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Dynamic changes in *nahAc* gene copy numbers during degradation of naphthalene in PAH-contaminated soils

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Abstract Many bacteria that degrade polycyclic aromatic hydrocarbons (PAHs) contain the *nahAc* gene that encodes a component of multimeric naphthalene dioxygenases. Because the *nahAc* gene is highly conserved, this gene serves as a potential biomarker for PAH degradation activity. The aim of this research was to examine the relationship between the rate of naphthalene degradation and the copy number of the *nahAc* gene in soils using conventional and real-time PCR. Four sets of degenerate primers for real-time PCR were designed based on the *nahAc* DNA sequences of 33 bacterial species. Before addition of naphthalene, copy numbers of the *nahAc* gene were below the detection limits of the assay at 5×10^3 copy numbers per gram of soil, but increased by over a thousand fold to 10^7 copies after 6 days of exposure to naphthalene vapors (approximately 30 ppm soil water concentration). Two unreported naphthalene dioxygenase homologs were found in the naphthalene-spiked soil by cloning and sequencing of the PCR products from the *nahAc* primers. Results of these experiments demonstrate the highly dynamic changes that occur in soil microbial communities after exposure to naphthalene and suggest that there is a direct relationship between gene copy numbers and degradation rates for naphthalene in PAH-contaminated soils.

Keywords Biodegradation · Biomarker · Environmental monitoring · Microbial ecology · Polycyclic aromatic hydrocarbon · Real-time polymerase chain reaction

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous toxic pollutants that comprise a group of over 100 different

chemicals [Agency for Toxic Substances and Disease Registry (ATSDR) 1996]. Environmental problems associated with PAH contamination are due to their ubiquity, toxicity, and recalcitrance (National Research Council 1983; Hall and Grover 1990; Cuny et al. 1999). PAHs have been found in at least 600 of the 1,430 National Priorities List sites identified by the Environmental Protection Agency (EPA) (ATSDR 1996). The elimination of these compounds from the environment involves biodegradation, which can be accomplished by many different bacterial species that use these substances as growth substrates (Cerniglia 1992). Among the 16 different PAHs that are on the EPA priority list, naphthalene has been commonly selected as a model compound because of its high aqueous solubility and the ease of isolation of naphthalene-degrading microorganisms (Laurie and Lloyd-Jones 2000). Since the first report on a naphthalene-degrading pathway by *Pseudomonas* species in 1964 (Davies and Evans 1964), there has been considerable research on the metabolic pathway, genes, and enzymes that are involved in naphthalene degradation (Ensley et al. 1982; Cerniglia 1984; Ensley and Haigler 1990; Eaton and Chapman 1992; Mason and Cammack 1992; Parales 2003).

Among the many different enzymes that are involved in PAH degradation, the initial dioxygenases that enable aerobic bacteria to attack the aromatic ring structures are key enzymes that serve as useful markers for PAH degradation activity. These enzymes are multimeric and are comprised of three components including a reductase, a ferredoxin, and an iron-sulfur protein (ISP_{NAP}) (Simon et al. 1993). The genes encoding the initial PAH dioxygenases of *Pseudomonasputida* G7 are located on the plasmid, NAH7 (Ahn et al. 1999). The ISP_{NAP} is composed of two non-identical subunits, alpha and beta; the *nahAc* and *nahAd* genes encode the alpha and beta subunits, respectively (Sanseverino et al. 1993; Stuart-Keil et al. 1998; Mavrodi et al. 2003). Regulation of these genes is controlled by *nahR*, which belongs to LysR family (Diaz and Prieto 2000).

The naphthalene dioxygenase gene is of particular interest as a marker for PAH degradation because the enzyme encoded by this gene not only degrades naphtha-

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lene, but also mediates degradation of phenanthrene, anthracene, dibenzothiophene, fluorine, and methylated naphthalenes (Ahn et al. 1999). The *nahAc* gene also is highly conserved among different Gram-negative bacteria, and it has been suggested that natural horizontal gene transfer has occurred between different species that carry this gene (Herrick et al. 1997; Stuart-Keil et al. 1998). This is supported by the observation that PCR with degenerate primers designed from the *nahAc* gene in *P. putida* PaW736 (NCIB 9816) can detect not only naphthalene-degrading *Pseudomonas* species but also strains of *Mycobacterium*, *Gordona*, *Sphingomonas*, *Rhodococcus*, and *Xanthomonas* that degrade naphthalene and higher molecular weight PAHs including phenanthrene, anthracene, fluoranthene, and pyrene (Hamann et al. 1999). At present, the majority of naphthalene degraders that have been characterized in pure cultures carry either the *nahAc* gene or the highly similar homologs (Habe and Omori 2003).

