

Abundance of Dioxygenase Genes Similar to *Ralstonia* sp. Strain U2 *nagAc* Is Correlated with Naphthalene Concentrations in Coal Tar-Contaminated Freshwater Sediments

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We designed a real-time PCR assay able to recognize dioxygenase large-subunit gene sequences with more than 90% similarity to the *Ralstonia* sp. strain U2 *nagAc* gene (*nagAc*-like gene sequences) in order to study the importance of organisms carrying these genes in the biodegradation of naphthalene. Sequencing of PCR products indicated that this real-time PCR assay was specific and able to detect a variety of *nagAc*-like gene sequences. One to 100 ng of contaminated-sediment total DNA in 25- μ l reaction mixtures produced an amplification efficiency of 0.97 without evident PCR inhibition. The assay was applied to surficial freshwater sediment samples obtained in or in close proximity to a coal tar-contaminated Superfund site. Naphthalene concentrations in the analyzed samples varied between 0.18 and 106 mg/kg of dry weight sediment. The assay for *nagAc*-like sequences indicated the presence of $(4.1 \pm 0.7) \times 10^3$ to $(2.9 \pm 0.3) \times 10^5$ copies of *nagAc*-like dioxygenase genes per μ g of DNA extracted from sediment samples. These values corresponded to $(1.2 \pm 0.6) \times 10^5$ to $(5.4 \pm 0.4) \times 10^7$ copies of this target per g of dry weight sediment when losses of DNA during extraction were taken into account. There was a positive correlation between naphthalene concentrations and *nagAc*-like gene copies per microgram of DNA ($r = 0.89$) and per gram of dry weight sediment ($r = 0.77$). These results provide evidence of the ecological significance of organisms carrying *nagAc*-like genes in the biodegradation of naphthalene.

Natural environments harbor diverse and often uncharacterized microbial populations able to degrade a wide variety of pollutants (36). Difficulties studying these microbial consortia arise because they are highly complex and because conventional cultivation methods can isolate only a small fraction of the microorganisms present in the environment (37). Real-time PCR provides the opportunity of monitoring and enumerating bacterial populations in contaminated environments without cultivation biases (4). This technique detects the accumulation of PCR products in each cycle of the process, which can be used to estimate the amount of target present prior to the start of the amplification (10).

Polycyclic aromatic hydrocarbons (PAHs), which are common constituents of petroleum, coal tar, and shale oil and which are formed in the combustion of fossil fuels can be toxic, mutagenic, and/or carcinogenic (30). Naphthalene is often used as a model compound to study PAH biodegradation (11), and the majority of the isolated naphthalene-degrading bacteria are species of *Pseudomonas* (41). In these organisms, naphthalene biodegradation genes (called *nah* genes) are located in two operons: the upper pathway encodes the conversion of naphthalene to salicylate, and the lower pathway encodes the conversion of salicylate to central metabolites via catechol

(18). The initial reaction in the degradation of naphthalene is catalyzed by a multicomponent enzyme system that adds both atoms of dioxygen to the aromatic ring to form *cis*-1,2-dihydroxy-1,2-dihydronaphthalene (6). This enzyme system consists of an iron-sulfur flavoprotein reductase, an iron-sulfur ferredoxin, and a catalytic oxygenase component (3). The catalytic component, called naphthalene 1,2-dioxygenase (NDO), is an $\alpha_3\beta_3$ hexamer (17). The α subunit (NDO $_{\alpha}$), containing the active site and frequently called *nahAc* when identified in archetypical *Pseudomonas*-type genes, is usually used as a target for molecular studies (22, 40) because it is believed to confer substrate specificity (28).

A more recently studied naphthalene biodegradation pathway is the one from *Ralstonia* sp. strain U2, isolated from oil-contaminated soil in Venezuela (8). *Ralstonia* sp. strain U2 converts naphthalene to central metabolites via gentisate, and the genes for the complete pathway (called *nag* genes) appear to be on a single operon carried on a large plasmid (9, 43). Genes encoding the conversion of naphthalene to salicylate in this strain are homologous to those of the archetypical *nah* upper pathway, suggesting a common ancestry, with the NDO $_{\alpha}$ gene being called *nagAc* (9). Two additional open reading frames (*nagGH*), encoding the salicylate 5-hydroxylase enzyme, which converts salicylate to gentisate, are located between the *nagAa* and *nagAb* genes (42).

Comamonas testosteroni GZ42, with the ability to grow on naphthalene as well as phenanthrene, also presents a *nag*-like arrangement of genes (44), with high sequence similarity with the *nag* operon (9). Genes *dntAc*, *ntdAc*, and *nbzAc*, involved in 2,4-dinitrotoluene (16, 34), 2-nitrotoluene (27), and nitroben-

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TABLE 1. Primers and probes used for the quantification of *nagAc*-like, bacterial 16S rRNA, and *luxE* genes

Target	Name ^a	Sequence (5'-3')	Concn (nM)	Amplicon size (bp)	Reference or source
<i>nagAc</i> -like genes	<i>nagAc</i> -like-F	GGCTGTTTTGATGCAGA	600	107	This study
	<i>nagAc</i> -like-R	GGGCCTACAAGTTCCA	600		
	<i>nagAc</i> -like-P	FAM-CCAGGCTGCATCACCCAGATA-BHQ-1	300		
<i>luxE</i> (<i>Vibrio fischeri</i>)	<i>luxE</i> -F	TATTCACGTCGATCGTCTTA	500	84	This study
	<i>luxE</i> -R	TGCTCAAACCAATCTCCA	500		
	<i>luxE</i> -P	FAM-ACGCTTGCTTGTTCTGTCAACTTC-BHQ-1	100		
16S rRNA genes (bacteria)	1055f	ATGGCTGTCGTCAGCT	600	352	12
	1392r	ACGGGCGGTGTGTAC	600		
	16STaq1115-BHQ	FAM-CAACGAGCGCAACCC-BHQ-1	125		

