriavidus*, *Luteimonas*, and *Rhizobium* may have been associated with BaP mineralization.

5.2. Introduction

Benzo[a]pyrene (BaP) is a 5-ring polycyclic aromatic hydrocarbon (PAH) that has been classified by the International Agency for Research on Cancer as carcinogenic to humans based on evidence of genotoxicity and carcinogenicity in animal models (32). The U.S. Environmental Protection Agency and other governmental agencies around the world have used BaP as a model PAH to establish regulations that aim to minimize human exposure to and protect the natural environment from PAH contamination. Engine exhaust is composed of a mixture of PAH compounds and is a major source of atmospheric BaP contamination, especially in urban areas (2, 3, 155-157), and soil is the main receptor of contaminated run-off following atmospheric deposition (2, 3, 20). Weathered coal gasification process waste from former manufactured-gas plants is also a source of BaP contamination in soil (23).

Bioremediation is a primary strategy for reducing PAH contamination in soil (37), but the efficacy of bioremediation may be limited by its ability to meet cleanup standards for the

carcinogenic PAHs at a given site. Many microorganisms can grow on PAHs containing 2, 3, or 4 rings, but no microorganisms are known to grow on PAHs containing 5 or more rings. Although there have been no reports of bacteria capable of utilizing BaP as a sole carbon and energy source, several bacteria can oxidize BaP when a suitable co-substrate is present or after growth on a suitable enrichment substrate. Individual PAHs (59, 62, 91, 92) and PAH mixtures (90) have been used as co-substrates in studies of BaP co-metabolism, while phenanthrene (93, 158) and pyrene (90) have been used as growth substrates to stimulate subsequent BaP mineralization or removal, respectively.

We previously used DNA-based stable-isotope probing (SIP) to describe the bacterial guild responsible for the degradation of naphthalene, phenanthrene, anthracene, pyrene, fluoranthene, and benz[*a*]anthracene in a PAH-contaminated soil obtained from the site of a former manufactured-gas plant (MGP) (Chapters 3 and 4). In the present study, we compared the effect of co-incubation or pre-enrichment with each of these PAHs on the ability of the bacterial community in a bioreactor used to treat the MGP soil to mineralize BaP. The primary objective was to identify those PAH growth substrates associated with BaP metabolism. We hypothesized that any organism capable of co-metabolizing BaP would enhance BaP mineralization if it grew in response to pre-enrichment with a PAH growth substrate. We also hypothesized that BaP mineralization would be inhibited in the simultaneous presence (co-incubation) of a PAH substrate that could serve as a growth substrate for an organism otherwise capable of co-metabolizing BaP. Pyrosequencing of 16S rRNA genes from the communities enriched on the various PAHs was conducted to identify those organisms that increased in relative abundance in response to the growth substrates associated with BaP mineralization. Identifying the organisms associated with BaP

metabolism in complex systems is a first step towards elucidating the genetic determinants of BaP metabolism and towards developing bioremediation strategies to improve the removal of BaP and other carcinogenic PAHs. A comprehensive investigation of the influence of 2- to 4-ring PAHs on BaP mineralization in a microbial community derived from a single field-contaminated soil has not been reported previously.

5.3. Materials and Methods

5.3.1. Growth substrates and chemical reagents

The natural abundance isotopomers (unlabeled versions) of naphthalene, phenanthrene, pyrene, fluoranthene, and benzo[*a*]pyrene were obtained from Sigma-Aldrich (St. Louis, MO), anthracene was obtained from Eastman Kodak (Rochester, NY), and benz[*a*]anthracene was obtained from Acros Organics (NJ). [7,10-^{14}C]Benzo[*a*]pyrene (68 mCi/mmol) was obtained from GE Healthcare UK Limited (Buckinghamshire, UK). All other reagents were the highest purity available. All solvents were molecular biology or high performance liquid chromatography (HPLC) grade.

5.3.2. Bioreactor conditions and maintenance

PAH-contaminated soil was obtained from the site of a former MGP in Salisbury, NC and processed as described previously (Chapter 3). In addition, the soil was sieved through #6 mesh (3.35 mm) prior to storage in the dark at 4°C. The processed soil (64% sand, 30% silt, 6% clay, 15% moisture, pH=7.6) was treated in a bench-scale, aerobic, slurry-phase bioreactor as described previously for the treatment of a different soil (159). Twenty percent of the treated soil slurry was replaced weekly with processed soil suspended in a buffer

containing 5 mM potassium phosphate (pH=7.5) supplemented with 5 mM NH_4NO_3

corresponding to a solids retention time of 35 days.

5.3.3. Mineralization experiments

The effects of co-incubation and pre-enrichment with 2-, 3-, and 4-ring PAHs on BaP

mineralization were tested using bioreactor-treated soil as inoculum. For the co-incubation

experiment, duplicate 125-mL Erlenmeyer flasks were spiked with 625 µg of unlabeled

naphthalene, anthracene, phenanthrene, pyrene, fluoranthene, or benz[*a*]anthracene in

acetone and 60,000 dpm of radiolabeled BaP in methanol. After evaporation of the carrier

solvents, 5 mL of treated soil slurry from the bioreactor and 25 mL of bioreactor buffer and a

CO_2 trap (101) were added to each flask. The flasks were capped with foil-covered, Teflon-

lined screw caps and agitated on an orbital shaker (150 rpm) in the dark and at room

temperature for 60 days. Periodically, the CO_2 trap was analyzed by liquid scintillation

counting and replaced with a new trap. A single analysis of each CO_2 trap was performed

after 24 h of agitation.

For the pre-enrichment experiment, duplicate flasks were spiked with a growth

substrate without BaP. Seven days was determined to be adequate for significant removal of

each growth substrate (Figure D1 in Appendix D). After seven days of agitation, the amount

of substrate remaining in each condition (Figure D2) was determined by HPLC (as described

in Chapter 3) and 60,000 dpm of radiolabeled BaP in methanol was added to clean flasks.

After evaporation of the methanol, slurries from the seven-day incubations were transferred

to the clean flasks containing the radiolabeled BaP, and a CO_2 trap was added to each flask.

The flasks were capped and agitated for 24 h, and each CO_2 trap was analyzed. Duplicate

flasks with no substrate added were also prepared for the co-incubation and pre-enrichment

mineralization experiments to serve as controls for the effect of substrate addition. Data from each experiment were compared using a two-tailed Student's t-test assuming unequal variance (Microsoft Excel 2010).

In each experiment, after removing the CO_2 trap (primary trap), flasks containing radiolabeled BaP were acidified with 200 ul of 85% phosphoric acid, a new CO_2 trap (secondary trap) was added, and the flasks were agitated for 3 h. Soil slurry (1 mL) from each flask was then mixed with 1 mL of ethyl acetate in separate 15-mL conical-bottom centrifuge tubes. The tubes were vortexed at maximum speed for 1 min and centrifuged for 5 min at 3,500 rpm. The secondary CO_2 trap and an aliquot of the organic layer of each resulting supernatant was analyzed by liquid scintillation counting. Radiocarbon recovery was determined by summing the activity measured in the primary CO_2 trap, the secondary CO_2 trap, and the ethyl acetate extract. There was no difference in radiocarbon recovery between flasks with or without added growth substrate compared to live and acid-inhibited flasks without added growth substrate (data not shown).

5.3.4. DNA extraction and pyrosequencing

For each of the PAH growth substrates (and the conditions with no substrate added), a set of duplicate flasks was set up as described for the mineralization experiments except that the BaP was added in unlabeled form (0.09 µg/flask, or 3 µg/L). For the pre-enrichment condition, two sets of duplicate flasks were prepared. In one set, after the 7-d pre-enrichment, an additional incubation for 24 h was conducted in the presence of unlabeled BaP at a concentration of 3 µg/L (corresponding to the concentration used in the mineralization experiments), and in the second set the additional incubation was conducted in the absence of BaP. DNA was extracted from soil pelleted from 2 mL of slurry (containing approximately

57 mg of dry soil) from each replicate using the FastDNA® Spin Kit for Soil (MP

Biomedicals, Solon, OH) according to the accompanying instructions, except that DNA was

eluted with Tris-EDTA buffer (TE, pH=8.0). Aliquots of DNA from each replicate extraction

were pooled. Each pooled sample was PCR-amplified in triplicate using a different pair of

barcoded primers targeting region 27F to 338R of the 16S rRNA gene. For DNA from the

pre-enrichment condition with phenanthrene in the presence of BaP, three sets of triplicate

PCR reactions were performed to evaluate the reproducibility of pyrosequence libraries. Each

primer consisted of an eight-base barcode (Table D1) and a two-base spacer (TC for the

forward primer, CA for the reverse primer) (160) followed by 27F

(AGAGTTTGATCCTGGCTCAG) (110) or 338R (TGCTGCCTCCCGTAGGAGT) (79).

Each set of triplicate PCR products was pooled, and the amplicon was recovered in TE using

a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) with the microcentrifuge protocol.

The DNA concentration in each cleaned amplicon was quantified with a NanoDrop 3300

fluorospectrometer (NanoDrop Products, Wilmington, DE) using the Quant-iT PicoGreen

dsDNA Assay Kit (Invitrogen, Eugene, OR). An aliquot containing 7.6 ng of DNA from each

sample was submitted to the UNC-Chapel Hill High-Throughput Sequencing Facility

(HTSF) for sequencing adapter ligation and multiplex pyrosequencing using the Life

Sciences 454 Genome Sequencer FLX Titanium platform (Roche Diagnostics Corp.,

Branford, CT).

5.3.5. Analysis of pyrosequence libraries

Sequences were analyzed as described in Appendix C. Briefly, the sequence data

were separated into libraries based on the eight-base barcode. Poor-quality sequences,

sequences < 250 bp in length, and sequences without detected barcodes were removed from

analysis using the Ribosomal Database Project's (RDP) pyrosequencing pipeline initial process tool (105). RDP Classifier (105, 115) was used to determine the phylogeny of each sequence (minimum 80% sequence similarity). The blastn application within BLAST+ (161) was used to search for sequences related to ($\geq$ 97% similarity) the unclassified bacteria within Anthracene Group 1 (Chapter 3) and Pyrene Group 2 (Chapter 4) in a local database of the pyrosequence data pool. After alignment and complete linkage clustering, the diversity and richness of each library was determined by the Shannon (H') and Chao1 (97% sequence similarity) indices, respectively (105). Replicate sequences within each library were removed and representative sequences were selected using RDP's dereplicate and fasta sequence selection tools, respectively (105). The selected sequences were aligned using RDP's pyrosequencing aligner (105), conserved blocks were selected from the alignment using the stand-alone version of Gblocks (162), and FastTree 2.1 (163) was used to generate the input trees for Fast UniFrac analyses (118). The category mapping variables used as input for the Fast UniFrac analyses and a description of each library are in Table 5.1.

5.3.6. Nucleotide sequence accession numbers

All sequences will be deposited in NCBI's Sequence Read Archive prior to submitting the manuscript for peer review.

5.4. Results

5.4.1. BaP mineralization

The bioreactor-treated soil (no substrate added) achieved 15% mineralization of BaP over a 24-h incubation (Figure 5.1), indicating that the microbial community in the reactor contained organisms able to metabolize BaP. The addition of naphthalene, phenanthrene, and

Table 5.1. Variables used to perform principal coordinates analysis and UniFrac analyses.

Library	Treatment[1]	Substrate[2]	BaP
BRS[3]	None	None	No
CNOB	Co	None	Yes
CNAB	Co	NAP	Yes
CANB	Co	ANT	Yes
CPHB	Co	PHE	Yes
CPYB	Co	PYR	Yes
CFLB	Co	FLA	Yes
CBAB	Co	BaA	Yes
PNOB	Pre	None	Yes
PNAB	Pre	NAP	Yes
PANB	Pre	ANT	Yes
PHB1	Pre	PHE	Yes
PHB2	Pre	PHE	Yes
PHB3	Pre	PHE	Yes
PPYB	Pre	PYR	Yes
PFLB	Pre	FLA	Yes
PBAB	Pre	BaA	Yes
PNON	Pre	None	No
PNAP	Pre	NAP	No
PANT	Pre	ANT	No
PPHE	Pre	PHE	No
PPYR	Pre	PYR	No
PFLA	Pre	FLA	No
PBAA	Pre	BaA	No

[1] Co, co-incubation; Pre, pre-enrichment.
[2] None, no exogenous substrate; NAP, naphthalene; ANT, anthracene; PHE, phenanthrene; PYR, pyrene; FLA, fluoranthene; BaA, benz[*a*]anthracene.
[3] BRS, bioreactor slurry (inoculum for all experiments).

pyrene inhibited BaP mineralization over a 24-h co-incubation compared to the control in which no exogenous substrate was added ($p < 0.01$) (Figure 5.1). Co-incubation with anthracene, fluoranthene, or benz[*a*]anthracene had no effect ($p > 0.1$).