One of the current challenges in bioremediation of PAHs is the ability to monitor microbial community responses to changing environmental conditions and various soil management practices. The application of real-time PCR methods is particularly useful for monitoring genes of interest that may serve as biomarkers associated with hydrocarbon biodegradation activity (Beller et al. 2002). Previous research successfully demonstrated that real-time PCR methods can be applied to monitor certain target genes in soil microcosms in relation to chemical enrichments. For example, quantitative real-time PCR methods have been used to monitor the increase of the *tfdA* gene during degradation of phenoxy acid herbicide 4-chloro-2-methylphenoxyacetic acid (Baelum et al. 2006). In another recent study demonstrating the utility of this method, Nakatsu et al. (2005) monitored the increase of biphenyl dioxygenase and phenol hydroxylase gene copy numbers in response to xylene exposure. These and other reports suggest that real-time quantitative PCR should have application for monitoring PAH degradation activity through quantification of gene copy numbers for key genes that are involved in naphthalene degradation.

In this research, we designed three different PCR primer sets that target the *nahAc* gene for use with real-time and conventional PCR. Experiments were then conducted to examine the relationship between naphthalene degradation rates and changes in the copy number of the *nahAc* gene. Results of these experiments demonstrated highly dynamic changes in gene copy numbers that occur in soil after exposure to naphthalene. The primer sets that were designed were also useful for examining the diversity of *nahAc* homologs that occurred within the microbial population that responded to the presence of naphthalene. The ability to rapidly quantify gene copy numbers for this key gene will facilitate monitoring of PAH degrader communities during biodegradation of PAHs in contaminated soils and in treatment systems for oil sludges using landfarming or bioreactors.

Materials and methods

Soil samples

The soils were collected from Port Hueneme, CA, Department of Defense National Test Site, and March Air Force Base site #34. The Port Hueneme soil samples had a history of previous contamination with diesel and heavy oil (http://enviro.nfesc.navy.mil/erb/erb_a/support/netts/pthueneme_history.htm). The soil samples from Port Hueneme had a pH of 7.1, an organic matter content of 3.8%, and contained 77% sand, 5% silt, and 18% clay (sandy loam). The March Air Force Base site #34 was a former aircraft fueling site that was contaminated with various PAHs, including benzo(α)pyrene, benzo(α)anthracene, chrysene, and other priority PAH pollutant compounds (<http://yosemite.epa.gov/r9/sfund/overview.nsf/0/10ae8e28eca0aafc8825660b007ee663?OpenDocument>). The soils from the March Air Force Base had a pH of 7.2, an organic matter content of 4.8%, and were composed of 49% sand, 8% silt, and 43% clay (sandy clay).

DNA extraction from soil samples

DNA extraction was performed with a Fast Spin kit for soil (Bio 101, La Jolla, CA, USA). The protocol was performed according to the manufacturer's instructions. DNA extracts were stored in distilled water at $-20\text{ }^{\circ}\text{C}$ until analysis.

Soil treatment

Soil samples consisting of 0.5 g of air-dried Port Hueneme soil and 150 μl of distilled water were mixed and placed into sterilized 1.5 ml screw cap tubes where they were exposed to naphthalene vapors. To keep the soils moist during incubation, the sample tubes without caps were placed in plastic Petri plates on top of tissue paper that was moistened with 1 ml of distilled water. Naphthalene vapors were supplied by placing 0.1 g of naphthalene granules in a small plastic cap in the middle of the plates. The plates containing the soils and naphthalene crystals were sealed with Parafilm, which is permeable to oxygen. The solubility of naphthalene in water is approximately 30 mg l^{-1} (<http://www.inchem.org/documents/pims/chemical/pim363.htm>). With vapor transfer, the naphthalene concentration in the water-saturated soil samples was thus 30 ppm and continually replenished by vapor transfer from the naphthalene crystals. Triplicate samples were collected every 24 h and were capped, frozen immediately, and stored at $-20\text{ }^{\circ}\text{C}$ until the end of the experiment when all of the samples were extracted for total DNA. Identical samples were collected for analysis of residual naphthalene concentrations in the soil.