^a F, forward primer; R, reverse primer; P, TaqMan probe; FAM, 6-carboxyfluorescein; BHQ-1, Black Hole Quencher.

zene (23) degradation, respectively, also present high sequence similarities with the NDO_α gene from *Ralstonia* sp. strain U2 (9). In addition, sequences with more than 90% similarity to the *nagAc* gene of *Ralstonia* sp. strain U2 were detected in mRNA extracted from groundwater samples (40). Zhou and coworkers suggested that the *nag*-like route of naphthalene biodegradation might be a relative rarity (42). However, the abundance of *nagAc*-like NDO gene sequences in libraries from environmental samples and the isolation of naphthalene-degrading strains with *nag*-type NDO_α sequences in several continents (8, 15, 38, 44) suggest that these organisms may be broadly distributed.

The objective of this study was to investigate the abundance of organisms carrying dioxygenase gene sequences with more than 90% similarity to that of the *Ralstonia* sp. strain U2 *nagAc* gene (*nagAc*-like genes) in the environment. To accomplish this goal, we designed a real-time PCR assay, which was applied to DNA extracted from surficial freshwater sediment samples obtained in or in close proximity to a coal tar-contaminated Superfund site. A positive correlation between the abundance of *nagAc*-like gene sequences and naphthalene concentrations measured in these sediments was found. This work represents a significant step in the elucidation of the ecological significance of indigenous bacteria carrying *nagAc*-like NDO_α gene sequences in aerobic naphthalene biodegradation.

MATERIALS AND METHODS

Bacterial strains. *Ralstonia* sp. strain U2 (ATCC BAA-423 [8]) liquid cultures were grown in BBL Trypticase soy broth (Becton Dickinson, Sparks, Md.), and *Pseudomonas putida* G7 was grown in the phosphate-buffered mineral salts medium PAS broth (2), with naphthalene added as solids to the liquid medium. All strains were cultivated at room temperature with constant shaking (150 rpm).

Sediment samples. Nine surficial sediment samples were collected in January 2002 from Chattanooga Creek, located in southeast Tennessee (Chattanooga, Tenn.). Samples were immediately frozen in dry ice and stored at -80°C for nucleic acid extraction.

Chemical analysis. PAHs were extracted from sediment samples with an accelerated solvent extractor (ASE-300; Dionex Corp., Salt Lake City, Utah). Sample extracts were analyzed with a Hewlett-Packard (HP) gas chromatograph (model 6890) equipped with mass-selective detector (model 5973) and an HP autosampler complying with Environmental Protection Agency (EPA) standard method 8270D.

DNA extractions. (i) **Pure cultures.** Total DNA was extracted from cells recovered from 10 ml of pure cultures of *P. putida* G7 and *Ralstonia* sp. strain U2 in stationary phase with the FastDNA kit (Q-BIOgene, Carlsbad, Calif.) by following the manufacturer's instructions.

(ii) **Sediment samples.** Total DNA was purified in triplicate from sediment samples with the FastDNA SPIN kit for soil (Q-BIOgene) with minor modifications as follows. In each DNA extraction 10⁹ copies of plasmid pUTK215 carrying the *Vibrio fischeri luxCDABE* cassette (13) were added to 0.5 g of wet sediment, in order to assess DNA recovery. Samples were homogenized twice for 30 s each at a speed of 5.5 m/s with a FastPrep FP120 instrument (Q-BIOgene). This treatment produced adequate cell lysis without producing excessive shearing of the DNA (data not shown). The washing of the binding matrix-DNA complex and elution were performed as described previously (5), except that DNA was eluted in a total volume of 100 μl of 10 mM Tris-HCl, pH 8.0. DNA recovery was calculated for each individual extraction from the recovery of the *luxE* gene measured by real-time PCR as described below. A recovery of 10⁹ copies of *luxE* gene per extraction corresponded to 100% DNA recovery. DNA recovery values were used to compensate for DNA losses during extraction only when copies of the target were expressed per gram of dry weight sediment (dw). DNA was quantified with a PicoGreen double-stranded DNA quantitation kit (Molecular Probes, Eugene, Oreg.), with lambda DNA as the standard, in accordance with the manufacturer's instructions.

Real-time PCR assays. Real-time PCR assays were carried out using 1× QuantiTect probe supermix (QIAGEN, Valencia, Calif.) in a total volume of 25 μl. Primers and probe were synthesized by Biosearch Technologies, Inc. (Novato, Calif.). Amplification and detection were performed with the DNA Engine Opticon continuous fluorescence detection system (MJ Research, Waltham, Mass.). All PCR runs included control reactions without template and standard DNA with concentrations between 10 and 10⁸ copies/reaction for *nagAc*-like gene and *luxE* assays or 10³ to 10⁸ copies for the 16S rRNA gene assay. The numbers of copies of each target per PCR was calculated by comparison of threshold cycles obtained in each PCR run from standard DNA.

