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Stable-isotope probing of the polycyclic aromatic hydrocarbon-degrading bacterial guild in a contaminated soil

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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 ¹³C-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.

Keywords

bacteria; functional diversity; metagenomics/community genomics; microbial communities; microbial ecology; pollution microbiology; uncultured microbes

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 (Agency for Toxic Substances and Disease Registry, 2007). PAHs are natural components of coal, petroleum, and other fossil fuels, and PAH contamination can result from the incomplete combustion of these and

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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 (US EPA Office of Solid Waste and Emergency Response, 2007), 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 (Kiyohara et al., 1982; Willison, 2004; Gaskin and Bentham, 2005; Zhou et al., 2008) and cultivation-independent (Jeon et al., 2003; Padmanabhan et al., 2003; Singleton et al., 2005; Singleton et al., 2006; Singleton et al., 2007; Jones et al., 2008; Huang et al., 2009) 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 (Oren, 2004) and fails to account for the complex interactions of the members of microbial communities with each other and with their native environment. Cultivation-independent techniques can help us better estimate the prokaryotic diversity of complex systems (Amann et al., 1995; Breznak, 2002; Rappe and Giovannoni, 2003) 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 (Radajewski et al., 2000). To date, SIP has been used to identify soil bacteria capable of degrading the PAHs naphthalene (Jeon et al., 2003; Padmanabhan et al., 2003; Singleton et al., 2005), phenanthrene (Singleton et al., 2005; Singleton et al., 2007), pyrene (Singleton et al., 2006; Singleton et al., 2007; Jones et al., 2008) and anthracene (M. D. Jones et al., submitted). 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-¹³C]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.

Results

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 6% of the added parent compound remained for each substrate except pyrene, for which 25% remained. The mineralization data for each PAH are shown in Fig. S1 of the supporting information. In general, mineralization occurred over the same time scale as the disappearance of the parent compound. For fluoranthene, mineralization reached a plateau after 17 d even though 95% of the added fluoranthene had been consumed by day 4. We observed a similar difference in the time frames over which mineralization and parent

compound disappearance occurred for anthracene in a separate SIP study on the same soil (M. D. Jones et al., submitted).

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. Because we do not have confidence that singleton sequences were associated with heavy DNA (Singleton et al., 2005), singleton sequences are not included in subsequent analyses but are listed in the supporting information (Table S1). The remaining sequences, along with sequences from our previous anthracene SIP experiment (M. D. Jones et al., submitted), were grouped into operational taxonomic units (OTUs) based on 97% sequence similarity (Table 1). Figure 1 shows how the representative for each OTU is related to selected reference sequences from GenBank.

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) (Singleton et al., 2006). Sequences related to PG2 were also present in the clone library generated from SIP with fluoranthene (12 of 91 clones), but 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).

Quantification of SIP-identified groups

Primers for quantitative PCR (qPCR) targeting the 16S rRNA genes of several SIP-identified groups were developed (Table 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 detection limit of each assay in the original soil sample (data not shown; see Table 2 for the detection limits). Several of these groups increased to above the detection 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 (Fig. 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 (Fig. 3). PG2-related bacteria associated with fluoranthene degradation were below the detection 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.

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 (M. D. Jones et al., submitted), 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.

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 1). Of the 16 OTUs reported in Table 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 1), suggesting a degree of specialization for degrading a particular PAH. Only one OTU associated with growth on naphthalene (*Rhizobium*) was associated with growth on a four-ring compound, and it was 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*. Four of the OTUs associated with anthracene degradation were also associated with naphthalene degradation, but several of the remaining OTUs did not grow on any other PAH evaluated.

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* (Jeon et al., 2003; Padmanabhan et al., 2003; Yu and Chu, 2005), *Acidovorax* (Jeon et al., 2003; Yu and Chu, 2005), and *Variovorax* (Jeon et al., 2003; Padmanabhan et al., 2003). Members of the genus *Sphingobium* have previously been associated with naphthalene degradation by other methods (Lafortune et al., 2009). *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 (Fig. 2). *Variovorax*-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 (M. D. Jones et al., submitted). The *Variovorax*- and *Pigmentiphaga*-related sequences associated with naphthalene were greater

than 99% (over 815 bp of aligned sequence) and 98.9% (817 bp) similar, respectively, to sequences recovered from SIP with anthracene (M. D. Jones et al., submitted).

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 (Singleton et al., 2005; Singleton et al., 2007), and *Sphingobium*-related sequences have been associated with phenanthrene degradation by other methods (Lafortune et al., 2009; Isaza and Daugulis, 2010). 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. The *Pigmentiphaga*-related sequence was identical (over 816 bp) to the sequences recovered from SIP with anthracene (M. D. Jones et al., submitted).