NAH7 plasmid DNA extraction of *P. putida* G7

Extraction of the *P. putida* G7 NAH7 plasmid DNA (GenBank accession number M83949) was performed by a modified alkali lysis method (Stuart-Keil et al. 1998). The concentration of the NAH7 plasmid DNA was calculated by measuring DNA absorbance at 260 and 280 nm with a spectrophotometer. The plasmid DNA was serially diluted and used as a standard for real-time PCR.

Primer development

To design degenerate *nahAc*-targeted primer sets, the DNA sequences of the *nahAc* gene from 33 different species were obtained from Entrez Medline Gene Bank (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>) and aligned by CLUSTAL X (V. 1.64b). Homologous protein-encoding regions of *nahAc* were selected from the aligned data, and eight sets of real-time PCR primers and probes targeting different regions of the *nahAc* gene were designed and verified using Primer Express (Perkin Elmer). Among these, four primer sets were evaluated for conventional PCR and SYBR Green real-time PCR analysis (Table 1).

PCR assay

PCR reaction mixtures contained 10 mM Tris-HCl (pH 8.3 at 25 °C), 2.5 mM MgCl₂, 0.25 mM deoxynucleotide triphosphates, 5 to 10 pmol of each primer, and 1 U of *Taq* polymerase. The temperature profile was 95 °C for 5 min followed by 94 °C with 35 cycles of 20 s, 55 °C for 20 s, and 72 °C for 30 s. A final extension step was carried out for 5 min at 72 °C, after which the DNA was stored at 4 °C. The PCR products obtained using the *nahAc* gene primers were cloned into pGEM-T vector using pGEM-T vector system I according to the manufacturer's instructions (Promega, Madison, WI, USA). The cloned PCR products were sequenced using a 16-capillary ABI PRISM 3100 Genetic Analyzer.

Real-time PCR assays

SYBR Green real-time PCR was performed on an iCycler IQ thermocycler (Bio-Rad) using a QuantiTect SYBR Green PCR Kit (Qiagen) following the protocols recommended by the manufacturer with slight modifications. Amplification was carried out in a total volume of 20 µl containing 10 µl of 2× QuantiTect SYBR Green PCR mixtures, 2.5 to 10 pM of each primer, and 1 µl of sample DNA. The temperature profile was 95 °C for 5 min followed by 94 °C with 50 cycles of 20 s, 55 °C for 20 s, and 72 °C for 30 s. Data analysis was carried out with iCycler software (version 3.0a; Bio-Rad).

Calculation of gene copy number from real-time PCR data

The concentration of standard DNA was measured by using a spectrophotometer and agarose gels. To quantify the gene copy number, *P. putida* G7 plasmid DNA (GenBank accession number M83949) was serially diluted using a 10× dilution series to generate a standard curve. The gene copy number was calculated as follows:

$$\text{Copy number (molecules}/\mu\text{l)} = \frac{\text{DNA concentration (g}/\mu\text{l)}}{\text{Plasmid length (bp)} \times 660^a} \times A^b$$

660^a = average molecular weight of one base pair

A^b = Avogadro's number

$$= 6.02 \times 10^{23} \text{ molecules/mole}$$

(Copy number is per chromosome equivalent)

The standard DNA was serially diluted to prepare five to six 10× dilution series, and the cycle of threshold (Ct) values were measured for every real-time PCR reaction to create a standard curve. The standard curve shows the negative correlation between Ct and copy number of standard DNA, through which the Ct value of samples was converted to the copy number of samples. The correlation