(i) ***nagAc*-like gene real-time PCR assay.** Sequences and concentrations of forward primer (*nagAc*-like-F), reverse primer (*nagAc*-like-R), and probe (*nagAc*-like-P) are shown in Table 1. The PCR program included 1 cycle at 50.0°C for 2 min, 1 cycle of 95.0°C for 15 min, and then 40 cycles of 95.0°C for 30 s and 59°C for 30 s. Standard DNA consisted of plasmid pCR4-TOPO (Invitrogen, Carlsbad, Calif.) carrying a PCR product of an NDO_α gene amplified from sediment sample DNA and sequenced to confirm its identity. The closest match of standard DNA was the *Polaromonas naphthalenivorans* strain CJ2 naphthalene dioxygenase gene (15) (GenBank accession number AY194932; 100% similarity). In addition, eight NDO_α clones from uncultured bacteria have 100% similarity with standard DNA (GenBank accession numbers AF099763, AF099764, AF099767, AF099770, AF099772, AF099773, AY194929, and AY250113). For the analysis of sediment DNA, triplicate real-time PCR measurements were performed at two different DNA concentrations (between 5 and 50 ng of sediment DNA/PCR). Specificity of the assay was analyzed by cloning real-time PCR products from three different sediment samples into pCR4-TOPO (Invitrogen) and randomly selecting and sequencing 10 clones from each library.

(ii) ***luxE* real-time PCR.** Forward primer (*luxE*-F), reverse primer (*luxE*-R), and probe (*luxE*-P) sequences and concentrations are shown in Table 1. The PCR program used for the *luxE* assay was as follows: 1 cycle at 50.0°C for 2 min, 1 cycle of 95.0°C for 10 min, and then 50 cycles of 95.0°C for 15 s and 61°C for 30 s. Standard DNA consisted of the pUTK215 plasmid (13), used as an internal spike. To calculate DNA recovery, template was added as 5 μl of a 1:100 dilution

of each sediment DNA (up to 3.7 ng of DNA/reaction) and measured in triplicate reactions.

(iii) **Bacterial 16S rRNA gene real-time PCR.** Bacterial 16S rRNA gene quantifications were performed by a previously described real-time PCR assay (12) with minor modifications, as follows. The concentrations of 1055f and 1392r primers and 16STaq1115-BHQ (carrying a Black Hole Quencher instead of carboxytetramethylrhodamine) are shown in Table 1. The PCR program used for the bacterial 16S rRNA gene assay included 1 cycle at 50.0°C for 2 min, 1 cycle of 95.0°C for 15 min, and then 45 cycles of 95.0°C for 30 s and 55°C for 45 s. Standard DNA consisted of dilutions of plasmid pCR2.1 carrying a bacterial 16S rRNA gene (GenBank accession number AF420301). The bacterial 16S rRNA gene target was quantified in sediment samples in triplicate with 1 ng of sediment DNA/PCR.

Multiple sequence alignments. Sequence alignments were performed with the ClustalX (version 1.64b) program, with all parameters set at their default values (35).

Statistical analysis. One-way analysis of variance was performed using SPSS, version 11.5 (SPSS Inc., Chicago, Ill.), with mean differences significant at the 0.05 level. The Tukey (b) post hoc test was used for multiple comparisons between groups. Data were normally distributed, and a \log_{10} transformation was used when the assumption of equality of variance was not met with untransformed data (copies of *nagAc*-like gene per gram dw and copies of 16S rRNA gene per microgram of DNA and per gram dw). Correlations between variables were analyzed with the bivariate two-tailed Pearson correlation in SPSS version 11.5.

RESULTS

Design of a real-time PCR assay for the quantification of *nagAc*-like genes. We designed a TaqMan real-time PCR assay to study the abundance and distribution of organisms carrying *nagAc*-like genes in environmental samples (Table 1). Target sequences of the assay were *Ralstonia* sp. strain U2 *nagAc* (GenBank accession number AF036940) (9), as well as 32 dioxygenase sequences publicly available by March 2002 and having similarities between 91.7 and 97.8% to *nagAc*. The majority of the target sequences represented NDO $_{\alpha}$ genes from uncultured bacteria cloned from groundwater by reverse transcription PCR (GenBank accession numbers AF099748, AF099749, AF099752, AF099753, AF099755 to AF099759, AF099761 to AF099767, AF099769, AF099770, and AF099772 to AF099774) (40). Target NDO $_{\alpha}$ genes from cultured representatives besides *nagAc* were *pahAc*, cloned from *C. testosteroni* (AF252550) (26), *nahAc* from *Burkholderia* sp. strain S1-17 (AF448048) and SOD-5b (AF448053), and *nahAc* from the PAH-degrading bacteria NJ2, NK2, NK3, and *Ralstonia* sp. strain NI1 (AB066442 to AB066445, respectively) (38). In addition, we targeted other closely related dioxygenase genes involved in 2,4-dinitrotoluene (U62430 and AF169302), 2-nitrotoluene (U49504), and nitrobenzene degradation (AF379638), with 93.1 to 94.6% similarities at the nucleotide level to the *nagAc* gene (16, 23, 27, 34). The most related nontarget sequences of the designed assay were *Pseudomonas*-type NDO $_{\alpha}$ genes, with similarities to the *nagAc* gene between 76.3 and 80.7%.

Specificity of the *nagAc*-like gene real-time PCR assay. The designed *nagAc*-like gene real-time PCR assay was optimized and applied to freshwater sediment samples. Cloning and sequencing of PCR products was used to analyze the specificity of the amplification. Three gene libraries were constructed from real-time PCRs of sediment samples with different degrees of naphthalene contamination (0.32, 52.8, and 106 mg/kg dw). Ten clones from each library were sequenced, and the closest match of each clone with BLAST (www.ncbi.nlm.nih

www.ncbi.nlm.nih.gov/BLAST) corresponded to a target *nagAc*-like sequence. In addition, sequences showed some degree of diversity in the 74 bp located between primers, suggesting that the assay is capable of detecting a variety of target sequences. For 20 clones, the closest cultured match corresponded to sequence AF252550 from *C. testosteroni* (97.3 to 100% similarity). In 10 clones the closest cultured match corresponded to the *nahAc* gene from the recently isolated *P. naphthalenivorans* strain CJ2 (15) (91.9 to 100% similarity). No sequence in the libraries was identical to the *nagAc* gene from *Ralstonia* sp. strain U2. In addition, 29 out of 30 clones had no mismatches with the probe sequence, and one clone carried one mismatch approximately in the center of the probe.

