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Quantification of *phnAc* and *nahAc* in Contaminated New Zealand Soils by Competitive PCR

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Unculturable polycyclic aromatic hydrocarbon (PAH)-degrading bacteria are a significant reservoir of the microbial potential to catabolize low-molecular-weight PAHs. The population of these bacteria is larger than the population of *nah*-like bacteria that are the dominant organisms in culture-based studies. We used the recently described *phn* genes of *Burkholderia* sp. strain RP007, which feature only rarely in culture-based studies, as an alternative genotype for naphthalene and phenanthrene degradation and compared this genotype with the genotypically distinct but ubiquitous *nah*-like class in different soils. Competitive PCR quantification of *phnAc* and *nahAc*, which encode the iron sulfur protein large (α) subunits of PAH dioxygenases in *nah*-like and *phn* catabolic operons, revealed that the *phn* genotype can have a greater ecological significance than the *nah*-like genotype.

It could mistakenly be inferred from available nucleotide sequence data that highly conserved *nah*-like gene clusters, which are isolated from polycyclic aromatic hydrocarbon (PAH)-degrading pseudomonads obtained from diverse geographic areas, are the dominant gene clusters involved in degradation of the low-molecular-weight PAHs naphthalene and phenanthrene. The results of probing and PCR amplification of *nah* sequences from contaminated soils and sediments also indicate that these sequences are ubiquitous (7, 17). Although PAH degraders with *nah* genotypes are easily isolated, it has been acknowledged that the *nah* catabolic cluster and closely related homologues may be present in only a small part of the PAH-degrading bacterial population (1, 4, 12, 20). Recently, the *phn* genes of *Burkholderia* sp. strain RP007 provided evidence that there is a different genotype that exhibits a low level of sequence homology with *nah* and has a different gene order yet encodes enzymes for an identical PAH degradation pathway (9, 10).

As increasingly diverse genes that encode enzymes for PAH catabolism are characterized (4–6, 14, 20), it is important not only to understand the function of these genes but also to determine their ecological significance in the context of environmental pollution. The objective of this study was, therefore, to compare the prevalence in aromatic hydrocarbon-contaminated soils of two distinct PAH catabolic genotypes. These genotypes were the divergent *phn* genes (9, 10), which are difficult to isolate by conventional microbiological methods (12), and the easily isolated *nah*-like genes (1, 7, 17). It has been shown that culture-based methods overemphasize *nah*-like genes and fail to detect bacteria with *phn* genotypes in contaminated soils in which both *nahAc* and *phnAc* are detected by PCR amplification and DNA hybridization (12). A molecular biological approach was required to overcome the disparity in the ease of culturing of host bacteria harboring these two genotypes. Highly specific primer combinations, which targeted genes that encode the iron sulfur protein large (α) subunits of the *nah*-like and *phn* PAH initial dioxygenases, allowed us to determine the number of copies of *phnAc* and *nahAc* present in soil by a quantitative competitive PCR (QC-PCR) technique (3, 11, 19). Although this approach did not allow us to determine relative activity or fluxes through *nah*-like and *phn* pathways, it did reveal the relative enrichment of populations after they were exposed to PAHs and allowed us to demonstrate that the *phn* genotype was enriched in response to the selective pressures exerted by specific PAHs in a range of soils.

MATERIALS AND METHODS

Soil samples and analysis. Two soils from the Waikato region of New Zealand (38°S, 175°E) containing different levels of PAHs were selected for analysis. One soil (soil A) was obtained from the site of a former town gas-generating plant and was severely contaminated with PAHs; the other soil (soil B) was contaminated with petroleum fuels. The PAH content of each sample was determined commercially by using U.S. Environmental Protection Agency methods 3545, 3540, and 3630. The number of culturable heterotrophs present in each sample was determined on R2A plates (Difco Laboratories, Detroit, Mich.) by spreading dilutions of a soil suspension that was prepared by shaking 10 g of soil in 90 ml of 0.1% pyrophosphate along with 30 g of glass beads for 1 h at 25°C. The R2A plates were incubated at 25°C for 48 h. Noncontaminated (pristine) soils from central Siberia (61°N, 89°E), Ross Island in the Antarctic (77°S, 166°E), and a native New Zealand forest (38°S, 175°E) were used to assess the ubiquity of the *phn* genes in different environments.