Table 1 Primer sets for PCR and real-time PCR

Primers	Sequences	Location	Target gene	Size of product (bp)
<i>nahAc</i> -1F	AAGAGCTGTACGGCGAGTC	362–380 ^a	<i>nahAc</i>	102
<i>nahAc</i> -1R	CCTGATCGAAGCAACCATAG	444–463 ^a		
<i>nahAc</i> -3F	GACGCTGCTTGGTACCTAGA	493–512 ^a	<i>nahAc</i>	94
<i>nahAc</i> -3R	TCCAGTTGGCCTTGATCA	569–586 ^a		
<i>nahAc</i> -6F	TGATCAAGGCCAACTGGA	569–586 ^a	<i>nahAc</i>	112
<i>nahAc</i> -6R	AGCGACGCGAAGATAGACTC	661–680 ^a		
<i>nahAc</i> -7F	ACTTGGTCCGGAGTTGATG	791–810 ^a	<i>nahAc</i>	136
<i>nahAc</i> -7R	CAGGTCAGCATGCTGTTGTT	907–926 ^a		
338F	ACTCCTACGGGAGGCAGCAG	338–357 ^b	16S rRNA	181
518R	ATTACCGCGGCTGCTGG	517–534 ^b		

^aBased on *P. putida* G7 NAH7 plasmid (GenBank accession number M83949, Region 2368...3717)

^bBased on *E. coli* numbering

coefficients of standard curves were 0.98 or higher in this study.

Q-PCR detection limit

Fully grown *P. putida* G7 was serially diluted with Luria–Bertani broth (LB) and spread onto LB plates. Samples were quantified by measurements of absorbance at 600 nm and were calibrated to colony-forming units (cfu) that grew on the LB plates. Spread plates were incubated at 30 °C overnight after which the *cfus* were counted. Identical samples from the same dilution series were spiked into 0.5 g of autoclaved Port Hueneme soil. Spiked soil samples were mixed well and then extracted for total soil DNA, after which *nahAc* copy numbers were measured using SYBR Green real-time PCR.

Naphthalene degradation assays

To measure the disappearance of naphthalene from the soil over time, Port Hueneme soils were spiked with 100 ppm of naphthalene dissolved in chloroform. Replicate samples were prepared by placing 4-g aliquots of the mixture into 20-ml glass vials that were sealed with Teflon film and incubated at room temperature. Triplicate samples were collected every 1 to 2 days and stored at –20 °C until the end of the experiment. At the time of extraction, 100 ppm of phenanthrene was added as an internal standard to the chloroform extractant. The samples were extracted by the addition of 5 ml of chloroform to each vial, after which the samples were shaken at 200 rpm overnight. The samples were centrifuged and the supernatants were collected for analysis of remaining naphthalene concentrations. The samples were analyzed using a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA) coupled to a flame ionization detector (GC-FID), with a HP-5 capillary column, 30.0 m length, 0.32 mm diameter, and 0.25 µm film thickness, with the following conditions: oven temperature at 175 °C; injector split at 250 °C (split ratio 1/20);

and FID at 300 °C with 40 ml/min of hydrogen flow and 450 ml/min of air flow.

Naphthalene gene sequence analysis

Homology searches were performed using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). DNA sequences of known genes were collected from the NCBI GenBank (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>). The programs CLUSTAL X (1.64b) and GeneDoc (2.0.004) were used to align the sequences and determine their homologies.

Results

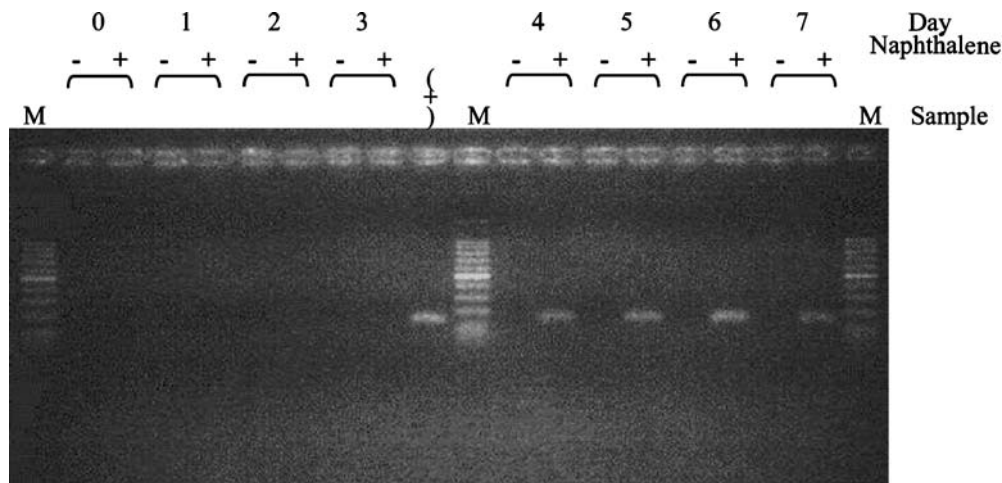
Primer design for detection of the *nahAc* gene