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 (Singleton et al., 2006). 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, Minnesota (Jones et al., 2008). The Salisbury, North Carolina 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, North Carolina soil did grow on phenanthrene (Singleton et al., 2007). 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 an extracellular metabolite derived from fluoranthene. *Sphingomonas* and *Sphingobium* are genera that are known to include fluoranthene-degrading species (Pinyakong et al., 2003; Story et al., 2004; Baboshin et al., 2008). The *Sphingobium*-related sequences were greater than 96.3% (over 790 bp of aligned sequence) and 95.9% (790 bp) similar to *Sphingobium*-related sequences from SIP with naphthalene and phenanthrene, 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 (Kazunga et al., 2001; Pinyakong et al., 2003; Basta et al., 2004; Stolz, 2009). What little is known about the bacterial degradation of benz[*a*]anthracene has resulted from studies of *Mycobacterium* isolates (Schneider et al., 1996; Moody et al., 2005;

Kim et al., 2006; Kim et al., 2008), 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* (Hilyard et al., 2008), as well as *Stenotrophomonas* (Juhasz et al., 2000), *Burkholderia* (Juhasz et al., 1997), and *Mycobacterium* (Zeng et al., 2010). 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 (Heitkamp et al., 1988; Kelley and Cerniglia, 1991; Moody et al., 2005) and other *Mycobacterium* species (Boldrin et al., 1993; Dean-Ross and Cerniglia, 1996; Schneider et al., 1996; Rehmann et al., 1998; Churchill et al., 1999; Gauthier et al., 2003; Miller et al., 2004; Pagnout et al., 2007; Zhou et al., 2008; Hennessee et al., 2009) 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. Preliminary efforts to isolate a representative PG2 culture have been unsuccessful to date.

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 (Jeon et al., 2003; Kasai et al., 2006; Singleton et al., 2009).

Experimental Procedures

Soil sample

PAH-contaminated soil from a former manufactured-gas plant site in Salisbury, Rowan County, North Carolina. 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. The total concentration of PAHs regulated by the U.S. Environmental Protection Agency was determined by high-pressure liquid chromatography (HPLC) as previously described (Singleton et al., 2008) 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.

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). [^{13}C] versions of each compound were synthesized by methods to be described elsewhere (Z. Zhang, L.M. Ball, and A. Gold, personal communication). Until the methods are published, details of the syntheses can be obtained by contacting MDA at mike_aitken@unc.edu. [^{14}C]Naphthalene (17.8 mCi/mmol), [^{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.

Identification and quantification of PAH-degrading bacteria

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 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 (0.7 mM $\text{CaCl}_2\cdot\text{H}_2\text{O}$, 0.2 mM $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 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). After two days of agitation without any added substrate to allow native PAH concentrations to decline, the soil in each flask was pelleted, the supernatants were discarded, and each pellet was resuspended in 30 ml of fresh nitrogen- and phosphorus-amended simulated groundwater. Each soil slurry was then added to a flask containing 625 μg of a [^{13}C]PAH ($t=0$). Flasks were 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. Mineralization was measured by liquid scintillation counting of $^{14}\text{CO}_2$ trapped in KOH-soaked filter paper (Singleton et al., 2008). The following endpoints were selected for the SIP incubations with [^{13}C]PAH based on the mineralization data shown in Fig. S1 in the supporting information: naphthalene, 8 h; phenanthrene, 16 h; pyrene, 12 d; fluoranthene, 17 d; and benz[*a*]anthracene, 21 d.

At each incubation endpoint for SIP experiments with ^{13}C -labeled naphthalene, phenanthrene, and pyrene, DNA was isolated via a single extraction of each of two 500 mg aliquots of the soil pellet 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 in the heavy DNA via clone libraries, and qPCR primers (Table 2) were developed as previously described (Singleton et al., 2006).

At each incubation endpoint for SIP experiments with ^{13}C -labeled fluoranthene and benz[*a*]anthracene, DNA was isolated from each of four 250 mg aliquots of the soil pellet 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 by ultracentrifugation (M. D. Jones et al., submitted). ^{13}C -enriched DNA was separated from unlabeled DNA, PAH-degrading bacteria were identified in the heavy DNA via 16S rRNA gene clone libraries, and qPCR primers (Table 2) were developed as previously described (Singleton et al., 2006), except that sequences were aligned within the myRDP personalized workspace (Cole et al., 2007).

