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Natural Horizontal Transfer of a Naphthalene Dioxygenase Gene between Bacteria Native to a Coal Tar-Contaminated Field Site

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Horizontal transfer of genes responsible for pollutant biodegradation may play a key role in the evolution of bacterial populations and the adaptation of microbial communities to environmental contaminants. However, field evidence for horizontal gene transfer between microorganisms has traditionally been very difficult to obtain. In this study, the sequences of the 16S rRNA and naphthalene dioxygenase iron-sulfur protein (*nahAc*) genes of nine naphthalene-degrading bacteria isolated from a coal tar waste-contaminated site, as well as a naphthalene-degrading bacterium from a contaminated site in Washington state and two archetypal naphthalene-degrading strains, were compared. Seven strains from the study site had a single *nahAc* allele, whereas the 16S rRNA gene sequences of the strains differed by as much as 7.9%. No *nahAc* alleles from the site were identical to those of the archetypal strains, although the predominant allele was closely related to that of *Pseudomonas putida* NCIB 9816-4, isolated in the British Isles. However, one site-derived *nahAc* allele was identical to that of the Washington state strain. Lack of phylogenetic congruence of the *nahAc* and 16S rRNA genes indicates that relatively recent in situ horizontal transfer of the *nahAc* gene has occurred, possibly as a direct or indirect consequence of pollutant contamination. Alkaline lysis plasmid preparations and pulsed-field gel electrophoresis have revealed the presence of plasmids ranging in size from 70 to 88 kb in all site isolates. Southern hybridizations with a 407-bp *nahAc* probe have suggested that the *nahAc* gene is plasmid borne in all the site isolates but one, a strain isolated from subsurface sediment 400 m upstream from the source of the other site isolates. In this strain and in the naphthalene-degrading strain from Washington state, *nahAc* appears to be chromosomally located. In addition, one site isolate may carry *nahAc* on both chromosome and plasmid. Within the group of bacteria with identical *nahAc* sequences, the Southern hybridizations showed that the gene was distributed between plasmids of different sizes and a chromosome. This suggests that plasmid modification after transfer may have been effected by transposons. Horizontal transfer of catabolic genes may play a significant role in the acclimation of microbial communities to pollutants.

Horizontal gene transfer—the transmission of DNA fragments between different organismal lineages—has been studied and exploited by prokaryotic geneticists and molecular biologists for decades in laboratory strains of bacteria (particularly *Escherichia coli*). It may seem logical to assume that horizontal gene transfer is commonplace in naturally occurring microbial communities (37, 39, 40) and that genetic exchange plays a significant role in the evolution and ecological impact of terrestrial and aquatic microorganisms (1). Yet evidence for horizontal gene transfer between microorganisms in their native habitats has traditionally been very difficult to obtain because of both uncontrolled aspects of field investigations and uncertainties about the genetics of naturally occurring microbial populations (4, 37, 40, 67). Many studies of horizontal gene transfer have utilized laboratory-based or other model systems to demonstrate that the potential for genetic exchange exists in nature. Thus, with defined donor and/or recipient microorganisms, exchange has been demonstrated in water (e.g., see references 6 and 51) and soil or sediment (18, 31–33, 47, 55, 61, 66) samples. More realistic field-incubated model systems involving defined donors and recipients have also been devised (38, 50, 73). These model systems provide investigators with interpretive control over their experiments, but experimental artifacts often compromise the environmental relevance of the resultant data (39, 41). When extensive genetic knowledge of either the microorganisms of interest (e.g., *E. coli* [20]) or of a phenotypic trait (e.g., antibiotic resistance [1, 8, 9, 48]) is available, convincing retrospective comparative phylogenetic arguments for gene transfer between naturally occurring microbial populations in their habitats (in situ) can be successfully assembled by detecting incongruent, independent patterns of gene distribution (10, 20, 28, 40, 49, 62, 65). Such studies often infer horizontal transfer by showing that a highly conserved gene is shared by a group of taxonomically diverse hosts.