Homologous regions of DNA that may serve as PCR primer sites for targeting the *nahAc* gene were determined by aligning the partial sequences of 33 known, different *nahAc* genes. Real-time PCR primers that target these regions were then designed using the criteria required for quantitative real-time PCR. Eight sets of degenerate primers were designed from which four were chosen for testing (Table 1). These four primer sets were tested using *P. putida* G7 plasmid DNA (NAH7) as a positive control. Each of the primer sets yielded DNA bands that were of the correct size for the target sequence (data not shown). We chose *nahAc*-7F/7R for further research, as the amount of PCR product that was produced with this primer set was greater than those with the other primer sets that were tested.

nahAc-targeted conventional PCR assay

DNA products of the desired size were amplified with *nahAc*-7F/7R in replicate soil samples from PAH-contaminated soils collected from Port Hueneme, CA and March Air Force Base, CA. Using conventional PCR, the

Fig. 1 Detection of the *nahAc* gene in soil exposed to naphthalene vapors by using conventional PCR using the *nahAc* 7F/R primer set. –, naphthalene-uns spiked; +, naphthalene-spiked; (+), positive control (NAH7 plasmid DNA); M 100 bp size marker



visual intensity of the amplified DNA band in the gel was very low or undetectable in the soils before exposure of the soils to naphthalene.

To determine the responsiveness of the indigenous naphthalene degraders to a naphthalene spike to the soil, samples of the Port Hueneme soil were exposed to naphthalene vapors continuously for 2 weeks during which time individual samples were collected every 24 h. A similar set of samples was maintained for a period of 2 months, after which they were analyzed to changes in gene copy numbers after longer term exposure to naphthalene. Soil DNA was extracted from both naphthalene-spiked samples and unspiked control samples. Both soil DNA samples were amplified with *nahAc*-7F/7R (Fig. 1). Results of this experiment showed that the *nahAc* gene was readily amplified after 4 days from the naphthalene-spiked soil, whereas little or no gene products were obtained from the unspiked soils.

nahAc-targeted real-time PCR assay

The DNA concentration of *P. putida* G7 plasmid DNA (NAH7) was 4.6×10^{-6} g/ μ l, and the plasmid length was approximately 83 k base pairs, thereby corresponding to a copy number that was 5×10^{10} molecules/ μ l. Real-time PCR was performed for two soil samples, three times each to generate a statistically representative dataset (Fig. 2). The linear correlation coefficient for the standard curve was 0.98 or higher. This graph shows that the amount of *nahAc* gene started to increase after 4 days in naphthalene-spiked soil, but that there was no significant changes in control soil without naphthalene. The copy number of the *nahAc* gene increased by over a thousand times after 6 days and remained at this level for over 60 days when the soil was exposed to naphthalene vapor (data not shown).

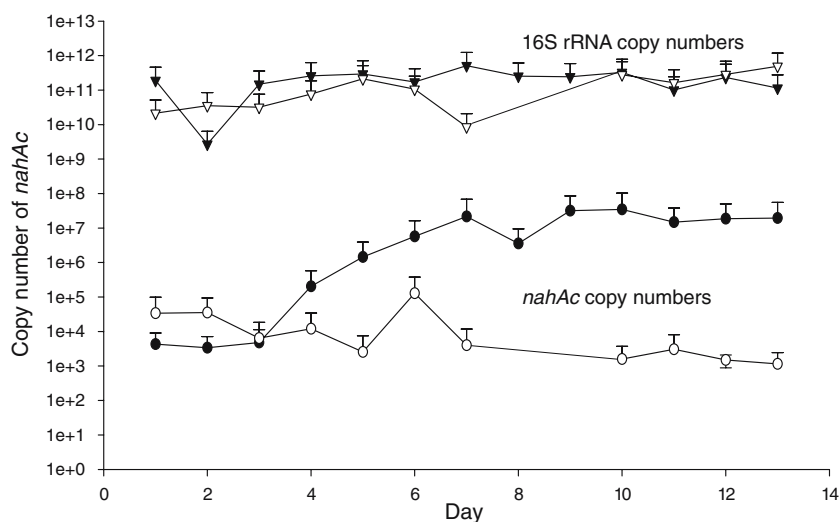
16S rRNA gene-targeted real-time PCR assay