To evaluate the effect of pre-enrichment with a PAH growth substrate, the microbial community in the bioreactor-treated soil slurry was incubated in the presence of a 2-, 3-, or 4-
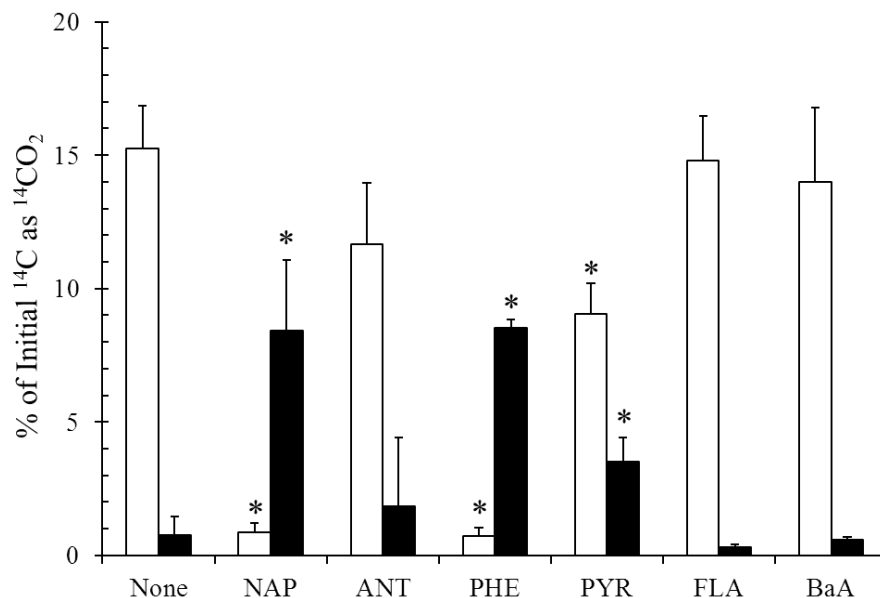
Figure 5.1. Percent of initial ^{14}C evolved as $^{14}CO_2$ after 24 h in the presence of ^{14}C-BaP (mean and standard deviation, n=3). Open and closed bars are data from co-incubation and pre-enrichment experiments, respectively, with the indicated growth substrate. Asterisks indicate that the value is significantly different from the respective incubation without exogenous substrate. Abbreviations are as in Table 5.1.

ring PAH as a growth substrate for 7 d, and then BaP mineralization was tested over an additional 24 h. Pre-enrichment with naphthalene, phenanthrene, or pyrene enhanced BaP mineralization ($p < 0.05$) compared to incubation for 7 d in the absence of exogenous substrate (Figure 5.1). The other PAH growth substrates had no effect ($p > 0.1$).

5.4.2. Analysis of pyrosequencing libraries

Pyrosequence libraries were generated using DNA recovered from the bioreactor slurry used as inoculum and the various incubations of the bioreactor-treated soil with or without an added growth substrate and with or without the addition of unlabeled BaP. Libraries for the co-incubation condition were obtained from DNA extracted after 24 h of incubation. Libraries for the pre-incubation condition were obtained from DNA extracted after an initial 7-d incubation with the PAH growth substrate followed by an additional 24 h-incubation with or without BaP added. The libraries and corresponding incubation conditions

evaluated are summarized in Table 5.1. After eliminating sequences that were too short, of poor quality or without detected barcodes, the 24 libraries contained 224,630 sequences.

The number of sequences and OTUs and the richness and diversity estimates for each library are listed in Table D1. The co-incubation libraries were generally richer and more diverse than the pre-enrichment libraries, and were similar in richness and diversity to the bioreactor slurry used as inoculum for the incubations; these observations are consistent with the extended period of incubation in the presence of a dominant growth substrate in the pre-incubation experiments, which would select for a niche bacterial community. The pairwise UniFrac significance test (weighted to account for differences in the number of sequences recovered) determined that there was no significant difference among the co-incubation libraries ($p > 0.1$) or between any co-incubation library and the bioreactor slurry inoculum library ($p > 0.1$). The UniFrac sample distance matrix (weighted) determined that there was no significant difference in the distance between pairs of pre-enrichment libraries generated after an additional 24 h of incubation with or without BaP ($p > 0.1$), suggesting that the presence of a trace concentration of BaP (3 µg/L) had no effect on the bacterial community. DNA from the bioreactor slurry pre-enriched on phenanthrene and then spiked with BaP was used to generate triplicate libraries (designated PHB1, PHB2, and PHB3; Table 5.1) to illustrate the reproducibility of the pyrosequencing results. Despite the differences in the number of sequences recovered from each replicate (Table 5.1), the pairwise UniFrac significance test (weighted) determined that there was no significant difference among these libraries ($p > 0.1$).

Principal coordinates analysis of 16S rRNA gene pyrosequence libraries (weighted and normalized) accounted for 85% of the variation among the libraries (Figure 5.2).

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Bacterial communities resulting from co-incubation (cluster 1 and the library from co-incubation with phenanthrene, CPHB) differed from those resulting from pre-enrichment (clusters 3 and 4). Although not indicated by the pairwise UniFrac significance test, principal coordinates analysis suggested that the library from co-incubation with phenanthrene (CPHB) was different from the other co-incubation libraries (Figure 5.2). In addition, the libraries from pre-incubation experiments in which a PAH growth substrate was not added (PNOB and PNON) were dissimilar from all other pre-incubation libraries. PNOB and PNON (incubated for 8 d) were also different from the library from co-incubation in the absence of exogenous growth substrate (CNOB, incubated for 24 h), suggesting that the longer incubation period alone led to a shift in the community. The libraries from pre-incubation with benz[*a*]anthracene, either with (PBAB) or without (PBAA) BaP addition, were also different from all other pre-incubation libraries (Figure 5.2). These libraries were most similar to the libraries from pre-incubation in the absence of exogenous growth substrate, which may have resulted from less growth on benz[*a*]anthracene compared to the other PAHs.

5.4.3. Taxonomic representation in pyrosequencing libraries

Proteobacteria accounted for the majority of the sequences recovered from each library (Table 5.2). Cluster 2 had a lower average relative abundance of Betaproteobacteria sequences and a higher average relative abundance of unclassified bacteria, Gammaproteobacteria, Nitrospira, and Acidobacteria sequences compared to cluster 4. Cluster 3 also had a lower average relative abundance of Betaproteobacteria sequences and a higher average relative abundance of Gamma- and Alphaproteobacteria sequences compared to cluster 4. Library CPHB had a higher relative abundance of Betaproteobacteria sequences

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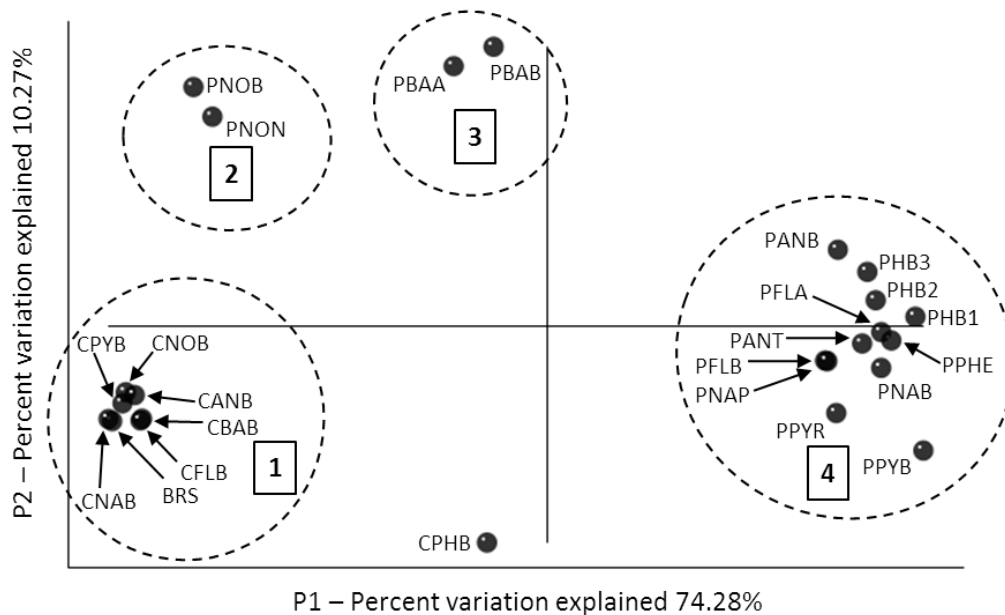
Figure 5.2. Weighted and normalized principal coordinates analysis of pyrosequence libraries of 16S rRNA genes. Clusters of libraries are circled and numbered. Libraries are as defined in Table 5.1.

Table 5.2. Percent abundance of major taxa present in pyrosequence library clusters.[1]

Phylum		Cluster Number			
Class	CPHB	1	2	3	4
Unclassified Bacteria	4	6 ± 1	10 ± 3	4 ± 3	2 ± 1
Proteobacteria	90	85 ± 2	75 ± 2	91 ± 9	97 ± 2
Alphaproteobacteria	20	36 ± 3	21 ± 9	38 ± 12	14 ± 5
Betaproteobacteria	55	26 ± 1	20 ± 7	29 ± 4	67 ± 5
Gammaproteobacteria	8	13 ± 1	22 ± 4	17 ± 2	10 ± 2
Nitrospira	< 0.5	< 0.5	2 ± 0.4	< 1	< 0.5
Acidobacteria	5	6 ± 1	12 ± 5	< 1	< 1

[1] Libraries are as defined in Table 5.1. Cluster designations are as in Figure 5.2. Except for CPHB (n=1), values are mean $\pm$ standard deviation (cluster 1, n=7; cluster 4, n=12) or range (clusters 2 and 3, n=2).

(mostly *Acidovorax*, Table D2) and a lower relative abundance of Alpha- and

Gammaproteobacteria sequences compared to cluster 1. These differences likely resulted in

the divergence of library CPHB and clusters 2 and 3 from cluster 1 and cluster 4, respectively

(Figure 5.2).

5.5. Discussion

Although BaP is known to be mineralized by bacteria, it is difficult to determine which organisms are responsible for BaP mineralization in complex, field-contaminated systems because no organisms have been isolated with BaP as a sole carbon source. Molecular techniques such as SIP can detect only those organisms capable of assimilating carbon during growth on a given substrate (11), not organisms that co-metabolize an organic compound in the absence of growth. On the assumption that BaP is removed from a contaminated system only via co-metabolism, our approach was to identify those PAHs that could influence the mineralization of BaP by organisms in a bioreactor treating soil from a field-contaminated site. These organisms would already have been highly enriched as a result of growth on PAHs and other carbon sources in the soil.

BaP mineralization in the bioreactor-treated soil slurry was inhibited in the presence of naphthalene, phenanthrene, and pyrene compared to incubation in the absence of added substrate over a 24-h period (Figure 5.1). Competitive inhibition of PAH metabolism has been observed in a number of cases when compounds were incubated together, and occurs when more than one substrate is metabolized by the same enzyme system (68-70). Co-incubation of anthracene, benz[*a*]anthracene, or fluoranthene with BaP did not inhibit BaP mineralization, suggesting that organisms capable of growing on or metabolizing these substrates either do not mineralize BaP or do so via pathways that are independent of the pathway(s) for metabolism of the other PAHs.

