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Stable Isotope Probing of an Algal Bloom To Identify Uncultivated Members of the *Rhodobacteraceae* Associated with Low-Molecular-Weight Polycyclic Aromatic Hydrocarbon Degradation▽

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Polycyclic aromatic hydrocarbon (PAH)-degrading bacteria associated with an algal bloom in Tampa Bay, FL, were investigated by stable isotope probing (SIP) with uniformly labeled [^{13}C]naphthalene. The dominant sequences in clone libraries constructed from ^{13}C-enriched bacterial DNA (from naphthalene enrichments) were identified as uncharacterized members of the family *Rhodobacteraceae*. Quantitative PCR primers targeting the 16S rRNA gene of these uncultivated organisms were used to determine their abundance in incubations amended with unlabeled naphthalene and phenanthrene, both of which showed substantial increases in gene copy numbers during the experiments. As demonstrated by this work, the application of uniformly ^{13}C-labeled PAHs in SIP experiments can successfully be used to identify novel PAH-degrading bacteria in marine waters.

Polycyclic aromatic hydrocarbons (PAHs) are an important class of chemical compounds commonly associated with petrochemicals. Based on their poor water solubility, toxicity, persistence, and potential to bioaccumulate, PAHs are recognized as high-priority pollutants in the environment and are of significant concern to human health (1, 4). In the marine environment, phytoplankton have been correlated with the removal of PAHs from the water column, particularly during periods of bloom (3, 5, 14, 18, 30). It has been inferred that phytoplankton adsorb PAHs from the surrounding seawater, subsequently transporting them to the sea floor by sedimentation (3, 14). With the potential of phytoplankton cells to accumulate PAH molecules onto their surface (2, 16), one might assume the formation of an enrichment zone for PAH-degrading bacteria. In this study, DNA-based stable isotope probing (SIP), a method that links function with identity in complex microbial communities (8), was used to identify PAH-degrading bacteria associated with an algal bloom in Tampa Bay, FL.

Water samples were collected (0.5-m depth) on 27 July 2009, near the Howard Franklin Bridge in Tampa Bay, FL. The samples were preserved in Lugol's iodine solution prior to taxonomic identification of the algal community and estimation of cell concentrations under the phase microscope (13). During that week, discoloration of the water, which was attributed to a bloom of the dinoflagellate *Pyrodinium bahamense* (ca. 3.7×10^6 cells/liter), was observed in a stretch of the bay extending from the Gandy Bridge to Oldsmar. Two other microalgae, *Rhizosolenia setigera* and *Gyrosigma* sp., were identified in significantly lower numbers, 9.3×10^4 cells/liter and 1.4×10^4 cells/liter, respectively. The dinocyst count was 4.6×10^3 per liter. Approximately 150 ml of water sample was passed aseptically through a stainless steel 224-μm-pore-size mesh to remove zooplankton. Using low vacuum pressure, the filtrate was then passed through a Whatman grade 1 filter to retain the microalgae. The microalgal fraction was then washed 6 times with naturally aged autoclaved seawater to remove as much of the free-living (i.e., non-algae-associated) bacterial population as possible and then resuspended in 40 ml of naturally aged autoclaved seawater to act as the inoculum for the SIP experiment.

All incubations were performed in 125-ml sterilized glass screw-top Erlenmeyer flasks with caps that were lined with aluminum foil to prevent sorption of PAHs. Each flask contained 19 ml of ONR7a synthetic seawater medium (9), 1 mg of labeled (^{14}C/^{13}C) and/or unlabeled substrate, and 1 ml of the washed algal suspension as the inoculum. For SIP, duplicate flasks were prepared with 1 mg of uniformly ^{13}C-labeled naphthalene ([U-^{13}C]naphthalene), and a second set of duplicates was prepared with 1 mg of unlabeled naphthalene. To determine the endpoint of the SIP experiment, mineralization of [U-^{14}C]naphthalene was measured periodically, starting at day 4, by liquid scintillation counting of $^{14}CO_2$ trapped in KOH-soaked filter paper (24). Mineralization was monitored in triplicate flasks containing unlabeled (1 mg) and radiolabeled (20,000 dpm) naphthalene, for which an endpoint of 14 days was determined (Fig. 1). An additional set of triplicate flasks was used to monitor the disappearance of unlabeled naphthalene by high-pressure liquid chromatography (HPLC). Samples were periodically taken from these flasks, and their DNA was extracted to subsequently measure for changes in the abundance of the target organisms identified through SIP. Triplicate flasks of acid-killed controls (pH < 2) containing