To monitor changes in the total bacterial population density over time, the amount of 16S rRNA gene was measured using real-time SYBR Green PCR with 338F/518R (Table 1). As shown in Fig. 2, the amount of 16S rRNA gene was relatively constant over time in both the control soil and in the soil that was exposed to naphthalene, indicating that there was little or no overall change in bacterial population densities within the total bacterial community.

Detection limit and correlation of *nahAc* gene with cell number

To examine the relationship between the copy numbers of *nahAc* target molecules with actual cell numbers, autoclaved Port Hueneme soil was spiked with a dilution series of *P. putida* G7 culture. The DNA extracts were analyzed three times by real-time SYBR Green PCR. The cfu of the same *P. putida* G7 culture was also measured and compared with the copy numbers of *nahAc* (Fig. 3). When the assay was calibrated using known cell numbers, the average copy number of target plasmid was approximately 10^1 to 10^2 copies per *P. putida* G7 cell. The results of the soil inoculation experiment showed that there was a strong correlation between the copy numbers of *nahAc* and cell numbers when the cells were over 10^4 cfu gram of soil, but that the standard deviation increased at cell densities below 10^3 cfu per gram of soil. The detection limit at which there was a significant difference over the background signal was thus approximately 1×10^4 copies per gram of soil, which is equivalent to approximately 1×10^2 cells per gram of soil. Our detection rate was more sensitive as compared to the results of previous studies, which detected 10^3 to 10^5 cells per gram of soil (Kolb et al. 2003; Okano et al. 2004; Duodu et al. 2005).

Fig. 2 Quantification of *nahAc* gene and 16S rRNA gene in naphthalene-spiked and unspiked (negative control) Port Hueneme soils by real-time PCR. \circ , naphthalene-unspiked and amplified with *nahAc*-7F/7R; \bullet , naphthalene-spiked and amplified with *nahAc*-7F/7R; ∇ , naphthalene-unspiked and amplified with 16S rRNA gene primer set 338F/518R; \blacktriangledown , naphthalene-spiked and amplified with 16S rRNA gene primer set 338F/518R



Naphthalene degradation

Naphthalene degradation rates in the Port Hueneme soil were determined separately by addition of 100 ppm of naphthalene to identical samples that were used for DNA analyses. Naphthalene degradation was measured over time by GC-FID (Fig. 4). In soils spiked with this concentration of naphthalene, degradation began after 6 days, whereas no naphthalene disappearance was observed in autoclaved, sterile Port Hueneme soil. The average extraction efficiency was approximately 80% based on the recovery of a phenanthrene spike which was added as an internal standard during the extraction procedure.

Cloning and sequencing of *nahAc*-targeted PCR products

Naphthalene-spiked Port Hueneme soil DNA was amplified with *nahAc*-7F/7R and the PCR products were cloned into a pGEM-T vector. From this library, 17 cloned PCR products were sequenced. All of the clones had identical sequences except for the two which varied at one nucleotide position. The DNA sequence of the first clone group was 5'-(*nahAc*-7F) GCATTCGGCGGCGCTAAGC AGGAAAGGCTGAACAAAGAAATTGGCGATGTTTCG CGCCCGGATTTACCGCAGCCACCTCAACTGCACCG TTTTCCCG (*nahAc*-7R)-3'. The second sequence varied by substitution of T for C at the position denoted by the underlined C. This substitution is the third position of protein codon, which is TAT or TAC. Because both codons encode tyrosine, this substitution is silent. Multiple DNA sequence alignment for these partial gene sequences showed up to 99% identities to other naphthalene dioxygenase genes. All of these homologous dioxygenase genes belonged to *Pseudomonas* spp.