Incubation of the bioreactor soil slurry in the absence of exogenous substrate for seven days (the pre-enrichment condition with no added substrate) led to almost a complete loss of BaP mineralization activity compared to the treated soil removed directly from the

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bioreactor (the co-incubation condition with no added substrate; Figure 5.1). This loss of activity could have resulted either from the decay of organisms associated with BaP mineralization under substrate-limited conditions, a decline in the metabolic capacity to mineralize BaP in one or more of those organisms, or both. The addition of naphthalene, phenanthrene or pyrene as a growth substrate over the seven-day pre-incubation period restored BaP mineralization activity, whereas anthracene, benz[*a*]anthracene, and fluoranthene did not.

The combined results of the co-incubation and pre-incubation experiments strongly suggest that one or more organism(s) capable of growing on naphthalene, phenanthrene, and/or pyrene are able to mineralize BaP, whereas bacteria that grew on anthracene, benz[*a*]anthracene, or fluoranthene are not. Accordingly, we anticipated that these differences would be manifested in the pyrosequencing libraries from pre-enrichment with the various PAH growth substrates. Although the libraries from pre-enrichment with all of the growth substrates other than benz[*a*]anthracene were grossly similar (Figure 5.2 and Table 5.2), we examined these libraries for differences in representation of individual sequences between pre-enrichments with an added growth substrate and pre-enrichments without an added growth substrate. For those sequences that increased in relative abundance in one or more libraries in which a growth substrate was added, we looked for much greater increases in response to naphthalene, phenanthrene, and/or pyrene than in response to anthracene, benz[*a*]anthracene, or fluoranthene.

The sequences and corresponding taxonomic groups that were at least 1% of the total sequences in one or more libraries from the pre-incubation experiments are summarized in Table 5.3, as are all of the groups that were identified previously in our SIP experiments with

the untreated soil (Table 4.1). Although there were a number of groups that increased substantially in response to naphthalene, phenanthrene and/or pyrene compared to the incubations with no added substrate, most of these also increased in response to at least one of the other growth substrates. One group, *Cupriavidus*, increased to more than 17% of the libraries in response to the addition of phenanthrene and to a lesser extent in response to naphthalene (3.4% of the naphthalene libraries); although this group also increased in response to fluoranthene (approximately 1% of the fluoranthene libraries), the increase was not as great as with naphthalene or phenanthrene. We therefore suggest that members of this group are the most likely candidates for association with BaP mineralization. Similarly, sequences associated with *Rhizobium* and *Luteimonas* increased in relative abundance in response to naphthalene and pyrene, respectively, to a greater extent than for any other growth substrate, suggesting that these groups may also be associated with BaP mineralization. Relative abundances of members of the genera *Methylibium* and *Rhodoferax* increased substantially in response to most of the PAH substrates (Table 5.3), indicating a broad response to PAHs but not likely an ability to co-metabolize BaP.

The genus *Cupriavidus*, formerly *Ralstonia* and *Wautersia* (164), is a member of the family Burkholderiaceae that contains many known PAH-degrading genera. *Cupriavidus* spp. have been isolated from petroleum-contaminated soil (165), have been associated with growth on phenanthrene (166) and with the degradation of substituted aromatic compounds (167), polychlorinated biphenyls (168), and humic substances (169). *Cupriavidus* spp. have also been suggested to be genetically equipped for survival in toxic environments (170). We recovered similar sequences from SIP experiments with salicylate and naphthalene in a different PAH-contaminated soil (13, 14), and these sequences were later associated with

Table 5.3. Relative abundances (in %) of bacteria that grew after pre-enrichment.[1]

Taxonomic Group	BRS	Growth Substrate						
		None	NAP	PHE	ANT	PYR	FLA	BAA
SIP-identified Groups								
Achromobacter	-	-	-	-	-	-	-	-
Acidovorax	7.10	5.25	5.36	11.09	12.15	13.19	11.01	2.38
Anthracene Group 1	< 1	-	-	-	-	-	-	-
Herminiimonas	-	-	-	< 1	-	-	< 1	-
Pigmentiphaga	< 1	-	-	-	-	-	-	-
Pseudomonas	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Pseudoxanthomonas	< 1	-	-	-	-	-	-	-
Pyrene Group 2	-	< 1	-	< 1	< 1	< 1	< 1	< 1
Rhizobium	< 1	< 1	**3.87**	< 1	< 1	< 1	< 1	1.73
Rhodobacter	-	-	-	-	-	-	-	-
Skermanella	-	-	-	-	-	-	-	< 1
Sphingobium	< 1	< 1	1.93	< 1	< 1	< 1	< 1	2.34
Sphingomonas	1.40	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Thiobacillus	8.33	2.72	< 1	< 1	< 1	< 1	< 1	1.33
Variovorax	< 1	-	< 1	< 1	< 1	< 1	1.05	< 1
Other Groups								
Acidobacteria Gp7	5.76	10.65	< 1	< 1	1.05	1.22	< 1	1.42
Actinobacteria	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Ancylobacter	< 1	-	1.06	3.68	1.77	< 1	3.16	7.28
Azoarcus	-	-	< 1	< 1	< 1	< 1	3.84	< 1
Cupriavidus	-	-	**3.38**	**17.28**	< 1	< 1	1.04	< 1
Firmicutes	< 1	-	-	< 1	-	< 1	-	1.28
Luteimonas	< 1	1.10	< 1	< 1	< 1	**3.31**	< 1	< 1
Mesorhizobium	< 1	< 1	< 1	< 1	1.92	1.38	1.74	4.46
Methylibium	< 1	-	10.72	5.19	6.30	12.54	7.69	1.07
Methylophilus	-	-	< 1	< 1	2.09	-	< 1	< 1
Nitrosomonadales	< 1	1.46	-	< 1	< 1	< 1	< 1	1.14
Nitrospira	< 1	2.44	< 1	< 1	< 1	< 1	< 1	< 1
Polaromonas	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Rhodoferax	< 1	-	< 1	3.69	7.12	< 1	4.28	6.13

[1] Each value is the average relative abundance of each group in the presence or absence of BaP. Bold values indicate groups tentatively associated with BaP mineralization as a result of this study and the growth substrate on which they were enriched. Abbreviations are as defined in Table 5.1.

[2] -, not detected in the library.

naphthalene mineralization and growth in response to the addition of nonionic surfactants to the bioreactor treating that soil (171). Sequences related to *Rhizobium* were recovered in heavy DNA clone libraries from SIP experiments with anthracene and benz[*a*]anthracene (Table 4.1), but they were among the least abundant sequences. *Rhizobium* spp. have been associated with phenanthrene degradation (172), but sequences similar to *Rhizobium* were not recovered from the phenanthrene clone library (Table 4.1). A *Luteimonas* isolate was recently described after being recovered from hydrocarbon-contaminated soil from an industrial site (173), and another isolate was recovered from a biofilter treating waste gas containing furan (174). Many of the other enriched groups have been associated with hydrocarbon degradation, but not necessarily PAH degradation (99, 168, 175-177).

The pre-enrichment experiments performed in this study mimicked the enrichment incubations performed in our previous SIP experiments with the untreated soil. However, the bacterial groups resulting from pre-enrichment experiments with the bioreactor-treated soil slurry are strikingly different from those resulting from SIP experiments with untreated soil. For example, Anthracene Group 1, the dominant anthracene-degrading group identified by SIP with anthracene in the untreated soil (Chapter 3), was not enriched after seven days of pre-enriching the bioreactor slurry on anthracene, even though this group was present in the inoculum (Table 5.3) and at least 80% of the added anthracene had been removed by this time point (Figure D2). In some cases, SIP-identified bacteria were enriched compared to their relative abundance in the inoculum (e.g., Pyrene Group 2; Table 5.3), but their relative abundances did not increase in response to the addition of a PAH growth substrate relative to the pre-incubation condition with no added substrate.

The approach we used in this study to identify PAH growth substrates capable of supporting BaP co-metabolism can complement efforts to isolate organisms capable of co-metabolizing BaP. Our collective work on SIP of PAH-degrading bacteria in field-contaminated systems (12-16 and Chapters 3 and 4) has indicated that a number of organisms associated with PAH degradation in these systems have not previously been isolated or characterized. Although our methods in this study provide only indirect evidence to associate one or more organisms with BaP mineralization, we are not aware of a cultivation-independent approach to identifying organisms that metabolize hydrocarbons that do not serve as carbon or energy sources. In turn, identifying bacteria capable of specific compound degradation in a complex system and associating those bacteria with other metabolic abilities can help to target isolation efforts. Ultimately, experimentation with bacterial isolates will lead to definitive evidence of the metabolic activity suggested by molecular results.

5.6. Acknowledgements

6. Conclusions and Recommendations for Future Research

6.1. Conclusions

Human exposure to PAHs, some of which may be carcinogenic, is unavoidable due to the global nature of PAH contamination, but bacteria can be used to reduce PAH contamination in soil. Therefore, the objective of my dissertation research was to identify bacteria capable of degrading specific PAHs and to associate specific PAH-degrading bacteria with the ability to mineralize BaP, a carcinogenic PAH. A better understanding of PAH-degrading microorganisms and their metabolic capabilities will lead to improved PAH remediation strategies, better-informed remediation decisions, potentially less costly remediation actions, and enhanced site monitoring capacities. Three specific objectives were fulfilled:

1. Determine the effect of multiple DNA extractions (performed on the same soil aliquot) on the identification and quantification of anthracene-degrading bacteria native to PAH-contaminated soil and identified by DNA-based stable-isotope probing.

Multiple DNA extractions of the same soil aliquot were necessary to maximize DNA yield and 16S rRNA gene copy number from soil samples. Although additional bacterial groups were not identified in later DNA extracts compared to those identified in the first extract, there was a shift in the abundance of SIP-identified bacteria in later extracts. Anthracene-degrading bacteria were defined as being present in the heavy DNA and increasing in abundance with anthracene removal. Of the five bacterial groups that were most

abundant in the heavy DNA clone libraries, sequences related to Anthracene Group 1 (an uncultivated member of the order Sphingomonadales), *Variovorax*, *Pigmentiphaga*, and *Herminiimonas* met both of these criteria; the growth pattern of sequences related to *Sphingobium* suggested that these bacteria were not primary anthracene degraders. *Pigmentiphaga* and *Herminiimonas* have not previously been associated with anthracene degradation. An assessment of the DNA extraction kit used in this dissertation research revealed the importance of optimizing DNA and gene target recoveries prior to extensive experimentation.

2.   *Use DNA-based SIP to identify 2-, 3-, and 4-ring PAH-degrading bacteria indigenous to PAH-contaminated soil, and design and validate quantitative PCR primers and standard curves to quantify SIP-identified groups.*

    This work included the first SIP experiments using [U-^{13}C] fluoranthene or benz[*a*]anthracene as a growth substrate. Pyrene Group 2, an uncultivated group of Gammaproteobacteria, was newly associated with growth on each of these substrates, and it was the only group that grew on pyrene. Bacteria related to *Sphingobium* and *Sphingomonas* also grew on fluoranthene. *Pigmentiphaga* was newly associated with naphthalene and phenanthrene degradation. The remaining bacteria identified in association with naphthalene (*Acidovorax*, *Pseudomonas*, *Sphingobium*, and *Variovorax*) or phenanthrene (*Acidovorax* and *Sphingobium*) were previously known to grow on those compounds. Naphthalene- and phenanthrene-associated *Pigmentiphaga*, *Sphingobium*, and *Variovorax* were similar to those identified by SIP with anthracene. Including anthracene, three of the six compounds investigated (anthracene, benz[*a*]anthracene, and pyrene) were primarily degraded by

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bacteria that are not closely related to any cultivated species, indicating the importance of the continued use of molecular methods to study complex environmental systems.

3.   Compare the effects of pre-incubation and co-incubation of 2-, 3-, and 4-ring PAH with benzo[a]pyrene on benzo[a]pyrene mineralization, and determine whether SIP-identified bacteria are associated with benzo[a]pyrene mineralization.