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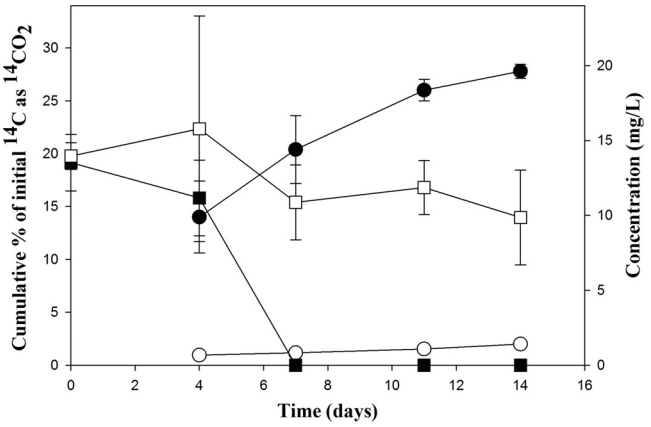

FIG. 1. Cumulative $^{14}CO_2$ recovered from incubations with [^{14}C]naphthalene (circles) and naphthalene removal from incubations with unlabeled naphthalene as measured by HPLC (squares) by the Tampa Bay algal field sample. Each data point is the mean of results from triplicate flasks ± standard deviations. Filled symbols represent live cultures (non-acid treated); open symbols represent acid-inhibited controls. Some error bars are smaller than the symbols.

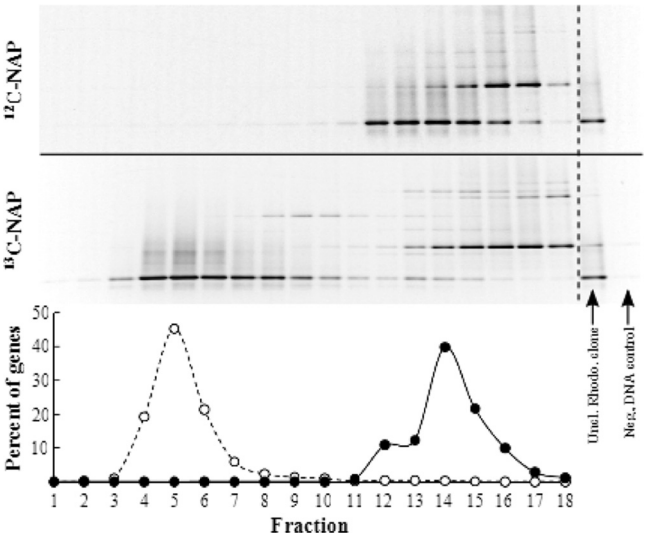

FIG. 2. Distribution of the uncharacterized *Rhodobacteraceae* (Uncl. Rhodo.) sequences in separated SIP fractions. (Top) DGGE image of bacterial PCR products from separated [^{12}C]naphthalene fractions with decreasing densities from left to right. (Middle) DGGE image of bacterial PCR products from separated [^{13}C]naphthalene fractions aligned to equivalent [^{12}C]naphthalene fractions. The representative clonal sequence used to generate the qPCR standard curve is indicated on the right. (Bottom) Distribution of qPCR-quantified, uncharacterized *Rhodobacteraceae* 16S rRNA gene sequences in fractions from [^{12}C]naphthalene incubations (closed circles with solid line) and from [^{13}C]naphthalene incubations (open circles with dashed line) presented as the number of genes in a fraction as a percentage of the total genes quantified in the displayed range of fractions. Data points are aligned with equivalent fractions of the DGGE images.