Gene transfer may play a key role in the acclimation of microbial communities to environmental pollutants in field sites, a phenomenon in which an increase in the rate of biodegradation of a contaminant is observed after exposure of the community to pollutant compounds (35). Although comparative phylogenetic analysis of biodegradation genes and plasmids has provided circumstantial evidence for the transfer and rearrangements of catabolic genes in situ between naturally occurring microorganisms, the retrospective nature of such genetic rearrangement studies (3, 22, 27, 52, 70, 74) has generally failed to address when and where the arrangements have occurred. Knowledge of in situ horizontal transfer of biodegradation genes at known times and locations (i.e., in specific field sites) has been difficult to document. Horizontal transfer of biodegradation genes was suggested when a transposon contributing to 3-chlorobenzoate catabolism was recovered from

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many diverse isolates in ground and bioreactor waters at a chemical landfill (76). Also, a recent study used comparative phylogenetic analysis of catabolic and 16S rRNA genes to demonstrate that two 3-chlorobenzoate catabolic genes had recently transferred between a gamma subclass and a beta subclass proteobacterium (77). However, those authors (77) were not able to determine whether this transfer had occurred in situ or during enrichment. Furthermore, Matheson et al. (46) recently provided evidence for the natural horizontal transfer of the 2,4-dichlorophenoxyacetic acid gene (*tfdA*) based on the occurrence of a highly similar *tfdA* allele in two phylogenetically divergent *Burkholderia* strains isolated from two different sites. In the present study, we compare nucleotide sequences from the 16S rRNA and naphthalene dioxygenase iron-sulfur protein (*nahAc*) genes of naphthalene-degrading bacteria isolated primarily from a coal tar waste-contaminated field site (42–45). Surprisingly, an identical *nahAc* allele was found in a group of phylogenetically distinct strains, indicating that relatively recent in situ transfer of this gene has occurred. Evidence is also presented for the occurrence of identical *nahAc* alleles on the plasmids of some host strains and on the chromosomes of others, suggesting that plasmid integration and/or transposons may have influenced the observed distribution of *nahAc*.

MATERIALS AND METHODS

Study site and bacterial strains. Bacteria were isolated from samples obtained from a coal tar-contaminated area located in South Glens Falls, N.Y. This site has been studied intensively, and details of site history, sample characteristics, and aseptic sampling procedures have been published previously (42–45).

The archetypical strains *Pseudomonas putida* G7 (gift from G. Sayler, University of Tennessee) and *P. putida* NCIB 9816-4 (gift from G. Zylstra, Rutgers University) were isolated previously from soils in Berkeley, Calif. (50a, 60), and Bangor, Wales, United Kingdom (13, 73a), respectively. These bacteria have served as model systems for elucidation of the biochemistry and genetics of naphthalene metabolism (57). *Pseudomonas fluorescens* N1 and *P. fluorescens* Nd9 were isolated in our laboratory from naphthalene-containing media inoculated with surface soil from the Pacific Northwest Laboratories (Richland, Wash.) and with subsurface sediment obtained from the contaminated source area (25) of the study site, respectively. Strains designated Cg were isolated from sediment obtained from the contaminated seep region of the site (42–45). Each aseptically gathered sediment sample was immediately placed on ice and maintained at 4°C. Dilution plates were prepared less than 24 h after the samples were gathered from the field site on mineral salts medium (MSB [60]) supplemented with naphthalene vapor as the sole carbon source. Colonies greater than 1 mm in diameter were selected randomly from replicate dilution plates having between 20 and 200 well-defined colonies. These were purified on MSB-naphthalene vapor plates and checked for purity by plating on a complex medium (5% PTYG [5]) to reveal colonies of associated non-naphthalene-degrading heterotrophs. Presumptive growth on naphthalene was confirmed by comparing growth on MSB with and without naphthalene vapor. Isolates were identified and characterized by standard microbiological procedures, enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprinting (24), modified from de Bruijn (9a), the Biolog identification system (Biolog, Inc., Hayward, Calif.), and the API-NFT kit (API Analytab Products, Plainview, N.Y.).