Naphthalene, phenanthrene, and pyrene each competitively inhibited BaP mineralization under co-incubation conditions, but BaP mineralization was enhanced by pre-enriching the bioreactor community on each compound. Anthracene, fluoranthene, and benz[*a*]anthracene did not affect BaP mineralization. None of the major PAH-degrading groups identified by SIP to address the previous objectives was associated with BaP mineralization, but pre-enriching the bacterial community on naphthalene or phenanthrene selected members of the genus *Cupriavidus* as the most likely bacteria to participate in BaP mineralization. Since the bacterial community in the bioreactor seems to be different from bacterial community in the untreated soil, an SIP investigation of the PAH-degrading bacterial guild within the bioreactor is warranted. Direct evidence of BaP metabolism cannot be obtained by presently available cultivation-independent methods because to date no organism is known to use BaP as a growth substrate. However, identifying the bacteria in a complex system that are capable of specific compound degradation and using indirect evidence to associate those bacteria with other metabolic abilities is a reasonable approach to investigating BaP metabolism.

6.2.   Recommendations for Future Research

To reduce the potential for human exposure to PAHs, it is imperative that researchers explore strategies to reduce environmental PAH contamination. Research goals should

include efforts to understand the mechanisms by which microorganisms remove PAHs from the soil environment. Identifying the microorganisms that are active against particular PAHs was the first step toward exploiting PAH-degrading bacteria for their metabolic capabilities at PAH-contaminated field sites. The next step is to determine what genetic elements facilitate PAH metabolism within each of the SIP-identified bacterial groups for which this information is not known.

The dioxygenase systems responsible for aerobic PAH metabolism (28) have been described for various bacteria capable of degrading lower molecular weight PAHs (178, 179). However, most of what we know about the genetic determinants of higher molecular weight PAH degradation by bacteria has resulted from the study of a single bacterium, *Mycobacterium vanbaalenii* PYR-1 (8, 141, 180-183), and its degradation genes are arranged in a different order and with low amino acid sequence homology to genes associated with lower molecular weight PAH degradation (142). It is possible that other bacteria capable of higher molecular weight PAH degradation, such as Pyrene Group 2, also have unique dioxygenase system gene arrangements or amino acid signatures.

The results of cultivation-independent approaches to studying microbial ecology have been used to target isolation efforts (7, 75, 99) and should continue to be employed to direct or complement studies of bacteria isolated from environmental systems. Identifying Pyrene Group 2 by SIP was the impetus for our as yet unsuccessful efforts to recover an isolate from this group. Similar efforts should be made toward recovering an isolate from Anthracene Group 1. Obtaining isolates from these previously uncultivated groups will allow for the investigation of the genes responsible for PAH metabolism. Gene expression studies can then be used to assess putative gene function and investigate gene regulation.

Appendix A: Multiple DNA extractions coupled to stable-isotope probing of anthracene-degrading bacteria in contaminated soil
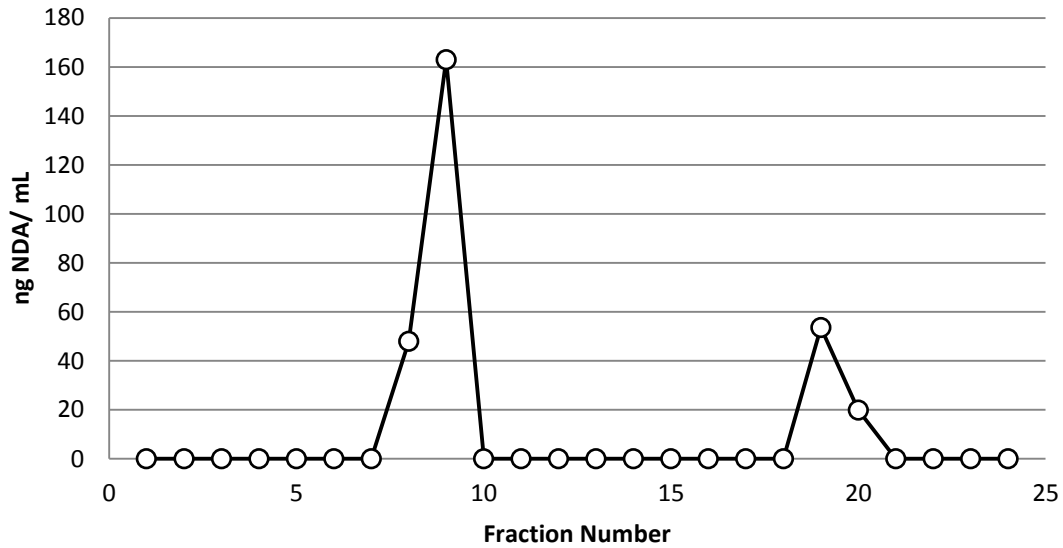
Figure A1. Mean DNA concentration (n=3) in each fraction of the control ultracentrifuge tube containing unlabeled *E. coli* K12 DNA and ^{13}C-labeled *P. putida* DNA as measured by fluorospectrometry. Error bars are not shown. Based on the volume in each fraction and the dimensions of the ultracentrifuge tube, the separation between the two peaks corresponds to approximately 27.8 mm in the tube.
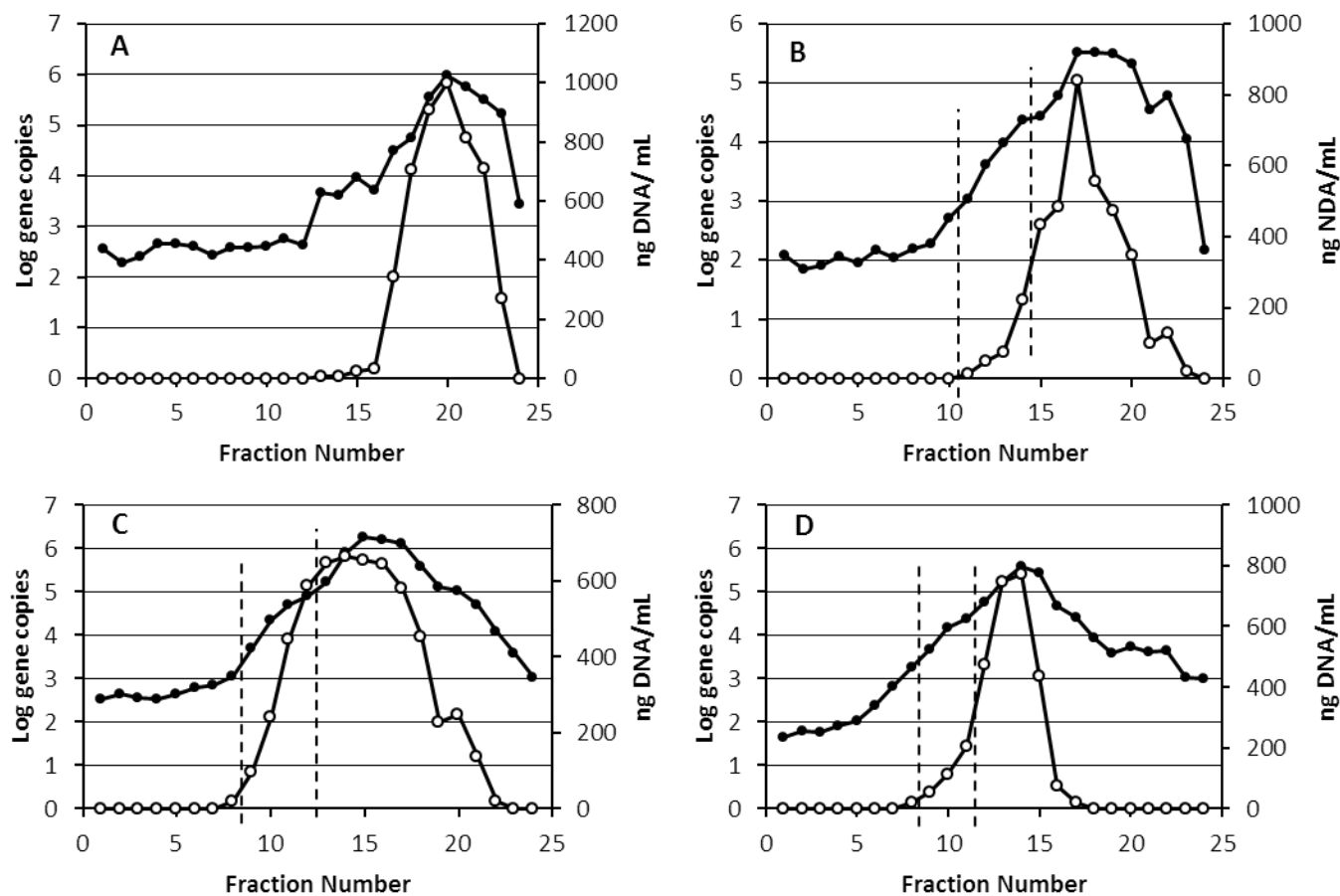
Figure A2. DNA concentration (open circles) and gene copy abundance (closed circles) in each fraction of the ultracentrifuge tubes containing DNA enriched with unlabeled (A) or ^{13}C-labeled anthracene (B, extract 1; C, pooled extracts 2-4; D, pooled extracts 5-7). The dashed lines bracket the range of fractions identified as containing primarily heavy DNA on the basis of DGGE analysis (see Figure A3).
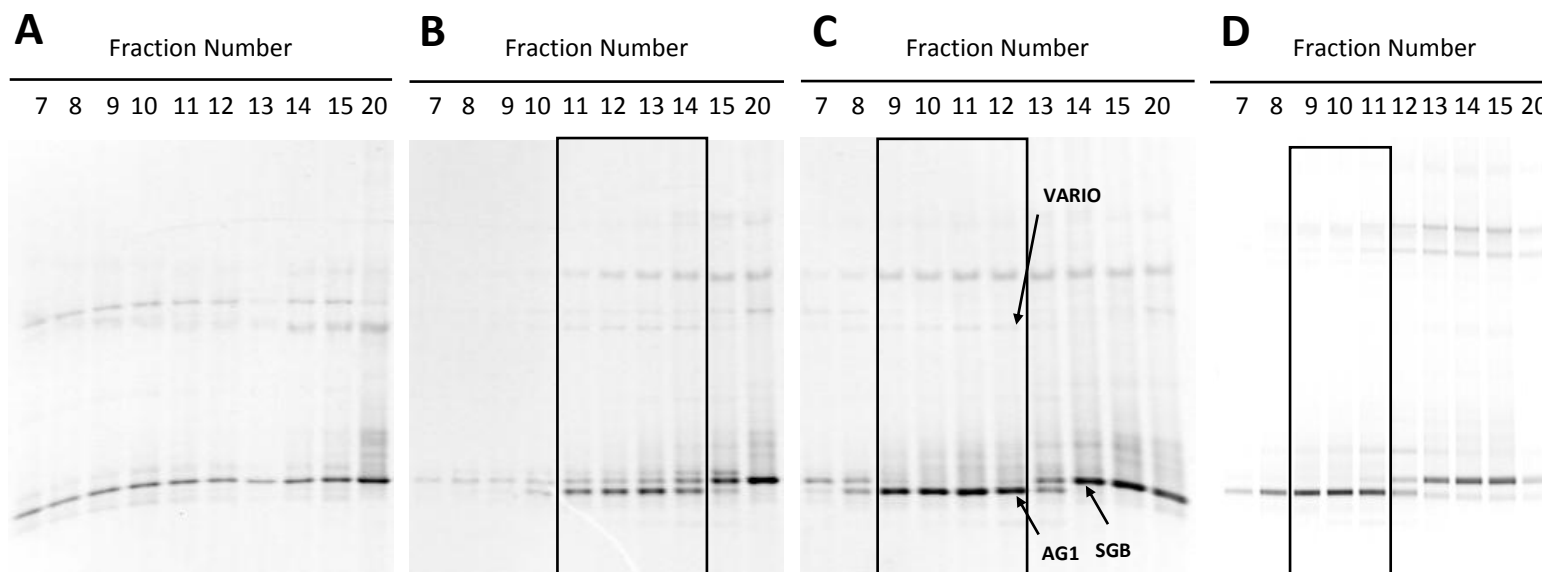
Figure A3. Negative DGGE images of community profiles from fractions 7-15 and fraction 20 (from left to right in each panel) in ultracentrifuge tubes containing DNA from soil slurry enriched with unlabeled (A) or ^{13}C-labeled anthracene (B, extract 1; C, pooled extracts 2-4; D, pooled extracts 5-7). The profile for fraction 20 represents the profiles observed for the range of fractions containing light DNA. The boxed lanes represent fractions that were pooled to serve as the composite heavy DNA fractions on which molecular analyses were performed. Arrows indicate bands corresponding to SIP-identified groups as determined by co-migration analysis (data not shown). Abbreviations are as in Figure 3.3.