unlabeled naphthalene were prepared by adding ca. 700 μl of 85% phosphoric acid. Finally, triplicate flasks containing unlabeled phenanthrene were incubated as described for incubations with naphthalene to assess whether naphthalene-degrading bacteria identified by SIP could also grow on phenanthrene. All flasks were incubated on an orbital shaker (250 rpm; 21°C) in the dark. Unlabeled naphthalene (>99%) and phenanthrene (>96%) were obtained from Sigma-Aldrich (St. Louis, MO). [U-^{13}C]naphthalene was synthesized by methods described elsewhere (32). [U-^{14}C]naphthalene (17.8 mCi mmol^{-1}) was obtained from Sigma-Aldrich.

At the 14-day endpoint, whole DNA from the total volume in the paired flasks amended with [U-^{13}C]naphthalene and the corresponding paired set with unlabeled naphthalene was extracted using the method of Tillett and Neilan (27), and isopycnic centrifugation was used to separate ^{13}C-enriched and unenriched DNA fractions as previously described (12). Denaturing gradient gel electrophoresis (DGGE) using 6.5% acrylamide gels containing a denaturant range of 30 to 60% was performed on each fraction to visualize the separation of DNA. PCR primers for DGGE were 63FGC (17) and 517R (20) with a PCR program as described by Yu and Morrison (31). Colors of the gel images were inverted and adjusted for contrast, and the images were cropped to only the regions displaying bands with the GNU Image Manipulation Program (GIMP; version 2.6.8). To identify naphthalene-degrading bacteria, a 16S rRNA clone library comprising 91 clones was prepared from the ^{13}C-enriched DNA fractions (25) and partially sequenced using primer 27f (29) at the Beckman Coulter Genomics sequencing facility (Danvers, MA). These ^{13}C-enriched heavy DNA fractions were selected based on the evidence provided in Fig. 2, which is discussed below. The clone sequences were grouped into operational taxonomic units (OTUs) based on a 3% distance cutoff. Using the complete linkage clustering and dereplicate tools available at RDP's Pyrosequencing Pipeline (6), one representative sequence was selected to represent the dominant OTU identified in the library. A near-complete 16S rRNA gene sequence for the organism represented by this clone was obtained at the University of North Carolina Chapel Hill Genome Analysis Facility. CLUSTAL_X (26) was used to align this sequence with close relatives and construct a bootstrapped (1,000 times) neighbor-joining tree.

Primers for real-time quantitative PCR (qPCR) were developed to quantify sequences in the dominant OTU. The representative sequence was imported and automatically aligned in the ARB-SILVA SSURef 94 database (22) and manually refined, taking into account the secondary structural information of the rRNA molecule. The Probe Design and Probe Match tools of the ARB software were used to design primers that were specific for this representative sequence. The primers were named Unc_57F (5′-AAC GTG CCC TTC ACT ACG-3′) and Unc_128R (5′-CCG ATC CTT CAC CGA AAT-3′). Primer specificity was confirmed with the Probe Check tool of RDP-II. The Unc_57F primer hit 184 sequences, of which 180 belonged to members within the *Rhodobacteraceae*, 3 to unclassified proteobacteria, and 1 to a gammaproteobacterium; the Unc_128R primer hit 108 sequences, all of which belonged to members within the *Rhodobacteraceae*. The optimal annealing temperature of the primer set (57°C) was determined using an Eppendorf Mastercycler gradient thermal cycler (Hauppauge, NY). The template for this reaction, and for construction of a standard curve for quantitative PCR, was a plasmid containing a representative sequence which had been linearized using PstI (New England BioLabs, Ipswich, MA) and