Development of QC-PCR protocol. The PCR primers used for specific probing of the *nahAc* and *phnAc* sequences were designed during a previous study (12). Primer nahAcfor (5′-TGGCGATGAAGAACTTTTCC) and primer nahAcrev (5′-AACGTACGCTGAACCGAGTC) amplify a 992-bp region encompassing nucleotides 63 to 1055 of *Pseudomonas putida* G7 *nahAc* (GenBank accession no. M83949). Primer P8073 (5′-TTCGAGCTGGAATGTGAGC) and primer P9047 (5′-AATAACCGGCGATTCCAAAC) amplify a 993-bp region encompassing nucleotides 82 to 1075 of *Burkholderia* sp. strain RP007 *phnAc* (GenBank accession no. AF061751). Since in this study we relied on accurate relative quantification of specific DNA templates, it was important that only homologous sequences were amplified. Therefore, an annealing temperature of 65°C was used; this temperature was determined empirically to be near the maximum optimum annealing temperature for the two primer sets. PCR amplification was carried out in 50-μl reaction mixtures that contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.25 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 μM, 2.5 U of PLATINUM *Taq* DNA polymerase (Gibco BRL, Gaithersburg, Md.), 0.2 μM forward primer, 0.2 μM reverse primer, and 0.1 μg of template DNA. The following PCR cycling conditions (Techne Cyclogene thermal cycler) were used: 94°C for 5 min; 35 cycles consisting of 94°C for 45 s, 65°C for 30 s, and 72°C for 60 s; 72°C for 10 min; and finally, cooling to 4°C. Maximum ramp rates were used throughout.

Each QC-PCR titration was performed by using six dilutions of standard DNA (prepared as described below), which resulted in a series of reaction mixtures that contained 10, 1, 0.1, 0.01, 0.001, and 0.0001 amol of pNΔSTD and pPΔSTD (equivalent to 6×10^6 to 60 copies of standard DNA template per reaction mixture). To each reaction mixture we added a known volume of target DNA

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TABLE 1. Soil properties

Soil	Type of contamination	Concn (mg kg [dry wt]$^{-1}$) of:			Concn of culturable heterotrophs (no. of cells g [dry wt]$^{-1}$)	Concn (no. of copies g [dry wt]$^{-1}$) of:	
		16 PAHs	Naphthalene	Phenanthrene		*phnAc*	*nahAc*
A	Town gas site PAHs	428	21.6	36.9	9.2×10^6	$1.0 \times 10^8 \pm 0.3 \times 10^8$[a]	$2.0 \times 10^8 \pm 0.9 \times 10^8$
B	Petrochemical	2.7	0.29	0.86	1.3×10^7	$6.2 \times 10^7 \pm 1.3 \times 10^7$	$1.3 \times 10^6 \pm 0.9 \times 10^6$

[a] Mean ± standard deviation ($n = 3$).

that was extracted from 0.5 g of soil by the method of Berthelet et al. (2). Each QC-PCR preparation contained 25 to 125 ng of DNA based on spectrophotometric determination of absorbance at 260 nm. The DNA extract volume, which included the aqueous phase added plus the soil moisture, was recorded in order to obtain an accurate dilution factor for subsequent calculation of the gene copy number per gram (dry weight) in each soil. Standard and target amplicons generated during the QC-PCR experiment were quantified by electrophoresing 10 μl of each reaction mixture through a 1% agarose gel in Tris-borate-EDTA buffer. After electrophoresis, the DNA was stained for 3 h with SYBR Gold (FMC Bioproducts, Rockland, Maine), and the gel was photographed. The intensities of the target and standard amplicons were then determined by processing images of scanned photographs with Scion Image software (Scion Corp., Frederick, Md.). A regression analysis, based on the amplicon intensities and sizes of target and standard DNAs and the initial concentration of standard DNA, was used to calculate the concentration of *nahAc* or *phnAc* genes present in each soil DNA extract; this concentration was subsequently adjusted to obtain a gene copy value on a soil dry weight basis. QC-PCR titrations for the contaminated soil samples were replicated with aliquots of each soil DNA extract; mean values and standard errors are reported.