SBANT182 (1)
Charlotte, NC soil pyrene SIP clone PYR10d11 (DQ123671)
St. Louis Park, MN soil pyrene SIP clone Slurry1-12 (DQ906944)
Salisbury, NC soil pyrene SIP clone sbpyr04 (GU266445)
Salisbury, NC soil pyrene SIP clone sbpyr03 (GU266444)
Thiothrix eikelboomii (AB042819)
Salisbury, NC soil naphthalene SIP clone sbnap62 (GU266332)
Pseudoxanthomonas spadix strain IMMIB AFH-5 (AM418384)
Salisbury, NC soil phenanthrene SIP clone sbphe2-50 (GU266439)
SBANT101 (4)
SBANT97 (3)
Pseudomonas fluorescens (AM900685)
Salisbury, NC soil naphthalene SIP clone sbnap45 (GU266321)
Pigmentiphaga litoralis strain JSM 061001 (EU583723)
Salisbury, NC soil phenanthrene SIP clone sbphe2-45 (GU266434)
SBANT140 (7)
Pigmentiphaga kullae strain K24 (AF282916)
SBANT22 (8)
Phenanthrene-degrading Ultramicrobacterium str. Um1 (AY367012)
Herminiimonas glaciei (EU489741)
Salisbury, NC soil naphthalene SIP clone sbnap02 (GU266293)
SBANT158 (43)
Variovorax ginsengisoli (AB245358)
SBANT115 (2)
Salisbury, NC soil naphthalene SIP clone sbnap83 (GU266349)
Salisbury, NC soil phenanthrene SIP clone sbphe2-30 (GU266421)
SBANT160 (4)
Sphingobium xenophagum strain AJ1 (AB099636)
SBANT43 (22)
Sphingobium herbicidovorans (AB042233)
Sphingobium yanoikuyae strain JAR02 (AY619556)
SBANT105 (1)
Novosphingobium resinovorum (X94103)
SBANT93 (81)
Rancho La Brea Tar Pits clone 91-78 (EF157133)
Porphyrobacter dokdonensis (DQ011529)
SBANT17 (5)
Rhizobium radiobacter (AJ295683)
SBANT23 (2)
Angulomicrobium amanitiforme (AJ535709)
SBANT71 (2)
Rhodobacter sphaeroides (X53853)
SBANT53 (2)
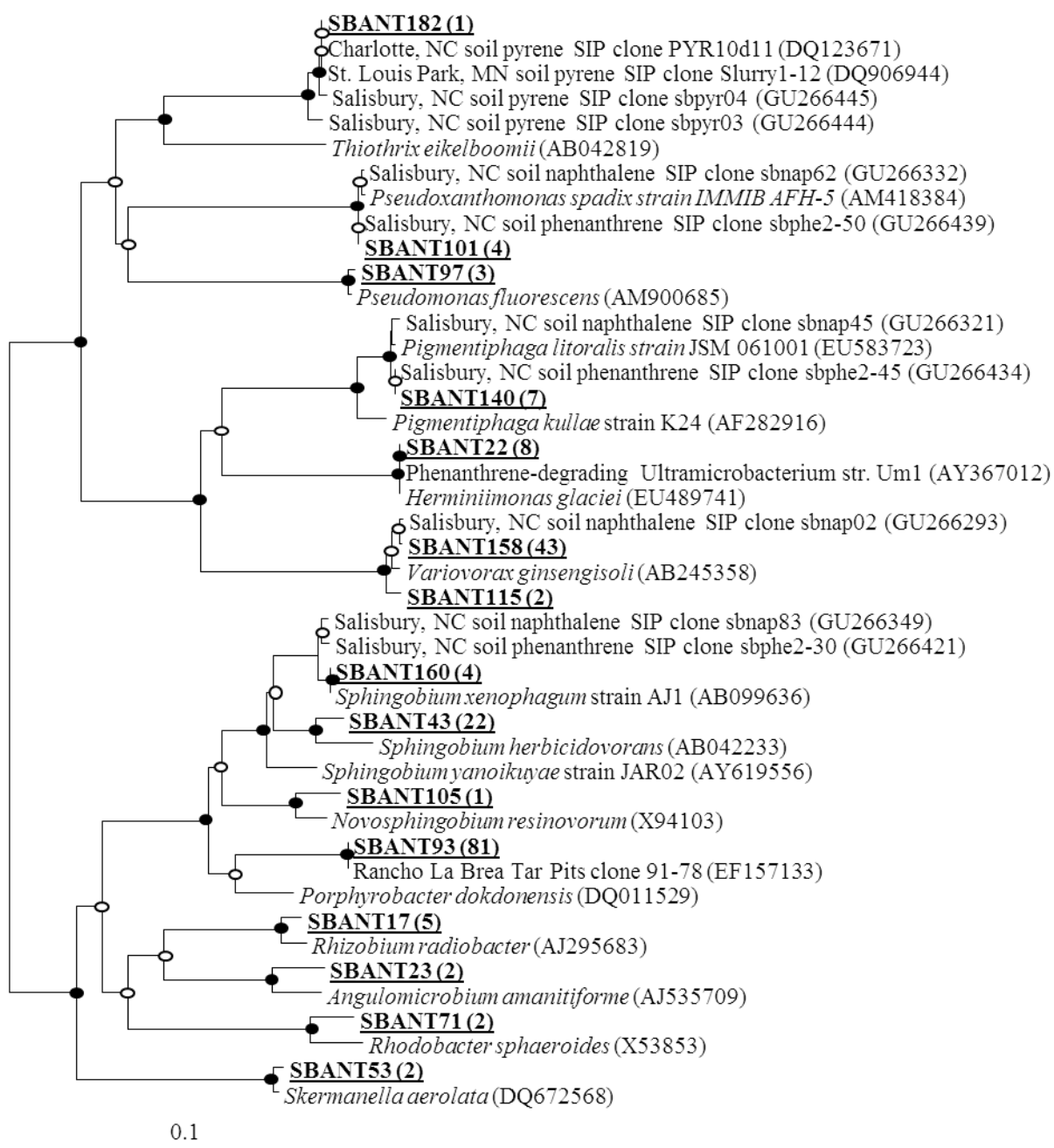*Skermanella aerolata* (DQ672568)

0.1

Figure A4. Phylogenetic tree of partial 16S rRNA gene sequences of SIP-identified anthracene-degrading bacteria recovered from the Salisbury soil (in bold and underlined) and selected reference sequences. The number of clone sequences represented and the GenBank accession number are in parentheses. The tree was rooted with *Mycobacterium vanbaalenii* PYR100 (AY636002, not shown). Open and closed circles at nodes indicate ≥ 50% and ≥ 95% bootstrap support, respectively.

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All Sequences

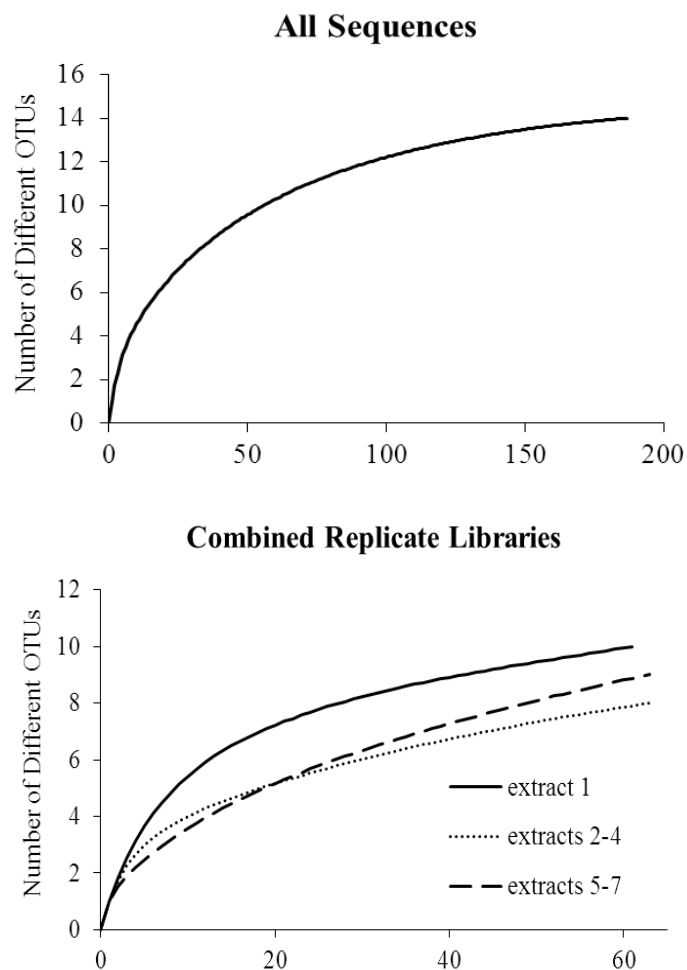**Combined Replicate Libraries**

Figure A5. Rarefaction curves (3% sequence distance) for all 187 sequences (top) and for the sequences recovered from each heavy fraction (bottom, 60-64 sequences per library).

Table A1. Permutation p-values testing the lack of correlation between copies of group 16S rRNA genes quantified in each of seven DNA extractions of anthracene-enriched soil.[1]

	BAC	AG1	VARIO	SGB	HERM	PIGM
BAC	-	**0.487**	0.000	0.001	0.017	0.003
AG1	-	-	**0.570**	**0.552**	**0.186**	**0.447**
VARIO	-	-	-	0.002	<u>0.096</u>	0.006
SGB	-	-	-	-	<u>0.078</u>	0.006
HERM	-	-	-	-	-	0.007
PIGM	-	-	-	-	-	-

[1] The permutation p-value is the proportion of permutations where we observed stronger correlations in the permuted data than in the non-permuted data. P-values ≥ 0.1 suggest pairs that are not well-correlated (values in bold). Underlined values indicate weakly correlated pairs.

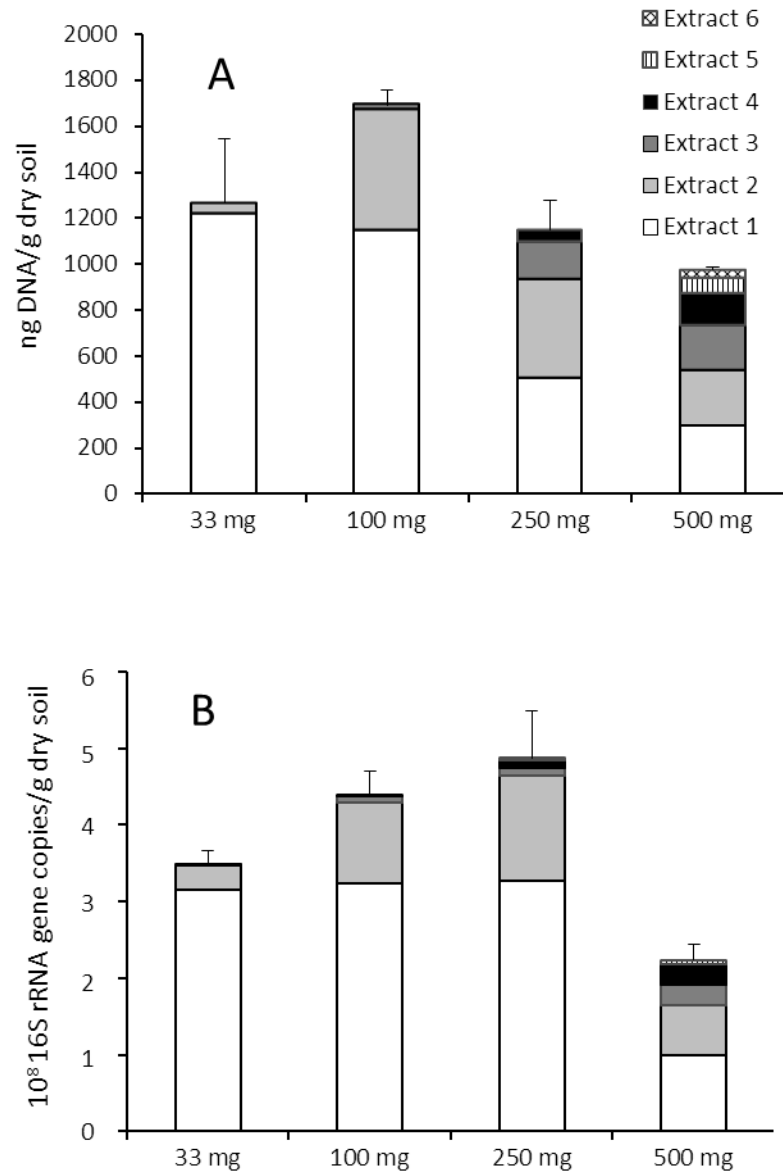[2] Abbreviations are as in Figure 3.3.