Discussion

Monitoring of PAH-degrading bacteria can be accomplished by culture-based methods, or by PCR methods that have been devised to detect catabolic genes that are known to be involved in PAH degradation. Potential target genes include those for the initial dioxygenases that carry out the first step in the biodegradation pathway, or genes such as those encoding catechol 2–3 dioxygenases that function for ring cleavage of salicylate in the pathway for PAH degradation. Another potentially important marker gene is the *phnAc* gene, which is not common among culturable PAH degraders but which can readily be detected in contaminated soils (Lloyd-Jones et al. 1999).

The methods that have earlier been devised for detection of *nahAc* include standard PCR methods that are semiquantitative (Mavrodi et al. 2003), and two quantitative methods that involve most-probable-number PCR (Chandler and Brockman 1996) and competitive PCR (Laurie and Lloyd-Jones 2000). Still another strategy has been used to target the mRNA transcripts encoding for *nahAc* by using reverse transcriptase PCR (Wilson et al. 1999). This latter method is particularly useful for examining gene expression. Recently, methods employing real-time PCR to target the *nahAc* gene also have been developed (Baldwin et al. 2003) but were not tested in actual environmental samples. The advent of real-time PCR methods has greatly simplified the ability to measure gene copy numbers in environmental samples. These latter methods employ fluorescent probes that are used to quantify the copy number of the target sequence at the end of each PCR cycle and can be easily calibrated.

Results of our experiments showed that both conventional and real-time PCR gave corresponding results and demonstrated that the copy number of the *nahAc* gene is highly dynamic in response to the presence of naphthalene. The real-time PCR assay proved to be extremely sensitive.

Fig. 3 Detection limits of *nahAc* gene and correlation between actual *P. putida* G7 cell number and the copy number of *nahAc*. The detection limit at which there was a significant difference over the background was approximately 10^3 cells per gram of soil

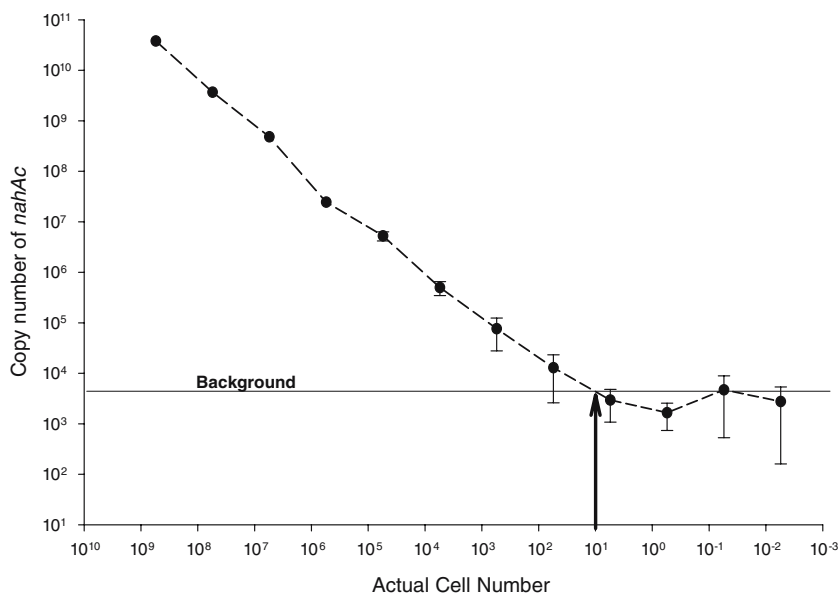
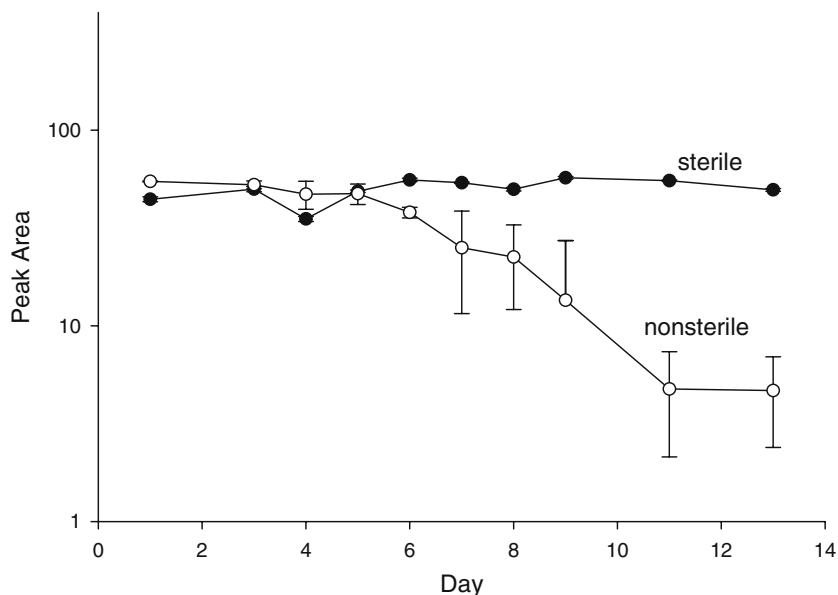