Construction of standard templates. Deletion derivatives of the G7 *nahAc* and RP007 *phnAc* genes were constructed and used as standards for QC-PCR. Klenow fragment-treated *nahAc* and *phnAc* amplicons were ligated into the *Sma*I site of pUC18 to form pNTGT and pPTGT, and restriction fragments situated between the primer binding sites were then removed before religation of constructs following S1 nuclease treatment. Thus, a 351-bp *Hpa*I-*Nsi*I deletion of the G7 *nahAc* amplicon was used to generate pNΔSTD, and a 324-bp *Nsi*I-*Apa*I deletion of the RP007 *phnAc* amplicon was used to generate pPΔSTD. The construct pNΔSTD yielded a 637-bp PCR product with primers nahAcfor and nahAcrev, and pPΔSTD yielded a 665-bp PCR product with primers P8073 and P9047. Each primer set exhibited identical amplification kinetics with the specific target and standard DNAs; therefore, an equimolar ratio of target and standard DNAs was used to calculate the QC-PCR titration. This ratio was determined by comparing the concentrations of the two PCR products in aliquots taken from PCR over a number of cycles (data not shown). Accurate standard solutions of pPΔSTD and pNΔSTD were prepared, and equimolar amounts based on a molecular mass of 660 Da/bp (15) were combined and diluted to obtain a stock preparation which contained 20 amol of both standard templates per μl. Tenfold dilutions of this mixture were used as standards in the QC-PCR experiments. Mixing the *phn* and *nah* templates ensured consistent dilution and did not interfere with subsequent PCR amplification.

Establishment of microcosms. Enrichment for *nahAc* and *phnAc* was evaluated in microcosms containing pristine soil amended with different PAHs. The soil was collected from a native New Zealand forest. Ten-gram samples of soil and 1-ml portions of basal salts [4 g of Na_2HPO_4 per liter, 2 g of KH_2PO_4 per liter, 1 g of $(NH_4)_2SO_4$ per liter] were placed in 100-ml glass flasks. Preparations were subjected to the following four treatments before they were incubated in the dark at 20°C for 60 days: no additional carbon source (blank); naphthalene supplied as a vapor from a vapor tube suspended above the soil; phenanthrene supplied as a vapor (as routinely used in our laboratory [9, 10, 12]); and 50 mg of fluoranthene added directly to the soil. Soil samples obtained from Siberia and Antarctica were also enriched in microcosms by using naphthalene and phenanthrene as supplementary carbon sources.

Sequencing of *phnAc* amplicons. Amplicons of *phnAc* derived from PAH- and petroleum-contaminated soils were cloned into the *Sma*I site of pUC18 by using a SureClone ligation kit (Amersham Pharmacia Biotech Inc., Piscataway, N.J.). The nucleotide sequences of selected amplification products were determined in both orientations by using the universal primers and a PRISM Ready Reaction DNA terminator cycle sequencing kit (Perkin-Elmer, Inc., Wellesley, Mass.). The Reaction mixtures were resolved by using an ABI model 377 sequencer at the Waikato DNA Sequencing Facility, and the sequences were analyzed by using Omiga 1.0 sequence analysis software (Oxford Molecular Group Ltd., Oxford, United Kingdom).

RESULTS AND DISCUSSION

QC-PCR analysis of *phnAc* and *nahAc* from contaminated soils. The two contaminated New Zealand soils used in this study were selected to represent high and low levels of PAH contamination and are described in Table 1. Figure 1 shows representative photographs of QC-PCR titration of *phnAc* and *nahAc* for the two soils (Fig. 1a), as well as an example of a QC-PCR calibration curve for *phnAc* (Fig. 1b). The lower limit of the QC-PCR titration series was 0.0001 amol of standard template (approximately 60 copies), which was equivalent to a detection limit of 2×10^5 copies of a given gene per gram (dry weight) of soil. By using PCR primers that were specific for a gene that encodes the iron sulfur protein large (α) subunit of the PAH dioxygenase from the recently described *phn* type of catabolic operon (10), we found that cells harboring *phn* genes are not as rare as microbiological culture techniques might lead us to believe (10, 12).

Replicate QC-PCR experiments ($n = 3$) revealed that the PAH-contaminated soil (soil A) contained $1.0 \times 10^8 \pm 0.3 \times$