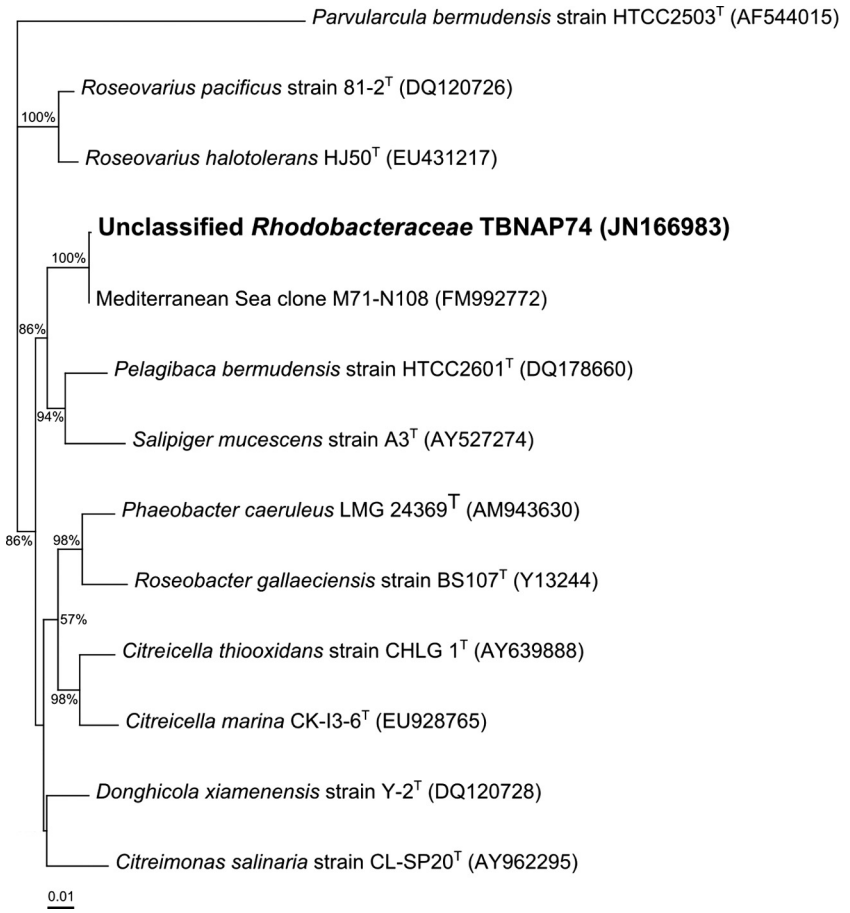

FIG. 3. Phylogenetic relationships among the unclassified *Rhodobacteraceae* TBNAP74 representative sequence recovered from heavy (^{13}C-enriched) fractions in this study (shown in bold) and closely related type strains of the family *Rhodobacteraceae*. GenBank accession numbers are in parentheses. The tree was constructed using the neighbor-joining algorithm. Only bootstrap values above 50% are shown (1,000 replications). Bar, 1% estimated sequence divergence.

purified using the QIAquick nucleotide removal kit (Qiagen, Valencia, CA). The amplification efficiency of the primers (21) was determined to be 1.92.

Purified DNA from time series incubations with unlabeled PAH was quantified using a NanoDrop ND-3300 fluorospectrometer (Thermo, Waltham, MA) and the Quant-iT Picogreen double-stranded DNA (dsDNA) kit (Invitrogen, Carlsbad, CA). As duplicates of the separated ^{12}C- and ^{13}C-labeled naphthalene incubations displayed similar distributions of DNA in the fractions, as well as similar DGGE profiles, only the replicate incubation whose fractions contained the highest total amount of DNA was used for further analyses. SIP-identified sequences were quantified in each separated SIP fraction using a single reaction by qPCR as described previously (25). Single reactions of triplicate DNA extractions were performed for samples from the time series containing either unlabeled naphthalene or phenanthrene.