In addition to sequence analysis, *Ralstonia* sp. strain U2 and *P. putida* G7 total DNA was used to test the specificity of the *nagAc*-like gene real-time PCR assay. *Ralstonia* sp. strain U2 total DNA generated a strong amplification signal with the assay, with detection of $1.3 \pm 0.1 \times 10^8$ *nagAc* copies per μg of total DNA. In contrast, total DNA from *P. putida* G7 produced in a very low and variable amplification signal and target quantifications of $(8.2 \pm 7.1) \times 10^2$ copies of the *nagAc* gene per μg of DNA. Overall, these results suggest that the designed real-time PCR assay is specific for *nagAc*-like sequences in sediment samples showing low levels of background signal from nontarget organisms, at least 5 orders of magnitude lower than specific amplifications.

Real-time PCR amplification efficiencies. Optimum template concentrations and amplification efficiencies for the *nagAc*-like gene assay were determined by using total DNA extracted from *Ralstonia* sp. strain U2 or a PAH-contaminated sediment sample. Linear relationships between the logarithm of the template concentration and threshold cycle values indicated that 1 to 100 ng of DNA per 25- μl reaction mixture resulted in accurate target quantification in both cases (Fig. 1). However, at least 25 ng per reaction was needed to surpass the detection limit of 10 copies/reaction when using DNA extracted from sediments with low levels of naphthalene contamination (data not shown). Amplification efficiencies for this assay calculated based on the slope of the regressions shown in Fig. 1 were 0.97 and 1.04 for sediment and *Ralstonia* sp. strain U2 DNA, respectively. These amplification efficiencies suggest the duplication of almost all target molecules in each cycle, in the conditions tested for the assay.

The *V. fischeri luxE* gene (Table 1) was used for spiking sediment samples in order to calculate DNA recovery. The amplification efficiency of the *luxE* assay was 0.96 for contaminated-sediment DNA at template concentrations between 1 and 50 ng per reaction (Fig. 1A). Amplification efficiencies obtained for 16S rRNA gene assay with pure culture and environmental sample DNA were 0.92 and 0.93, respectively. It has been previously reported that the 16S rRNA gene assay shows low amplification efficiency (12). However, because of high target concentration in most environmental samples, this assay was proven to be reliable as a measure of total bacterial biomass (5, 12). An inhibitory effect of real-time PCRs at high DNA concentrations was observed when using environmental samples, but not *Ralstonia* sp. strain U2 total DNA (Fig. 1). This effect may be associated with a low level of inhibitory-substance carryover from the sediment sample during the DNA extraction.

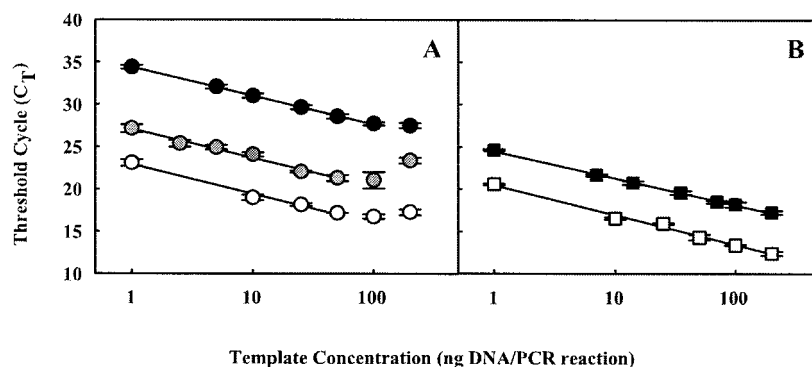


FIG. 1. Effect of template concentration in real-time PCR assays. (A) Total DNA extracted from contaminated sediments; (B) total DNA extracted from *Ralstonia* sp. strain U2. Black symbols, *nagAc*-like gene assay; gray symbols, *luxE* assay; white symbols, bacterial 16S rRNA gene assay. Equations of the regressions are as follows: $y = 34.40 - 3.38x$, $r^2 = 0.999$ (*nagAc* assay plus sediment DNA); $y = 24.51 - 3.22x$, $r^2 = 0.998$ (*nagAc* assay plus *Ralstonia* sp. strain U2 DNA); $y = 27.11 - 3.42x$, $r^2 = 0.982$ (*luxE* assay plus sediment DNA); $y = 22.92 - 3.49x$, $r^2 = 0.986$ (16S rRNA gene assay plus sediment DNA); $y = 20.48 - 3.53x$, $r^2 = 0.992$ (16S rRNA gene assay plus *Ralstonia* sp. strain U2 DNA). Error bars indicate the standard deviations of triplicate real-time PCRs. C_T , threshold cycle.