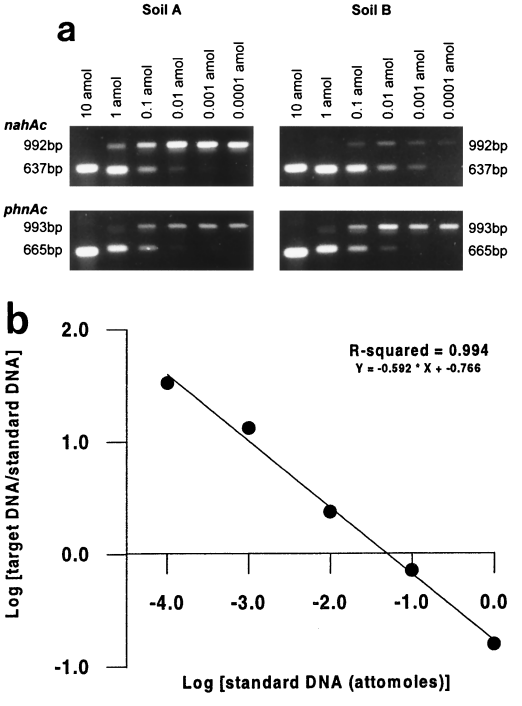

FIG. 1. (a) QC-PCR of *phnAc* and *nahAc* amplified from two contaminated soils. Soil A was derived from a former town gas-generating site and contained high levels of PAHs. Soil B was contaminated with petrochemicals and contained low levels of PAHs. The upper band in each QC-PCR titration series contained the target amplicon (unknown); the lower band consisted of amplicons derived from the standard DNA. The amounts of standard DNA used (10 to 0.0001 amol) are indicated above the lanes. (b) Representative plot of the ratio of target DNA intensity to standard DNA intensity plotted against the initial concentration of standard DNA on logarithmic scales. Band intensities were quantified by processing images of Polaroid photographs of SYBR Gold-stained gels. The plot is not a representation of panel a.

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10^8 copies of *phnAc* per g of soil and $2.0 \times 10^8 \pm 0.9 \times 10^8$ copies of *nahAc* per g of soil, while the petroleum-contaminated soil (soil B) contained $6.2 \times 10^7 \pm 1.3 \times 10^7$ copies of *phnAc* per g of soil and $1.3 \times 10^6 \pm 0.9 \times 10^6$ copies of *nahAc* per g of soil. The numbers of copies of both *phnAc* and *nahAc* in these contaminated soils were significantly greater than the numbers of copies in a pristine soil, which did not contain detectable levels of either gene (i.e., less than 2×10^5 copies per g). The greater numbers of copies in the PAH-contaminated soil than in the petroleum-contaminated soil may reflect higher naphthalene and phenanthrene levels. Similar observations have been made in previous studies of the molecular ecology of *nah*-like genotypes in PAH-contaminated soil (16, 17). For the two contaminated New Zealand soils that we analyzed, the *phn* and *nah*-like genotypes were present (assuming that there was one copy of each gene per cell) at levels that were greater than or equal to the total numbers of heterotrophs determined for the same soil samples by culture techniques (Table 1). Although the presence of *phnAc* or *nahAc* genes in a soil does not a priori indicate that PAH-degrading activity is present, our data revealed elevated levels of the *phnAc* and *nahAc* genes in soils in which it can reasonably be expected that selection pressures exerted by PAHs would enrich populations able to degrade these substrates.

Diversity of *phnAc* amplicons. Previous studies have shown that primers based on *nah*-like genes target a group of sequences that exhibit more than 75% sequence homology and form the phylogenetically distinct *nah*-like and *dnt-ntd* groups (7, 12, 18). We sequenced both strands of *phnAc* amplicons to determine the diversity of homologous sequences targeted in soil DNA by primers P8073 and P9047. The *phn* primers P8073 and P9047 amplified a very highly conserved group of *phnAc* genes that exhibit more than 98% sequence identity with the RP007 *phnAc* sequence. In this study two sequences derived from petroleum-contaminated soil and one sequence derived from PAH-contaminated soil were identical to the RP007 *phnAc* sequence, while an additional three sequences derived from PAH-contaminated soil samples differed at only 2, 12, and 15 nucleotide positions. Similarly high levels of sequence conservation were also found for six *phnAc* amplicons derived from two other contaminated soils in a previous study, which also exhibited more than 98% nucleotide homology with the RP007 *phnAc* sequence (9). Sequence errors due to *Taq* DNA polymerase or PCR amplicon contamination during cloning were statistically improbable since the majority of base substitutions (24 of 29 substitutions; >80%) resulted in synonymous (silent) substitutions. In addition, the *Taq* DNA polymerase error rate, <0.05%, as determined by sequencing two cloned *phnAc* amplicons derived from the *Burkholderia* sp. wild-type strain RP007, was also too low to account for these substitutions.