Fig. 4 Quantification of naphthalene concentration in naphthalene-spiked Port Hueneme soils by GC-FID.

●, naphthalene concentration in autoclaved sterile control soil;
○, naphthalene concentration in nonsterile soil



Using spiked cells of *P. putida* G7 to calibrate the assay (Fig. 3), the detection limit was determined to be approximately 1×10^4 copies per gram of soil. After exposure to naphthalene vapors, the initially low population density of bacteria carrying this gene increased by 3 orders of magnitude after 6 days (Fig. 2). At the same time, in soils spiked with a fixed concentration of 100 ppm naphthalene, naphthalene concentrations began to decrease rapidly with the majority of the contaminant being degraded over the next 7 days. In the samples that were used for DNA analysis, which were constantly exposed to naphthalene vapor in the headspace, there was no further increase in the gene copy number of 10^8 per gram of soil, which was achieved after 6 days (Fig. 4) or in another set of samples maintained for 60 days (data not shown).

In addition to monitoring the copy number of the *nahAc* gene, we used real-time PCR methods to monitor changes in the copy number of the bacterial 16S rRNA gene. In this manner, we were able to monitor changes in the numbers of total bacteria in the soils in conjunction with exposure to naphthalene. As shown in Fig. 2, the amount of 16S rRNA gene was relatively stable over time regardless of the addition of naphthalene. This indicates that there was no significant change in the overall population density of the total bacteria in soil exposed to naphthalene. The relatively constant value for this parameter as an estimate of bacterial biomass of course does not rule out changes in the species composition of the bacterial community that may have occurred after exposure to naphthalene.

One of the concerns in developing a real-time PCR assay for the *nahAc* gene is the verification of its specificity for the target gene. Real-time PCR assays can use internal fluorescent probes that are designed to detect specific gene sequences within the DNA fragment that is being

amplified, or, alternatively, may use the fluorescent dye SYBR Green which binds to any double-stranded DNA product that is generated by PCR. The use of SYBR Green real-time PCR analysis has many advantages and is relatively inexpensive, but is potentially subject to artifacts caused by nonspecific amplification of nontarget sequences and the formation of primer dimers. Thus, it was essential to verify the specificity of the assay by cloning and sequencing the PCR products that were amplified with the *nahAc*-7F/7R primers. Our results showed that all of the cloned sequences were naphthalene dioxygenase homologs, which showed up to 99% identities to other naphthalene dioxygenase genes. Based on their sequence homologies, the two clone groups that were identified contained *nahAc* sequences that were the same as those reported for *Pseudomonas* dioxygenase genes. However, caution should be observed in characterizing the diversity of *nahAc*-like genes that occurred in these soils because the fragment size is very small as compared to the total length of the *nahAc* gene sequence.

To fully characterize PAH-degrading bacterial populations in soils, it will eventually be necessary to devise methods to target marker genes for all of the predominant pathways that are used by PAH-degrading bacterial species that occur in soils (Moser and Stahl 2001), including, for example, the *phnAc* gene, which has been suggested to have high ecological relevance (Laurie and Lloyd-Jones 2000). As new quantitative methods are developed for the predominant genes, it should be possible to undertake comprehensive analyses of PAH-degrading bacterial populations that will greatly facilitate bioremediation methods that are being devised for the cleanup of PAH-contaminated soils.

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