Complete removal of naphthalene occurred by day 7, whereas mineralization of ^{14}C associated with naphthalene continued up until day 14 (the selected endpoint of the experiment) (Fig. 1). All live (non-acid-treated) incubations were observed to produce a pale-yellow coloration after 4 to 6 days, which is indicative for the formation of a muconic semialdehyde intermediate(s) from the degradation of naphthalene via a catabolic pathway involving a meta-ring cleavage enzyme(s) (7). The yellow coloration faded by day 11 and was not detectable by day 14, indicating that further metabolism of the intermediate(s) had occurred. Degradation and mineralization of the naphthalene can be attributed solely to bacterial activity, because the incubations were conducted in the dark, which would not support the growth of the algae or, rather, suppress their activity for prolonged periods. Supporting this, microscopic examination of the incubations revealed that the algal cells had undergone extensive intracellular bleaching and, essentially, appeared nonviable.

DGGE analysis of the fractions derived from the labeled and unlabeled incubations showed clear evidence of isotopic enrichment of DNA in [^{13}C]naphthalene incubations, separation of ^{13}C-labeled and unlabeled DNA, and different banding patterns between the ^{13}C-enriched and unenriched DNA fractions (Fig. 2). One band in particular was especially dominant in fractions containing ^{13}C-enriched DNA. For the ^{13}C incubation shown in Fig. 2, fractions 4 to 8 were combined and used in the generation of the 16S rRNA gene clone library. Fractions 5 to 9 of the duplicate gradient were combined in a similar fashion (data not shown). After excluding vector se-

quences, poor sequence reads, chimeras, and singleton sequences, the clone library constructed from pooled ^{13}C-enriched DNA comprised 90 sequences. These 90 sequences exhibited 99.1% sequence identity and, hence, represented a single OTU. Classifier analysis in RDP-II (28), at the 80% confidence threshold, indicated that this OTU represented a clade of uncultivated and unclassified bacteria within the family *Rhodobacteraceae*. The PCR-DGGE product of the representative sequence of this OTU aligned with the dominant PCR product present in ^{13}C-enriched DNA fractions (Fig. 2). Using 18S rRNA-specific primers EK-1F, EK-82F, and EK-1520R under previously described conditions (15), the pooled ^{13}C-enriched DNA was not found to contain any algal DNA, thus further supporting the unlikelihood that the algal community from the Tampa Bay water sample contributed to the degradation and mineralization of the naphthalene.

The near-complete 16S rRNA gene sequence (1,427 bp) that was selected to represent this OTU (designated TBNAP74) was used to construct a phylogenetic tree with related sequences as determined by BLASTN searches of GenBank (Fig. 3). The closest relative to this unclassified *Rhodobacteraceae* was to a single environmental clone, M71-N108 (GenBank accession number FM992772; 99.8% sequence similarity), an alphaproteobacterium recently isolated from the deep oligotrophic Eastern Mediterranean Sea (10). The next highest levels of similarity (≤96% sequence identity) were to environmental and cultivated strains that belonged predominantly to the *Roseobacter* clade. Members within this clade are widely recognized for possessing multiple ring-cleaving pathways (19) and are almost always found in high abundance during algal blooms (11). However, the ≥3% sequence difference for members of this clade to the newly identified OTU (i.e., unclassified *Rhodobacteraceae*) identified in this study is sufficiently distinct as to possibly represent a new phylogenetic taxon within this clade. The dominant representation of these unclassified *Rhodobacteraceae* in the clone library suggests that they may possess a competitive advantage (i.e., specialization) for degrading naphthalene over other bacteria in this Tampa Bay algal field sample.