The 16S rRNA gene sequences derived from ^{13}C -enriched DNA 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 (Cole et al., 2009). A neighbor-joining phylogenetic tree was constructed with representative sequences and close relatives using Clustal X 2.0 (Larkin et al., 2007) and bootstrapped 1000 times.

DNA extracted from triplicate incubations with unlabeled growth substrates performed in parallel to the SIP experiments 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 (M. D. Jones et al., submitted). Detection limits for qPCR, as a product of both the detection limits of the primer sets and dilution of the sample, are identified in Table 2.

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).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 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 (M. D. Jones et al., submitted) 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 1. Open and closed circles at nodes indicate 95% and 50% bootstrap support, respectively. PG2, Pyrene Group 2; AG1, Anthracene Group 1

Fig. 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; $t = 0$ 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 detection limit of the assay. PIGM, *Pigmentiphaga*; SGB, *Sphingobium*; VARIO, *Variovorax*; ACI, *Acidovorax*; BaA, benz[a]anthracene. Error bars represent one standard deviation and are shown in only one direction for clarity.

Fig. 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 Fig. 2.

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

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

¹ NAP, naphthalene (65 sequences total); ANT, anthracene (185); PHE, phenanthrene (85); PYR, pyrene (96); FLA, fluoranthene (91); BaA, benz[a]anthracene (91).

² Assigned using RDP Classifier (Wang et al., 2007) with an 80% confidence threshold.

³ From M.D. Jones et al. (submitted). Percentages do not add to 100 because two sequences found in the clone libraries from heavy DNA were subsequently concluded not to have grown on the ¹³C-labeled anthracene in the SIP experiment.

⁴ -, Not found or was a singleton sequence in that clone library, except as noted otherwise.;⁵ Found in the clone libraries from heavy DNA but subsequently concluded not to have grown on the ¹³C-labeled anthracene in the SIP experiment.

Table 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)	Detect. Limit ⁵	RDP Hits ⁶
Bacteria	341F	CCTACGGGAGGCAGCAG	60	--	--	--	--	--
	517R	ATTACCGCGGCTGCTGG						
<i>Pigmentiphaga</i> ⁷	PigmF	CAGCGGTTCTGGAAAG	56	SBNAP45	63	1.91; 2.03	8.86×10^6	17
	PigmR	TGACATACTCTAGTTCGGGA						
<i>Sphingobium</i> ⁸	SGBF	ACGTAGGCGGCGATT	59	SBNAP83	70	2.03; 2.03	1.44×10^7	329
	SGBR	CCTCTCCAAGATTCTAGCAA						
<i>Sphingobium</i> ⁹	SGB.5F	ACAGTACCGGGAGAATAAGCTC	56	SBANT43	158	1.98; 1.92	2.32×10^7	128
	SGB.5R	CAAGCAATCCAGTCTCAAAGGCTA						
<i>Variovorax</i>	VarioF	AGCTGTGCTAATAACCGCATAA	61	SBNAP02	279	2.05; 1.99	8.10×10^7	65
	VarioR	GAGACTTTTCGTTCCGTAC						
<i>Acidovorax</i>	AcidF	TAACGGAGCGAAAGCTT	55	SBPHE2-37	60	1.98; 2.01	2.08×10^7	331
	AcidR	GTCCGCGCAAGGCCTT						
Pyrene Group 2	PG2.4F	CCAAAGCCGACGACGGGTAG	59	SBPYR03	94	2.02; 1.99	8.17×10^7	900
	PG2.4R	TTCCCCACTGCTGCCTC						
<i>Sphingomonas</i>	SPH.1F	CGGTACGGAAATAACTCA	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 and Pigm primers are from Jones et al. (M. D. Jones et al., submitted), *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 Fig. 1.

⁴ Amp. Eff., Amplification efficiency (Pfaffl, 2001) with eubacterial (Bac) and group-specific (Group) primers.

⁵ Detection limit of each qPCR assay (number of 16S rRNA gene copies/ g dry soil).

⁶ Number of sequences returned by the Ribosomal Database Project II release 10.18 (Cole et al., 2009) (excluding sequences from this study) with no mismatches to primer pairs.

⁷ *Pigmentiphaga*-related sequences were identical to those recovered from the SIP experiment anthracene (M. D. Jones et al., submitted).

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⁸Targets naphthalene- and phenanthrene-associated *Sphingobium* sequences.

⁹Targets fluoranthene-associated *Sphingobium* sequences. The clone used for qPCR was from a highly similar sequence recovered in clone libraries from an earlier SIP experiment with anthracene (M. D. Jones et al., submitted).