Analysis of freshwater sediment samples. (i) Sediment samples and DNA extractions. Nine sediment samples from three different sections of Chattanooga Creek were selected for molecular analysis (Fig. 2C). Zone I is located upstream of the PAH source, and chemical analysis of these samples indicated the presence of 0.18 to 0.32 mg of naphthalene/kg dw. The EPA recently remediated zone II by removing coal tar deposits and dredging contaminated sediments along a 1-mile section of the creek (phase I remediation plan, completed in November 1998). Naphthalene concentrations in analyzed samples of this section were 1.13 to 44.8 mg/kg dw. EPA is currently considering remediation of zone III. Samples of this section contained 44.4 to 106 mg of naphthalene/kg dw in samples 5 to 7, but much lower naphthalene concentrations downstream, in samples 8 and 9 (7.8 and 6.9 mg/kg dw, respectively). All samples showed, in addition, various levels of higher-molecular-weight PAHs (data not shown).

Total DNA was extracted in triplicate from each sediment sample. Observed differences in DNA yields (Table 2) may be due to different amounts of biomass in the samples or, alternatively, to uneven losses of DNA during the extraction. We estimated the percentage of DNA recovery in each extraction by adding 10^9 copies of plasmid pUTK215, which contains the *luxCDABE* operon from *V. fischeri* (13), and analyzing its recovery by real-time PCR of the *luxE* gene. Total DNA recoveries varied significantly among samples ($P < 0.001$), although they were fairly consistent in triplicate extractions from the same sample (Table 2). Control DNA extractions without spiking with pUTK215 had very low *luxE* background signals (Table 2). Percentages of *luxE* recovery obtained in each DNA extraction were used to compensate for differences in nucleic acid recoveries when concentrations of the target were calculated as copies per gram dw.

(ii) Target quantification. Concentrations of *nagAc*-like gene targets calculated as copies per microgram of DNA are shown in Fig. 2A. Multiple comparisons of these data indicated that the concentration of the *nagAc*-like gene was significantly higher in samples 5 to 7 ($P \leq 0.008$). After compensating for differences in DNA recovery among extractions, it was found that the concentration of the *nagAc*-like gene target varied

between $(1.2 \pm 0.6) \times 10^5$ and $(5.4 \pm 0.4) \times 10^7$ copies per g dw (Fig. 2B). Multiple comparisons of \log_{10} -transformed data indicated that copies of *nagAc*-like gene per gram dw were also significantly higher ($P \leq 0.002$) in samples 5 to 7 than in the rest of the analyzed samples.

As a way of estimating total bacterial biomass in sediment samples, a real-time PCR assay targeting most of the bacterial 16S rRNA gene (12) was applied to Chattanooga Creek total-DNA sediment samples. Copies of 16S rRNA gene per microgram of DNA were not significantly different among samples (\log_{10} -transformed data, $P > 0.05$; Fig. 2A). This was not surprising, as we expected to find approximately the same average chromosome size and average 16S rRNA gene copy number per cell in different sediment samples, assuming similar amounts of eukaryotic DNA. Similar amounts of 16S rRNA gene target per microgram of DNA were also observed in activated sludge samples from bench scale reactors operating at different retention times, with different amounts of suspended biosolids (5). In contrast, significant differences among samples in 16S rRNA gene copies were found when amounts were calculated as copies of this target per gram dw (\log_{10} -transformed data, $P < 0.001$; Fig. 2B). This result suggests that differences in DNA yields were due not only to uneven DNA recoveries but also to different amounts in initial biomass.

(iii) Correlation with naphthalene concentrations. A positive correlation between naphthalene concentrations and the number of copies of the *nagAc*-like gene target measured in sediment samples was found (Fig. 3A and B). The correlation coefficient for these two variables was higher when the target was calculated as copies per microgram of DNA ($r = 0.89$) than as copies per gram dw (\log_{10} -transformed data, $r = 0.77$). Both correlations were significant at the 0.01 level. This difference may be due to inaccuracies of the measurement of DNA recovery introduced to the values of the *nagAc*-like target during normalization. Alternatively, copies per microgram of DNA may be a better indicator, indirectly evaluating the proportion of the population carrying the measured gene, since no significant differences among samples in 16S rRNA gene target per microgram of DNA were found (Fig. 2A). In contrast, there was no significant correlation between naphtha-