During incubations of the algal sample with unlabeled naphthalene or phenanthrene, the 16S rRNA gene copy number for this unclassified *Rhodobacteraceae* OTU increased by several orders of magnitude (Fig. 4), thus providing further confirmation of its enrichment on at least two low-molecular-weight PAHs as growth substrates. By day 7 of the naphthalene enrichment, the gene copy number increased by ca. 5 orders of magnitude, coinciding with the time frame for the disappearance and mineralization of this compound (Fig. 1). As the gene copy number did not substantially increase after day 7, there was no evidence for growth of these organisms on the colored metabolite detected in the medium, although they may have aided its disappearance. In flasks amended with unlabeled phenanthrene, the gene copy number increased by ca. 3 orders of magnitude from days 1 to 9. This increase in gene copy number for both sets of incubations with unlabeled substrate coincided with an increase in the total concentration of DNA, an indicator of cell growth. Collectively, the low bacterial diversity identified in the heavy DNA fractions, which is almost exclusively represented by the unclassified *Rhodobacteraceae* OTU (Fig. 2), and the dramatic increase in the abundance of

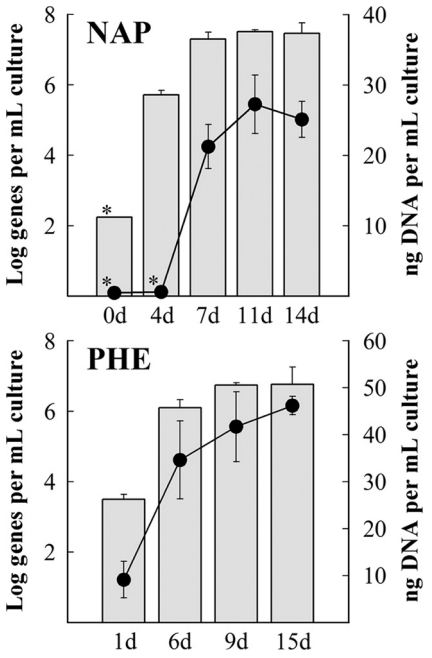

FIG. 4. Abundance of uncharacterized *Rhodobacteraceae* 16S rRNA genes during incubation with either unlabeled naphthalene (top) or phenanthrene (bottom). Bars are the averages and standard deviations of results from triplicate qPCRs measuring the abundance of group-specific 16S rRNA genes. Circles are the means and standard deviations of triplicate measurements of the total mass of DNA per sample. Bars or data points with asterisks represent numbers with one or more readings below the quantification limit of the assay and are presented as the largest possible value for that point.

these organisms by day 7 in the naphthalene incubations and by day 6 in the phenanthrene incubations strongly support that this newly identified OTU was solely responsible for degradation of these PAHs. Additionally, the growth of these organisms coinciding with PAH disappearance and the appearance of their 16S rRNA genes only in the most heavily enriched ^{13}C-DNA fractions of incubations containing a labeled substrate suggest that their presence in clone libraries was not due to cross-feeding on a PAH metabolite. However, we cannot disregard the possibility that other bacterial taxa in the Tampa Bay algal bloom also possessed the capacity to degrade either naphthalene (or phenanthrene) or its metabolites; they were merely not strongly represented in the most highly ^{13}C-enriched fractions analyzed.

To our knowledge, this is the first study employing SIP with a U-^{13}C-labeled PAH to identify the PAH-degrading bacterial community in a marine water sample. We identified a novel PAH-degrading clade which was found associated with a toxic algal bloom in Tampa Bay, FL. These organisms, which have low taxonomic affiliation to any characterized strain, represent unclassified members of the family *Rhodobacteraceae*. Considering that the majority of microorganisms in our biosphere are resistant to cultivation (23), this work highlights the importance of pursuing cultivation-independent methods to reveal further information on PAH-degrading microorganisms, particularly in ocean environments for which SIP experiments employing hydrocarbon substrates have been scarcely employed.

Nucleotide sequence accession number. The 16S rRNA representative gene sequence was deposited in GenBank under the accession number JN166983.

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