Figure A6. DNA mass (A) and 16S rRNA gene recovery (B) in successive DNA extracts of the original (untreated) soil. Values for each extract are means of duplicate analyses. Error bars represent the range of cumulative DNA mass or total number of gene copies recovered. The data for the 500-mg aliquot of soil are the same as are shown for the untreated soil in Figure 3.2.

Appendix B: Comprehensive stable-isotope probing of the polycyclic aromatic hydrocarbon-degrading bacterial guild in a contaminated soil
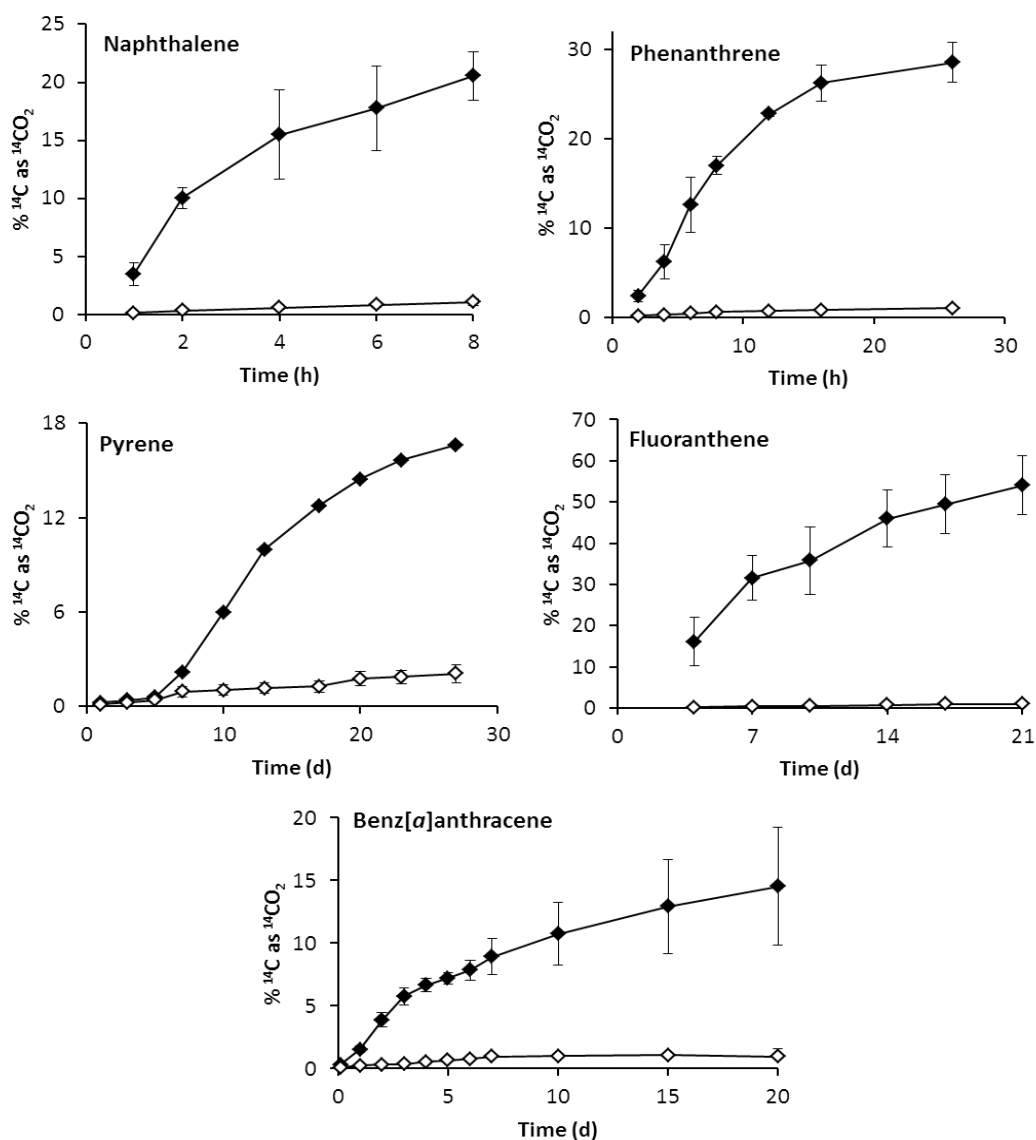

Figure B1. Mineralization data used to determine the endpoints for SIP experiments with naphthalene (8 h), phenanthrene (16 h), pyrene (12 d), fluoranthene (17 d), and benz[*a*]anthracene (21 d). Each data point is the cumulative mean ± standard deviation measured from triplicate flasks; filled symbols represent uninhibited flasks and open symbols represent acid-inhibited flasks. Low activity in the ^{14}C-pyrene stock resulted in poor reproducibility in live replicates, hence the data from a single live replicate is shown without error bars.

Table B1. Singleton 16S rRNA gene sequences recovered from heavy DNA clone libraries.

SIP Substrate	Clone Name (GenBank Accession Number)	Closest Cultivated Genus
Phenanthrene	SBPHE2-03 (GU266396)	*Variovorax*
	SBPHE2-26 (GU266417)	*Pseudomonas*
	SBPHE2-50 (GU266439)	*Pseudoxanthomonas*
Fluoranthene	SBFLA14 (HM640120)	*Acidovorax*
	SBFLA42 (HM640150)	*Pseudoxanthomonas*
	SBFLA62 (HM640170)	Unclassified *Comamonadaceae*
	SBFLA71 (HM640180)	*Rhizobium*
	SBFLA84 (HM640194)	*Thiobacillus*
Benz[*a*]anthracene	SBBAA7 (HM640087)	*Pigmentiphaga*
	SBBAA53 (HM640071)	*Thiobacillus*
	SBBAA77 (HM640095)	*Sphingobium*
	SBBAA86 (HM640105)	*Herminiimonas*

Appendix C: Analysis of Pyrosequencing Libraries

David R. Singleton and Maiysha D. Jones

1. **Transfer of files to local computer.** Download the sequence files from the server address provided by UNC-HTSF (right-click the link on the server page and Save As…). There are three files for each region of the 454 plate; a fasta file (FNA), a quality file (QUAL), and a SFF file. If multiple regions were ordered, separate files for each region will be provided. The number preceding the file name indicates the plate region for those sequences (e.g., '7' in the example below).

Name ▲	Size	Type	Date Modified
7.TCA.454Reads	23,486 KB	FNA File	3/23/2010 11:56 AM
7.TCA.454Reads	62,767 KB	QUAL File	3/23/2010 11:57 AM
GD6H2VJ07	162,931 KB	SFF File	3/23/2010 11:58 AM

2. **Merging of multiple 454 plate regions.** If you have data from only a single region, skip to the next step.

   When you have data from multiple regions of a 454 plate, it is easiest to combine the results from each of those regions now into a single fasta (FNA) and quality (QUAL) file prior to processing. While cutting and pasting each of the regions in a text editor *may* work (they are very large files and could crash the program), the easiest method I've found to join these text files is to use a command from the DOS prompt written into Windows.

   Bring up the DOS window by running the program 'cmd' from the Start menu. Navigate to the directory containing the files using the 'cd' command to change directories and the 'dir' command to list directory contents.

   Once in the directory with all of you data (all regions must be in the same directory), use the following command to join the various fasta files together:

   > for %f in (*454Reads.fna) do type "%f" >> allseqs.fna

   Likewise, the quality files can be joined together with:

   > for %f in (*454Reads.qual) do type "%f" >> allseqs.qual

   There should now be two new files in your folder: 'allseqs.fna' and 'allseqs.qual'. The SFF files will not be used in any of the described analyses. **Important:** Double check that the new file is approximately the size of the sum of the input files before proceeding. You <u>must</u> make sure that your combined file name does not have "454Reads" in the title, or it will be included in the new combined file as well, duplicating the data.

3. **Software for pyrosequencing analysis.** Open the RDP-II website: http://rdp.cme.msu.edu/index.jsp and select the Pyro tab at the top. This will bring up a suite of tools for analyzing data from pyrosequencing runs.

4. **Segregation of barcoded data into libraries.** Under the "Data Processing Steps", select the "Pipeline initial process". This tool will remove poor quality sequences and segregate the sequences into libraries based on the barcode sequence.

Upload the FASTA file (FNA) and the quality file (QUAL) where indicated. In order to segregate the libraries, you will also need a tag file. The tag file is created with a text-editing program (e.g., notepad or Wordpad). Here is the tag file used for the bioreactor samples. It is two, tab-separated columns, the first being the unique 8-bp barcode incorporated into the primers and the second being the name of the library into which the sequence will be deposited.

Aagcaacg A1
Aagcatgg A2
Aagcgcaa A3
Aagctagg A4
Aaggaagg A5
Aaggccaa A6
Ttgcaacg B1
Ttgcatgg B2
Ttgcgcaa B3
Ttgctagg B4
Ttggaagg B5
Ttggccaa B6
Ccggatat F1
Ccgcataa F2

I recommend keeping the library name short (a few characters) and each, similar library beginning with a unique letter. In this instance, A = Monthly-fed reactor, B = Weekly-fed reactor, F = Feed soil. This will ease the renaming of sequences later. Next cut and paste both the forward and reverse primers used to generate the PCR amplicons into BOTH the

forward and reverse primer boxes. Include the 2 base linker, but not the barcode. You should only have two primers in each box: one Bacterial F and one Bacterial R. Since the sequencing direction was random, either the reverse or forward primer could have been read by the machine as the "forward" primer. These are also the bases that will be trimmed to not appear in the final sequence.

Finally, set the filter to a minimum sequence length of 250 bp and set the reverse primer max to 2.
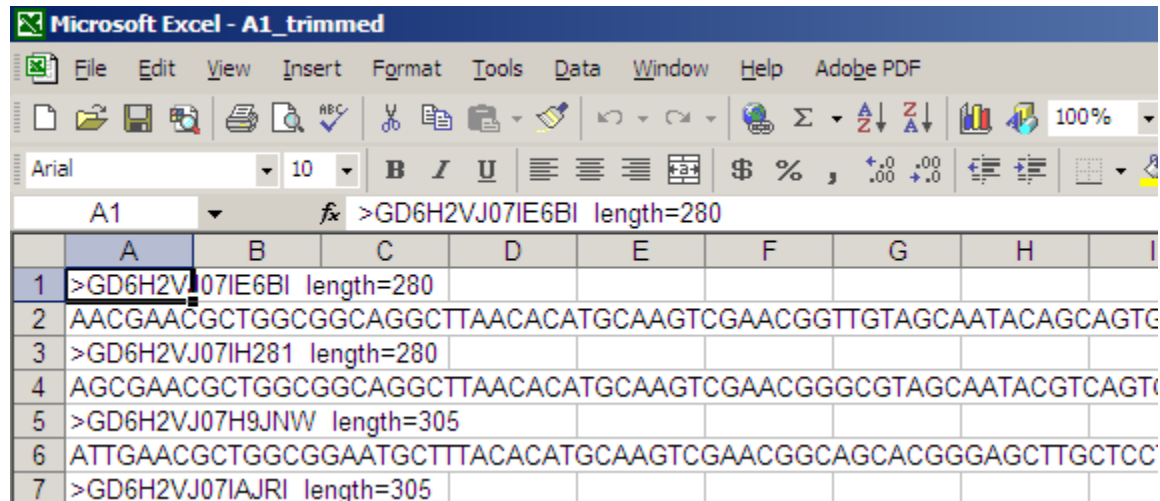
Select the "Perform Initial Processing." It may take quite some time to upload the files. Be patient. When the analysis is complete, a download link will be provided. Uncompress the resulting file (using a program such as WinZip-available for free at shareware.unc.edu) to see the segregated libraries.
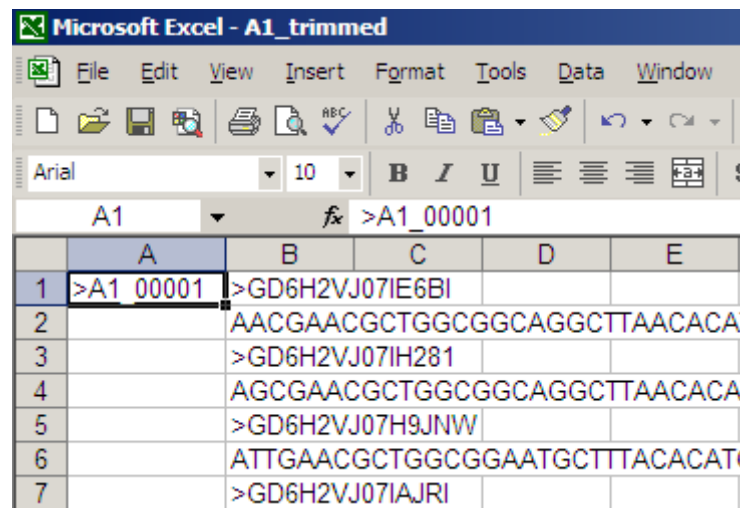
5. **Renaming of sequences.** Now that the sequences are segregated into their respective libraries, it is time to give them a more meaningful name. This requires a bit of creativity and Microsoft Excel.