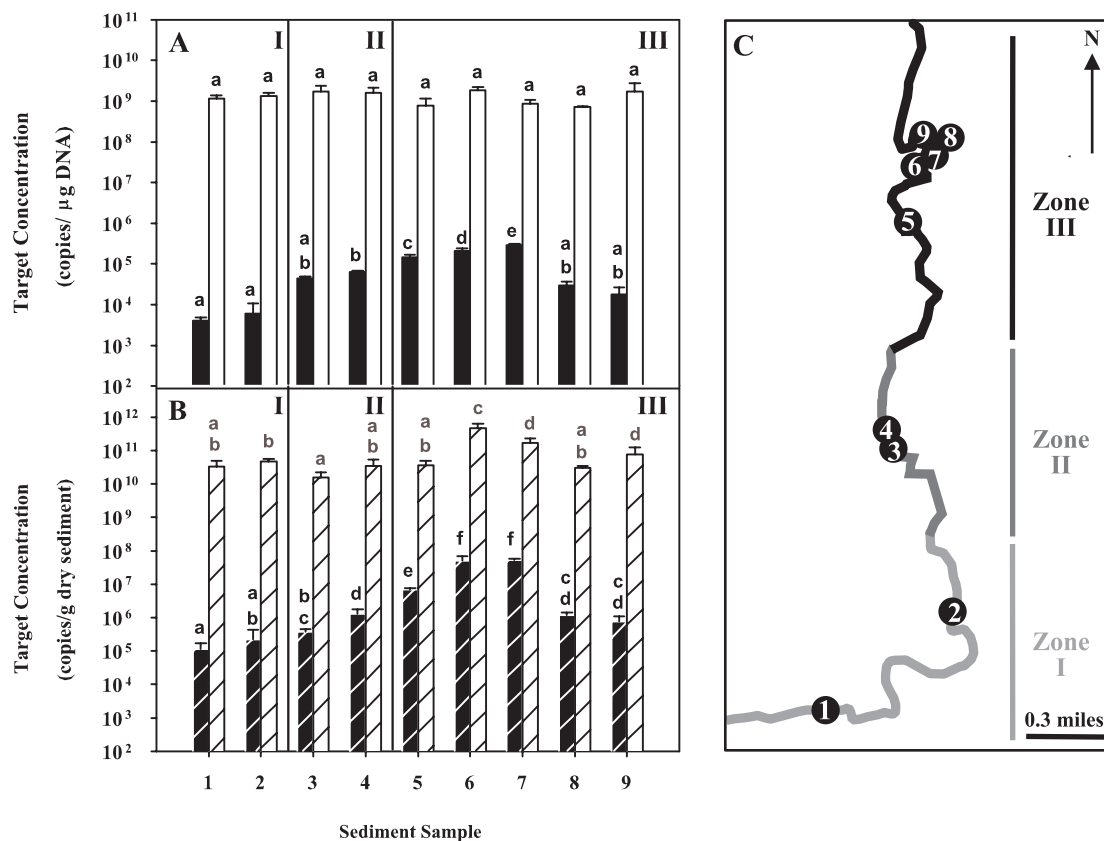


FIG. 2. Molecular analysis of sediment samples. (A) Copies of target per microgram of DNA of the *nagAc*-like gene (black bars) and bacterial 16S rRNA gene (white bars) in Chattanooga Creek sediment samples. (B) Copies of target per gram dw of sediment of the *nagAc* gene (black bars with white stripes) and bacterial 16S rRNA gene (white bars with black stripes). Values of copies of the target per gram dw were corrected by DNA recovery calculated based on real-time PCR quantification of the spiked *luxE* gene, as indicated in Materials and Methods. Letters above bars indicate homogeneous subsets by the multiple-comparison Tukey (b) post hoc tests of untransformed data (copies of the *nagAc*-like gene per microgram of DNA) or \log_{10} -transformed data (copies of the *nagAc*-like gene per gram dw and copies of the 16S rRNA gene per microgram of DNA or per gram dw). Error bars indicate the standard deviations of values measured in triplicate DNA extractions. (C) Map of sampling area. I to III represent different segments of the creek. Zone I, background section upstream of the contaminated area of the creek (sampling sites 1 and 2); zone II, remediated section of Chattanooga Creek (sampling sites 3 and 4); zone III, section of the creek considered for remediation (sampling sites 5 to 9). Arrow, direction of the creek, as well as north (N).

TABLE 2. DNA yields and *luxE* recovery in surficial sediment samples of Chattanooga Creek

Zone ^a	Sample	DNA yield ^b (μg of DNA/g dw)	<i>luxE</i> recovery ^b (%)
I	1	4.5 \pm 0.8 (4.3)	18.4 \pm 8.0 (0.001)
	2	28.4 \pm 1.5	82 \pm 13.7
II	3	9.6 \pm 0.2	107.9 \pm 3.3
	4	10.3 \pm 0.2	50.8 \pm 10.8
III	5	26.4 \pm 4.1	55.2 \pm 14.9
	6	30.8 \pm 1.3 (29)	65.4 \pm 16.5 (0.0004)
	7	20 \pm 0.9	54 \pm 9.0
	8	15.7 \pm 0.6	37.9 \pm 3.4
	9	17.4 \pm 0.2	37.8 \pm 6.7

^a Zones I, II, and III correspond to background upstream from the source of contamination, remediated, and contaminated nonremediated sections of the creek, respectively (Fig. 2C).

^b Averages \pm standard deviations for triplicate extractions are indicated. Values in parentheses for samples 1 and 6 indicate DNA yields and *luxE* recoveries for DNA extractions without *luxE* addition.

lene concentrations and copies of 16S rRNA gene target per microgram of DNA (\log_{10} -transformed data, $r = -0.213$, $P = 0.285$; Fig. 3C). A low but significant correlation (\log_{10} -transformed data, $r = 0.409$, $P = 0.034$; Fig. 3D) between the number of copies of 16S rRNA gene per gram dw and naphthalene concentrations was found. These results suggest that naphthalene concentration may influence the sizes of populations carrying the *nagAc*-like gene in surficial freshwater sediments but has a small effect on the general bacterial population.

DISCUSSION

Biodegradation usually modifies the microbial community, in particular if targets promote cell growth, and these secondary effects (e.g., population expansion or shifts in the relative proportion of cell types) can be used to evaluate this process (32). We were interested in studying the importance of organisms carrying *nagAc*-like genes in the biodegradation of naphthalene. We designed a real-time PCR assay able to recognize NDO_α gene sequences more than 90% similar to that of the