The highly conserved nature of the *phnAc* amplicons obtained with primers P8073 and P9047 may be an artifact of the high degree of primer specificity and stringent amplification conditions. We have not determined whether this observation is a true reflection of the diversity of *phn* genes in the environment, which would require the use of degenerate primers allied with less stringent annealing conditions during PCR amplification. What is certain is that the original isolation of the *phn* genes was fortuitous since bacteria harboring this genotype appear to be very difficult to isolate yet are ubiquitously distributed and may be present in relatively high numbers in PAH-contaminated soils.

Distribution of *phn* genes. Having determined that *phn* genes were enriched in PAH-contaminated New Zealand soils, we were also interested in evaluating whether the *phn* genes are ubiquitous. Noncontaminated (pristine) soils obtained from central Siberia (61°N, 89°E), Ross Island in the Antarctic (77°S, 166°E), and a native New Zealand forest (38°S, 175°E) were used to assess the ubiquity of the *phn* genes in different environments. Enrichment of the PAH-degrading populations in these uncontaminated soils was necessary before we screened for the *phnAc* genotype since the levels of analogues of *phnAc* were initially below the limits of detection (i.e., less than 2×10^5 copies per g of soil) in these soils. Soil microcosms were therefore established, and PAH-degrading bacteria were enriched by using the low-molecular-weight PAHs naphthalene and phenanthrene. The broad geographic distribution of the *phn* genotype was confirmed when *phnAc* was amplified from a central Siberian soil, from an Antarctic soil from Ross Island, and from various New Zealand soils. The *phn* genes were enriched in soil microcosms that were incubated for 1 week in the presence of either naphthalene or phenanthrene, and after enrichment the levels were above our limit of detection (2×10^5 copies of *phnAc* per g [dry weight] of soil). Again it is particularly interesting that we detected *phn* genes in soils from areas as far afield as Siberia, Antarctica, and New Zealand and yet to our knowledge only one confirmed strain with a *phn* genotype has been isolated from the environment (9, 10, 12).

TABLE 2. Microcosm enrichment

Microcosm enrichment	Concn (no. of copies g of soil^{-1}) of:	
	phnAc	*nahAc*
Preincubation[a]	ND[b]	ND
Control incubation	2.0×10^8	ND
Naphthalene	1.8×10^9	ND
Phenanthrene	5.5×10^8	ND
Fluoranthene	4.9×10^8	ND

[a] Soil sample obtained before microcosms were established.
[b] ND, not detected ($<2 \times 10^6$ copies per g [dry weight] of soil).

In situ specificity of the *phn* genes. We also examined the effects of the selection pressures exerted by different low-molecular-weight PAHs on the *phnAc* and *nahAc* genotypes in an uncontaminated New Zealand soil. The levels of both *nahAc* and *phnAc* were initially below the detection limit (2×10^6 copies of *phnAc* per g [dry weight] of soil) in subsamples of this soil. This detection limit was higher than the detection limit for the contaminated soils due to the higher humic content of this soil, which required greater dilution of target DNA to a level which did not inhibit PCR amplification. As expected, selection pressures within the microcosms enriched for a PAH-degrading phenotype (8, 13) but interestingly favored the *phn* genotype and not the *nah*-like genotype, which we were not able to detect in any microcosm (Table 2). These findings imply that the *nah*-like genotype is not always ecologically dominant and confirm that it is not always realistic to represent PAH degradation by one genotype, as this genotype may not be present at a detectable (or significant) level in all environments (1, 12).

Enrichment of *phnAc* from uncontaminated soils appeared to be greatest with naphthalene (nine times greater than enrichment in the control microcosm), which has the greatest volatility, solubility, and, hence, availability of the three PAH substrates used for enrichment. Since previous studies have shown that the *phn* genes are induced by both naphthalene and phenanthrene (10), it is likely that phenanthrene also enriches for the *phn* genotype. Although the QC-PCR data for phenanthrene enrichment of *phnAc* may not be conclusive in the New

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Zealand soil microcosm (the level was only two to three times the level observed in the control microcosm), we have demonstrated that *phnAc* was enriched in both Siberian and Antarctic soils when they were exposed to phenanthrene. It remains to be established whether fluoranthene is degraded in situ by *phn* gene products or is able to induce transcription of the *phn* genes, since the evidence for enrichment in response to fluoranthene was not conclusive. Whether the increase observed in the control microcosm was real or due to variability and biases inherent in using small soil samples was not determined. A real increase may have occurred in response to natural soil substrates as a consequence of moisture, temperature, and inorganic nutrient level adjustments made while the control microcosm was being established.

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