Open the 'trimmed' file of a library in Excel. You should see a document that looks like this:

Add a new column just before column A by selecting all of column A (just press the 'A' at the top of the column) and the Insert and Column option on the toolbar.

In the first row of the "new" column A, type the new name of the first sequence with a number designation at the end. Use as many zeros in the first number to account for the number of sequences in that library after trimming (see each file called libraryname_stats). Don't forget to add the '>' symbol. In the second row, adjacent to the sequence data, enter the formula '=B2' without quotes.

After hitting [Enter], cell A2 should contain the same sequence information as B2. Highlight both cells A1 and A2, and drag the bottom right hand corner of that highlight box down the entire column until you reach the end of your data. This should have renamed every other cell sequentially based on the formula in the first cell (A1), while copying the sequence data exactly. Unfortunately, as the sequence data in column A is the result of a formula, we can't just erase the other columns (B) and save this file.

Alternatively, you may be able to highlight cells A1 and A2, and then double click on the lower right-hand corner of the highlight box to fill in the entire column to the end of your data set.

Once every sequence has been renamed, select all of column A, hit Copy (Ctrl-C), and open a <u>new</u> worksheet. Under the <u>E</u>dit menu, use the 'Paste <u>S</u>pecial…' command with the

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'Values' option selected. *Alternatively you can use the next tab in that same worksheet. Only the active tab will be saved in the tab delimited file.*
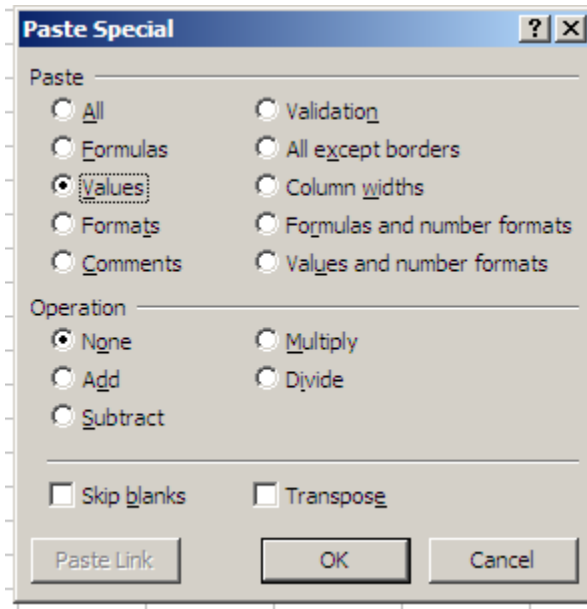
You should now have a clean worksheet with renamed sequences, and sequence data that doesn't refer to any other cell. To keep this data readable by other programs (and retain the Fasta format), do a "Save as…" and make sure that the file type is 'Text (Tab delimited)'. Save the file as something distinctive and representing the renamed feature of the sequences (e.g., 'A1_allseqs_renamed.fasta').

Repeat this process for the other libraries, making sure that no two sequences are ever identically named.

6. **Classification of sequences.** The RDP Classifier tool (available from the main page or the pyro page) accepts as input a fasta file of sequences. Enter each fasta file from a library, one at a time, into the classifier program. Results can be downloaded to be opened in Excel from the results page.

For each spreadsheet, type a dash into cell F4, and in cell E8 type:

$$=IF(C8=0,\$F\$4,C8/\$C\$8*100)$$

Fill to the bottom of the column (shift+control+end). This will give you the percent representation of each organism.

As all of the bacterial (and archaeal if you used bacterial and archaeal primers to generate libraries) phylogenetic groups will be presented in the text file, including those that were

not found in the library, it is a simple matter to select, copy, and paste the Classifier results from multiple samples into a single spreadsheet. Insert rows for your SIP-identified groups if they are unknown (for example, pyrene group 2). Delete all rows that have no data.

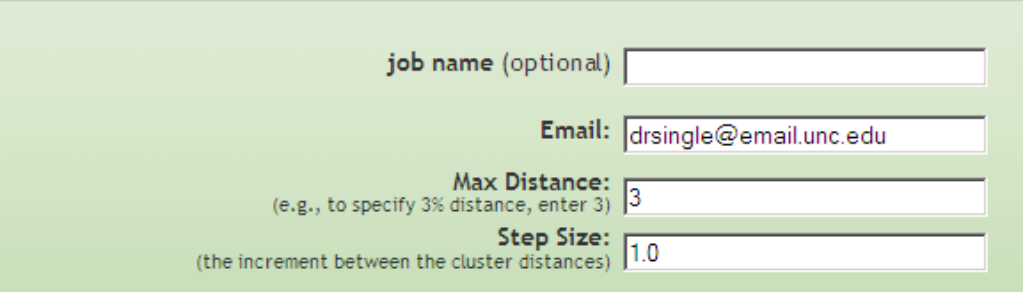7. **Alignment of Pyrosequences.** The next step is to align the sequences in each of the libraries. Returning to the Pyro homepage of the RDP, select the option for 'Pyrosequencing aligner'. You can load multiple libraries into the pipeline at once, and after uploading all of them you will be given the option to combine the samples into one alignment or to keep them separate. For now, keep the libraries separate (through step 11). The upload and alignment may take some time.

8. **Clustering of Sequences.** Using the aligned sequence files as input, the "Complete Linkage Clustering" tool of RDP can be used to group sequences together based on sequence similarity. A dissimilarity of 3% (97% similarity) is commonly used with 16S rRNA gene data, although these values can change depending on the user and analysis to be run. Using the parameters illustrated in the image below will give four sets of clusters; 0, 1, 2, and 3% dissimilarity. The default is up to 15% dissimilarity.

   If you put all the alignment files in at once, your output cluster files will be numbered. They will not be named according to the library name. If you put them in one at a time, you can name each job and each output cluster file will be named accordingly, but this will be time consuming depending on how many libraries you have.

9. **Diversity Estimates.** The clustered sequence files can be used to obtain a variety of diversity estimates, including chao1, Shannon, and rarefaction data. Data are returned as text files that are more easily visualized in Excel. Interpretation of these results is an exercise left to the analyzer. *This step is not necessary to proceed, but you might as well have the data.*

10. **Dereplication.** RDP provides a tool (Dereplicate) that takes as input both the cluster file and fasta alignment of sequences from a library to provide a file which lists representative sequences of operational taxonomic units (OTUs) at a distance specified by the user.

The output looks like this:

1	47	A1_01335	0.02724	0.00163
2	49	A1_01202	0.02692	0.00099
3	1	A1_01610	0	0
4	1	A1_01619	0	0
5	160	A1_04182	0.02308	0.00206
6	1	A1_01618	0	0

The columns are (from left to right): a number designating an OTU, the number of sequences from that library in the OTU, the sequence selected as a representative of that OTU, the maximum distance of any other sequence from the representative sequence (in this example, < 0.03), and the minimum sum of squares for each cluster.

11. **FASTA Sequence Selection**. In order to prepare the data for Fast UniFrac analysis, we must first create a fasta file containing only the representative sequences from each cluster.

The first step in this process is to open the result files from the previous (dereplication) step. Copy the entire third column (the one containing sequence names) into a text document (in WordPad, for example) and save as it as a text-only file with a distinctive name (e.g., 'A1_dereplicated_seqs_list.txt').

This text file is one file required for input into the 'FASTA Seqeuence Selection' program of RDP's pyro pipeline (sequence ID list file); the other being the fasta file of your renamed sequences from step 5. The output file is simply an edited fasta file containing only the representative and singleton sequences.

Unzip the output files. Cut and paste the sequences in each of the output files into a single fasta file called allseqs_fasta-selection.fasta.

12. **Align fasta-selection sequences**. Go to http://www.drive5.com/muscle/downloads.htm and save the appropriate file to a folder called 'PAP' (pyro analysis programs) on your desktop. Copy allseqs_fasta-selection.fasta to this folder. Using MS DOS prompts, move to the PAP directory (type: cd desktop\pap). Close all open windows and programs before running the alignment. To run the alignment, hit enter after typing:

    -in filename.fa –out filename.afa –maxiters 1 -diags1 -sv

    Alternatively, you can run your alignment online with RDP or by uploading the fasta selection file to the webserver: http://mafft.cbrc.jp/alignment/server/. *Keep the default settings and input your email address to get results.* <u>*If you used RDP, open the output file and scroll down to the bottom. There may be lines that begin with > that are not your sequence data. Delete these lines before running gblocks.*</u>

13. **Get blocks.** Gblocks is a program that cleans up sequence alignments by extracting the most useful aligned sequence data. Download Gblocks from http://molevol.cmima.csic.es/castresana/Gblocks.html. Put the program in the PAP folder. This program also runs in MS DOS, but will open by double clicking on the icon. Read http://molevol.cmima.csic.es/castresana/Gblocks/Gblocks_documentation.html to decide what parameters to adjust. Under option b., 1. and 2. are default values based on the data in your alignment input. The other parameters on this menu, the extended menus, and the save menu should be adjusted to maximize the number of positions returned. The input file should be in the same folder as the gblocks application.

14. **Build a tree.** FastTree is a program that will build a phylogenetic tree from the selected sequence blocks output by gblocks. Download fasttree from http://www.microbesonline.org/fasttree/#Install. Put the program in the PAP folder. The gblocks output file should also be in this folder in fasta format. The fasttree output will be in Newick format. Using MS DOS prompts, move to the PAP directory (type: cd desktop\pap). *Closing all open windows and programs before building the tree might speed up the tree building process.* To build the tree, hit enter after typing:

    fasttree -nt inputfilename.fasta > outputfilename.nwk

15. **Run FastUniFrac.** Read through the tutorial at http://128.138.212.43/fastunifrac/. Generate the necessary mapping files. Register to use the site. Upload the required files, choose your preferred analyses, and voila! You have successfully analyzed you pyrosequence data. Have fun interpreting the output!

SEARCHING LIBRARIES FOR SPECIFIC DEGRADERS

1. **Compile sequences from all libraries.** Make a folder containing all files from Step 5 above. Using DOS, move to that folder and type this:

    for %f in (*.fasta) do type "%f" >> allseqs.fasta

All fasta sequnces should now be in a single file called allseqs.fasta, and the size should be similar to the sum of the sizes of each individual file.

2. **Download the BLAST+ executable.** Go to http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_TYPE=Download, and run the appropriate file. The program will make a folder in C:\Program Files called NCBI. Within NCBI will be a folder called bin. Make a folder on the desktop called BLAST+. Copy the blastn and makeblastdb applications from the bin folder to the BLAST+ folder. These applications run in DOS and use command line prompts.