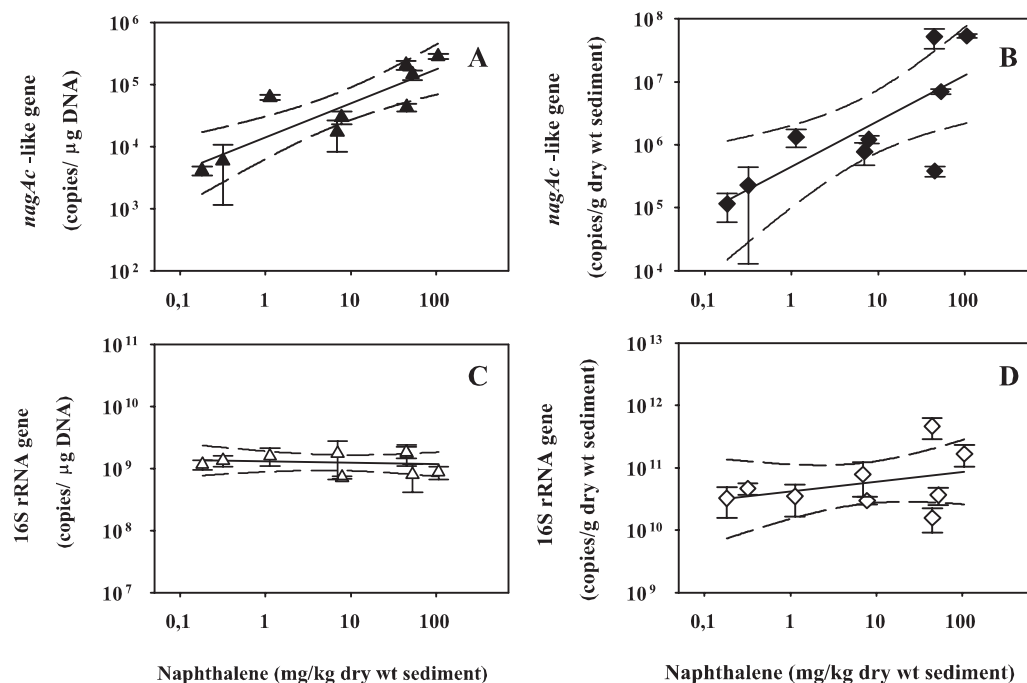


FIG. 3. Correlations between target and naphthalene concentrations in Chattanooga Creek sediment samples. (A) Copies of the *nagAc*-like gene target per microgram of DNA ($y = 4.14 + 0.55x$, $r^2 = 0.747$); (B) copies of the *nagAc*-like gene target per gram dw ($y = 5.65 + 0.72x$, $r^2 = 0.586$); (C) copies of the bacterial 16S rRNA gene per microgram of DNA ($y = 9.11 - 0.023x$, $r^2 = 0.021$); (D) copies of the bacterial 16S rRNA gene per gram dw ($y = 10.62 + 0.16x$, $r^2 = 0.128$). Values of copies of the target per gram dw were corrected by DNA recovery calculated based on real-time PCR quantification of the spiked *luxE* gene, as indicated in Materials and Methods. Dashed lines, 95% confidence intervals; error bars, standard deviations of triplicate DNA extractions.

Ralstonia sp. strain U2 *nagAc* gene. Sequences cloned from both uncultured organisms and pure cultures were used for the design of this assay, as a way of obtaining a more realistic representation of this group's diversity. The assay was optimized and applied to PAH-contaminated freshwater sediment samples.

Since the design of the assay, new *nagAc*-like sequences have been incorporated into the databases. An analysis of NDO $_{\alpha}$ genes from uncultured bacteria as well as pure cultures publicly available to date indicated 0 mismatches of the designed *nagAc*-like real-time PCR primers and probe with 37 sequences with similarities of between 91.7 and 97.8% to the *Ralstonia* sp. strain U2 *nagAc* gene. In addition, six sequences of the naphthalene dioxygenase gene with more than 91.7% similarity to the *nagAc* gene had one mismatch in a primer or the probe, but none of them in a critical position, such as in the 3' end of a primer. The designed forward primer has one mismatch with the *Ralstonia* sp. strain U2 *nagAc* gene in the third base close to its 5' end, where this gene carries a T instead of a C, as do the rest of the available *nagAc*-like sequences. No sequences with similarities higher than 91.7% to the *nagAc* gene in the databases has more than one mismatch with the designed real-time PCR system.

Due to their high similarity to *nag*-type NDO $_{\alpha}$ gene sequences, *dntAc*, *ntdAc*, and *nbzAc* genes, involved in the degradation of nitroaromatic compounds, can also be recognized by the *nagAc*-like gene real-time PCR assay. It has been suggested that the gene encoding the initial dioxygenase involved in the biodegradation of these compounds recently diverged

from a naphthalene degradation pathway similar to that of *nag* of *Ralstonia* sp. strain U2 (16). Although strains carrying these genes are not able to grow with naphthalene or salicylate as a carbon source, their initial dioxygenases present relaxed substrate specificities and are able to transform naphthalene (23, 24, 28).

All organisms isolated so far carrying *nag*-like genes for the degradation of naphthalene or nitroarenes belong to the beta subdivision of *Proteobacteria*, although some of them were formerly classified as *Pseudomonas* spp. It has been suggested that these distinctive *nag*-type NDO $_{\alpha}$ genes may be related to the use of a gentisate pathway such as that for the well-characterized strain *Ralstonia* sp. strain U2 (26), although this important aspect remains to be determined. *C. testosteroni* CZ42 presents genes homologous to *nagG* and *nagH* encoding salicylate 5-hydroxylase, the enzyme that converts salicylate to gentisate (44). In addition, in *C. testosteroni* strain H part of the gene encoding salicylate 5-hydroxylase has been sequenced (26) and catechol 2,3-dioxygenase activity could not be measured (25). It will be of interest to know if the recently isolated *P. naphthalenivorans* strain CJ2 (14), a beta proteobacterium with an NDO $_{\alpha}$ gene that belongs to a subcluster separate from *nagAc* (15) and the only cultured representative of this group of sequences (called clade I in reference 15), follows this alternative biodegradation pathway.