3. **Build a local blast database.** Copy allseqs.fasta to the BLAST+ folder. Open the DOS workspace. The database output files will be in the BLAST+ folder (nhr, nin, and nsq).To build the database type:

    makeblastdb -in allseqs.fasta -dbtype nucl -title allseqs_db -out allseqs_db

4. **Search a local blast database.** Generate a separate fasta file for each sequence you want to find in your database, and run this program once for each sequence you want to find. For example, I am looking for PG1, PG2, and AG1 so I will have three fasta files in the BLAST+ folder, and I will run blastn three times. To search your database for PG2 type:

    blastn -db allseqs_db -query pg2.txt -out allseqsfound_pg2.txt -perc_identity 97 -num_descriptions 120000 -num_alignments 120000

    Definitions:
    blastn: application to search for nucleotide sequences
    -query: file for sequence you want to find
    -perc_identity: tells blastn you only want results that are at least 97% similar to the query
    -num_descriptions: number of sequence IDs to return (max = the number of seqs in your database)
    -num_alignments: number of alignments to return (max = the number of seqs in your database)

5. **Interpreting sequences found.** Open allseqsfound_pg2.txt in Notepad. Scroll down and look for a break point in the scores (decrease) and E values (increase). The lower the E value, or the closer it is to "0", the higher is the "significance" of the match. Search for the first sequence ID after the break point using Edit, Find to go to that sequence's alignment with the query sequence. The length of this alignment (and those that follow it) will likely be shorter than those before it. You cannot be confident in these matches. Control+Home to the top of the page and copy sequence IDs (…and scores and E values—you can delete these later if you want to) above the break point into a new Notepad file and save as found_pg2.txt. The location of the breakpoint is your call to make. Repeat for each query sequence.

6. **Count the number of sequences found in each library.** Open found_pg2.txt in excel as a SPACE DELIMITED file. List the name of each library in column D. In cell E1 type:

$$=COUNTIF(\$A\$1:\$A\$787, "FS1*")$$

Fill column E to the bottom of the library names. Change 787 to the last row number for your data, and FS1 is the library name. Save as an excel file. If you save this as a text file you will lose you formulas. For the other found_query.txt files: Open the file in excel. Copy columns D and E from found_pg2.xlsx, and paste them into the same columns in the open found_query.txt file. Double check that the formulas are working correctly and that all rows are included in the formula. Incorporate this data into the Classifier results tables generated above, and adjust percentages accordingly.

Appendix D: Association of polycyclic aromatic hydrocarbon-degrading bacteria in contaminated soil with benzo[*a*]pyrene mineralization
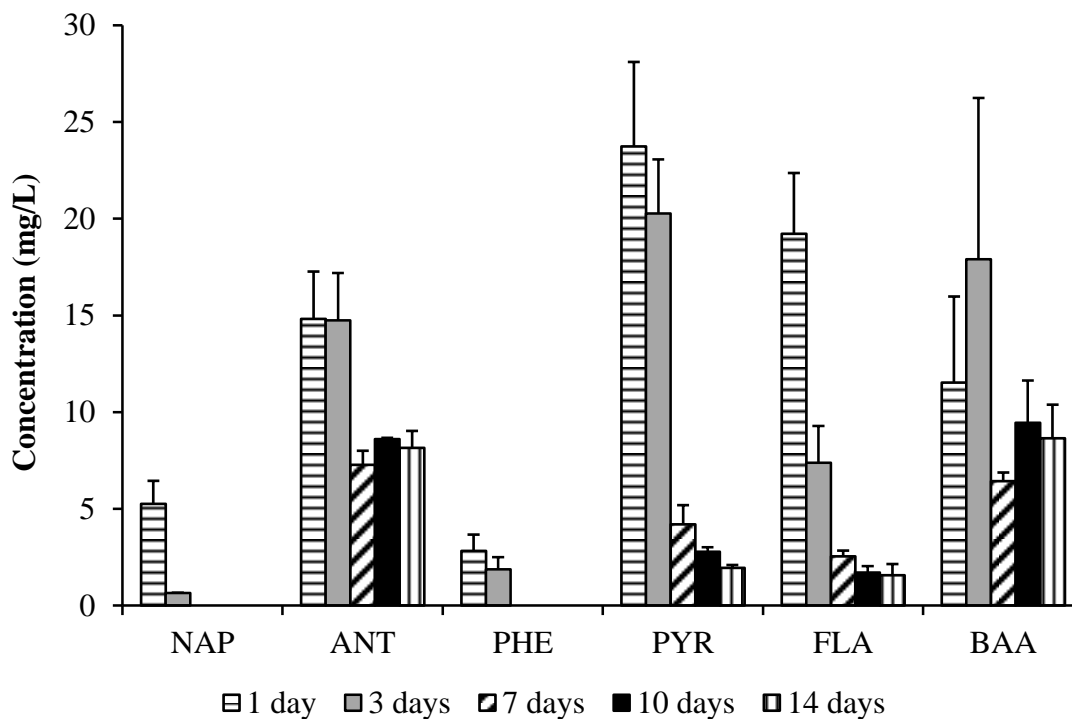
Figure D1. Results of preliminary pre-enrichment experiment. The bioreactor soil slurry was incubated with one of the indicated PAHs (21 mg/L initial concentration) over a 14-day period. Aliquots were removed from each flask at the indicated time point and extracted with ethyl acetate. Each value is the mean ± standard deviation of a single HPLC measurement of the extract from each of triplicate flasks (n=3).
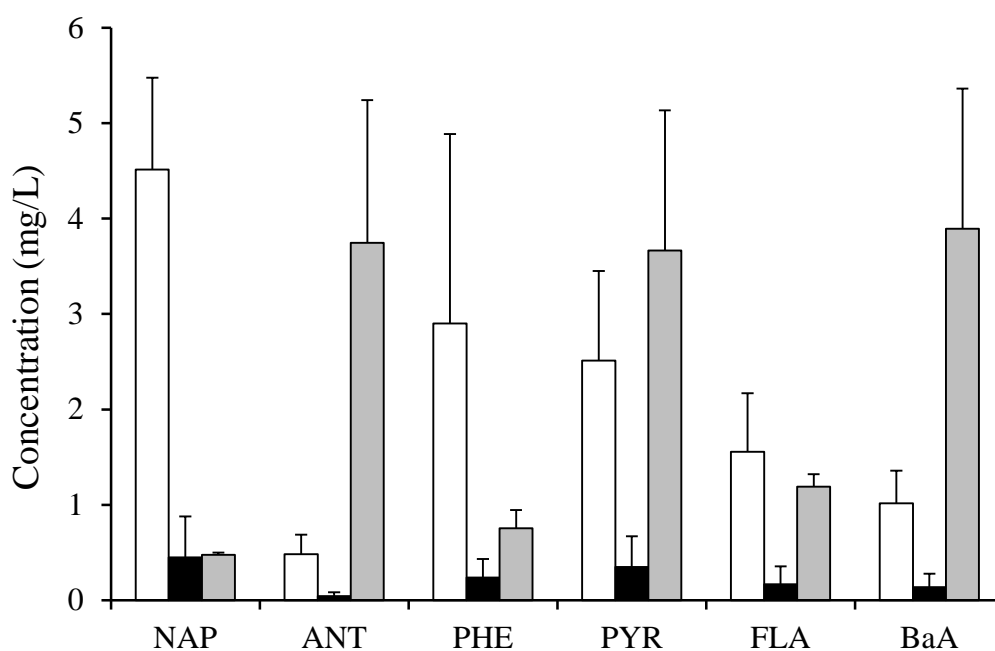
Figure D2. PAH present in inoculum (open bars, t=0) and substrate remaining after 7 d of pre-enrichment without an added substrate (black bars) or with the substrate indicated (gray bars). Each value is the mean ± standard deviation of a single HPLC measurement of an ethyl acetate extract from each of two sets of duplicate flasks (n=4).

Table D1. Pyrosequencing library barcodes, sequence/cluster counts, and community estimates.

Library[1]	Barcode	No. of Sequences	No. of OTUs	Chao1	LCI95[2]	UCI95	H'[3]
BRS	AAGCAACG	17184	3053	5283	4999	5607	6.26
CNOB	AAGCATGG	17758	3207	5363	5093	5671	6.39
CNAB	AAGCGCAA	17359	2938	4840	4590	5127	6.20
CANB	AAGCTAGG	10904	2233	3715	3497	3969	6.19
CPHB	AAGGAAGG	12634	1817	3085	2876	3335	5.29
CPYB	AAGGCCAA	22373	3804	6208	5929	6524	6.43
CFLB	TTGCAACG	993	354	662	565	802	5.13
CBAB	TTGCATGG	2153	669	1197	1070	1366	5.60
PNOB	TTGCGCAA	452	186	398	314	538	4.45
PNAB	TTGCTAGG	412	107	177	142	246	3.63
PANB	TTGGAAGG	1243	252	500	407	650	4.18
PHB1	TTGGCCAA	1135	190	352	284	468	3.95
PHB2	GAGAACAC	40398	2093	3044	2884	3236	4.76
PHB3	GAGACACA	14280	1070	1714	1571	1898	4.57
PPYB	TTGCTTCG	738	144	323	241	472	3.46
PFLB	TTGGTACG	373	111	166	139	220	3.87
PBAB	TTGGTTGG	946	269	439	379	532	4.61
PNON	TTGGCGTA	404	147	385	277	582	4.25
PNAP	TTGGATGC	1485	249	391	338	477	3.95
PANT	TTGCGGAT	1074	199	335	280	429	3.83
PPHE	CCGGATAT	16159	1115	1792	1645	1978	4.34
PPYR	CCGCATAA	8866	960	1560	1428	1728	4.31
PFLA	CCGGTTAA	12980	1146	1788	1653	1958	4.62
PBAA	GACTTCAG	21900	2733	4219	4011	4461	5.81

[1] Libraries are defined in Table 5.1 of the manuscript.
[2] Lower (L) and upper (U) 95% confidence intervals around the chao1 index.
[3] Shannon index. Each variance is less than 0.01.

Table D2. Relative abundances of bacteria that grew during co-incubation.[1]

Classification	BRS	CNOB	CNAB	CANB	CPHB	CPYB	CFLB	CBAB
			Co-incubation Libraries					
SIP-identified Groups								
Achromobacter	-	-	-	-	-	-	-	-
Acidovorax	7.10	6.79	6.25	7.42	32.58	6.57	7.05	7.57
Anthracene Group 1	< 1	< 1	< 1	< 1	< 1	< 1	-	< 1
Herminiimonas	-	< 1	< 1	< 1	-	< 1	-	< 1
Pigmentiphaga	< 1	-	-	-	< 1	< 1	-	-
Pseudomonas	< 1	< 1	1.75	< 1	< 1	< 1	< 1	< 1
Pseudoxanthomonas	< 1	< 1	< 1	< 1	< 1	< 1	-	< 1
Pyrene Group 2	-	-	-	-	-	< 1	-	-
Rhizobium	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Rhodobacter	-	-	-	-	-	-	-	-
Skermanella	-	-	-	-	-	-	-	-
Sphingobium	< 1	< 1	9.64	1.96	3.40	2.44	1.31	3.62
Sphingomonas	1.40	< 1	< 1	< 1	< 1	< 1	2.11	< 1
Thiobacillus	8.33	7.03	6.45	6.76	3.72	6.51	3.63	5.16
Variovorax	< 1	< 1	< 1	< 1	< 1	< 1	-	< 1
Other Groups								
Acidobacteria Gp7	5.76	6.30	5.79	6.36	3.90	6.21	5.24	4.04
Methylophilus	-	< 1	1.04	1.01	< 1	< 1	< 1	< 1
Phenylobacterium	< 1	1.04	< 1	< 1	< 1	< 1	2.01	1.39
Polaromonas	< 1	< 1	< 1	< 1	< 1	< 1	< 1	1.07
Rhodococcus	< 1	< 1	< 1	< 1	< 1	< 1	1.01	< 1

[1] Libraries are defined in Table 5.1 of the manuscript.

[2] -, not detected in the library.

7. Literature Cited

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