The closest nontarget sequences of the *nagAc*-like gene assay are NDO $_{\alpha}$ genes cloned from *Pseudomonas* spp. and uncultured bacteria closely related to them, with similarities to the *nagAc* gene between 76.3 and 80.7%. The total number of

mismatches of the *nagAc*-like gene real-time PCR assay with archetypical *nah*-type sequences (such as M83949) is 13 to 16 out of a total of 54 nucleotides present in the primers and probe set. Low unspecific amplification from pure cultures of *P. putida* G7 indicated that these sequences will not affect the quantification if both populations are present at approximately equal concentrations. In addition, no *Pseudomonas*-type sequences were found in 30 analyzed clones from libraries constructed from real-time PCRs of sediment samples. The designed primer and probe set has 12 or 13 mismatches with *phnAc*-type dioxygenase sequences, such as the one cloned from *Burkholderia* sp. strain RP007 (21) and related sequences from uncultured bacteria (GenBank accession numbers AY032935, AY032936, and AY032937) (22). On the other hand, the *narAa* gene cloned from the gram-positive bacterium *Rhodococcus* sp. strain NCIMB12038 (20) has 26 mismatches with the *nagAc*-like primers and probe.

The area chosen for testing the *nagAc*-like gene real-time PCR assay is located in an EPA Superfund site in Tennessee. Chattanooga Creek has historically been subjected to gross pollution by industrial waste discharges mainly, but not only, from a former coal carbonization facility. The main contaminant compounds found in the stream are PAHs, chlorinated solvents, organic solvents, pesticides, polychlorinated biphenyls, metals, and phenols (www.epa.gov/region4/waste/npl/npltn/tennprtn.htm). Naphthalene concentrations in Chattanooga Creek surficial sediment samples retrieved in January 2002 varied between 0.18 and 106 mg/kg dw. The lowest naphthalene concentration was found in sample 1, the furthestmost sampling point upstream of the main source of contamination (zone I; Fig. 2B). The highest naphthalene concentration was found in sample 7, belonging to the nonremediated zone III. This sample also showed the highest phenanthrene, fluoranthene, fluorene, anthracene, pyrene, benzo[*a*]anthracene, chrysene, and benzo[*b*]fluoranthene concentrations of all analyzed samples (11,453, 8,426, 4,651, 2,658, 5,287, 2,144, 1,277, and 1,042 mg/kg dw, respectively) and highest *nagAc*-like gene target concentrations (Fig. 2). PAH concentrations in the remediated section of the creek (zone II) were in general lower than in section III, indicating that the excavation of sediments up to the bedrock reduced the level of contamination present in this Superfund site. However, fresh sediments covering section II still show considerable PAH contamination, and it is therefore of interest to study the intrinsic bioremediation capacity at this site.

A strong positive correlation between naphthalene and *nagAc*-like gene target concentrations was found (Fig. 3A and B). A lower but significant correlation (0.01 level) between phenanthrene and *nagAc*-like target concentrations was also found ($r = 0.74$ and 0.57 for copies per microgram of DNA and \log_{10} -transformed copies per gram dw, respectively; data not shown). These results suggest a strong influence of the levels of these contaminants on population size for organisms carrying *nagAc*-like genes. Such a high correlation was surprising, considering other factors that can affect population size, for instance, toxicity of naphthalene or other contaminants present in the sediment, oxygen and nutrient concentrations, and substrate bioavailability (30). In addition, populations of bacteria carrying *nagAc*-like NDO_α genes should compete for the same growth substrate with other populations of this guild with dif-

ferent aerobic as well as anaerobic naphthalene biodegradation pathways.

Results from Laurie and Lloyd-Jones's work (22) suggest that the *Pseudomonas*-type genotype may not always be ecologically dominant. Quantitative studies using competitive PCR showed that *phnAc* genes similar to the one cloned from *Burkholderia* sp. strain RP007 were present at equivalent or greater concentrations than *nahAc*-like genes in contaminated soils (22). In the same study, neither target was detected in pristine soils, while the addition of naphthalene enriched *phnAc* but not *nahAc* genes (22). In contrast, other studies indicated an effect of naphthalene concentrations on *nah*-type populations. In one of these studies, *nahAc* transcript levels correlated positively with [^{14}C]naphthalene mineralization rates, soil naphthalene concentrations, and *nahAc* gene frequency in soil samples (7). Furthermore, Sanseverino et al. (31) established a threshold level of naphthalene of approximately 100 mg/kg of soil, below which the *nahAc*-like naphthalene-degrading population is relatively inactive.

As found in this study with *nagAc*-like populations, higher concentrations of the *nahAc* gene were found in PAH-contaminated freshwater sediments than in sediments with only trace amounts of these compounds (19). In addition, transient responses of indigenous microorganisms to hydrocarbon exposure were detected in groundwater at a field scale study, where an enrichment of *nah*-like genes was followed by a population crash (33). In bioslurry treatments of dredged harbor sediments contaminated with PAHs the microbial community structure has been correlated with the presence of catabolic genes (such as the naphthalene dioxygenase gene) and PAH loss (29). Recently, a primer set for the detection and quantification of a larger group of NDO_α sequences belonging to both *nah*- and *nag*-type organisms using SYBR green I has been published (1), and its application to environmental samples can provide important information of the distribution of these gene types in the future.

The quantification of distinct naphthalene dioxygenase genes in environmental samples is starting to provide important information concerning the ecological significance and the distribution of different naphthalene-degrading populations. The quantification of *nagAc*-like genes in sediment samples of Chattanooga Creek suggests an important role of bacteria carrying these genes in naphthalene biodegradation. Efforts are under way to quantify other important naphthalene-degrading populations and also to implement the use of the technique reverse transcription real-time PCR to allow the analysis of the expression of different types of NDO_α genes in environmental samples.

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