^{14}C-labeled naphthalene mineralization assay. Metabolism of naphthalene by each of the isolates except *P. putida* NCIB 9816-4 was confirmed by assaying [^{14}C]naphthalene mineralization (42). Cells grown on MSB agar medium plus naphthalene or 0.1% salicylate were suspended in 2 ml of double-strength MSB until turbid (optical density at 600 nm of >0.25). Each cell suspension was aseptically transferred to 25-ml screw-cap vials (Pierce, Rockford, Ill.) containing a glass marble. Unlabeled sterile saturated naphthalene solution (approximately 30 mg/liter; 2 ml) and radiolabeled naphthalene (2×10^4 dpm of [1-^{14}C]naphthalene; 10.1 mCi/mM; >98% radiopurity; Sigma Radiochemicals, St. Louis, Mo.; prepared from crystals allowed to dissolve in sterile deionized water) were added to the vials. These were sealed with Teflon-backed silicone septa after addition of 0.4 ml of 0.5 N NaOH as a CO_2 trapping solution to an inner shell vial (15 by 45 mm; Kimble, Vineland, N.J.) resting on top of the marble. The assay vials were incubated with agitation (135 rpm) in the dark at 22°C. The NaOH was removed after 2 days, and the ^{14}C was trapped as CO_2 counted in a scintillation counter (model 5000CE; Beckman Instruments, Inc., Fullerton, Calif.). Uninoculated controls produced no significant $^{14}CO_2$.

DNA sequencing of *nahAc* and 16S rRNA genes. *nahAc* DNA was prepared for sequencing by growing strains at room temperature on MSB-naphthalene plates for 3 to 7 days. A small number of cells from a single colony was transferred into 10 μl of deionized H_2O by using a sterile platinum inoculating needle, and the cells were subsequently heated in an MJ Research (Watertown, Miss.) Minicycler at 95°C for 5 min to aid in cell lysis. PCR amplification was carried out in a 100-μl volume with the Minicycler and reagents as previously described (25), but with primer concentrations of 0.15 μM and 1.0 U of *Taq* polymerase (Gibco-BRL, Gaithersburg, Md., and Promega Corp., Madison, Wis.) and primers *nahAc*-F (5′-CGCGGAACTGGCTTTTTCTCACTCA nucleotides [nt] 152 to 176 of the *nahA* sequence of Simon et al. [57]) and *nahAc*-R (5′-ACCGAAACCAAGGTTTGA AAGCAGA [nt 1221 to 1197]). In addition, hot-start conditions were employed, with deoxynucleoside triphosphates added after the tubes were heated to 80°C. Tubes were cycled 30 times at 92°C for 40 s and at 65°C for 1 min and 30 s and once at 75°C for 5 min. Two 100-μl reaction mixtures were pooled and concentrated, agarose gel purified by electrophoresis in molecular-grade preparative agarose (Bio-Rad Laboratories, Hercules, Calif.), and extracted from the agarose with a Spin-Bind DNA extraction cartridge (FMC BioProducts, Rockland, Maine) according to the manufacturer's specifications. Extracted DNA was diluted to 25 ng/μl and sequenced with internal nested primers *nahAc*-1 (25) and *nahAc*-5 (5′-GGAGGTCATTTGCAAGCCTG [nt 783 to 764]) and the *Taq* DyeDeoxy terminator cycle sequencing procedure on an Applied Biosystems (Foster City, Calif.) 373 DNA sequencer at the Cornell DNA Sequencing Facility. Both DNA strands were sequenced, and data from a consistently readable 373-bp portion of the 440-bp amplicon are reported here.

16S DNA was prepared for sequencing by PCR amplification from colonies and primers, reagents, and conditions as previously described (25), with the following exceptions: (i) primer concentration was 0.3 μM, (ii) 2.0 U of *Taq* polymerase per 100 μl of reaction mixture was used, and (iii) hot-start conditions were employed as described above. DNA was pooled, concentrated, purified, and extracted as described above. A 305-bp fragment was sequenced as described above with primer 16Sp-5 (25) and nested primer 16Sp-339R (5′-TGCTGCCTCCCGTAG GAG; [nt 339 to 322 described by Brosius et al. [7]). Both DNA strands were sequenced; however, due to difficulties encountered sequencing from a nonnested primer (16Sp-5), the plus strand was sequenced twice in all cases for verification. Sequence data from both the 16S and *nahAc* genes were compiled with version 1.0.3 of the SeqEd DNA sequence analysis program (Applied Biosystems).

DNA sequence alignment. *nahAc* and 16S rRNA gene sequences were aligned manually. No complications were encountered in aligning the *nahAc* sequences, because there was general conservation of sequence and because differences consisted entirely of single nucleotide substitutions. Alignment of one portion of the 16S sequence—helix 6 (34), comprising positions 61 to 106—was more complex due to its evidently rapid rate of substitution relative to the rest of the molecule. This variable region was, therefore, aligned by using a group of 18 aligned sequences retrieved from the Ribosomal Database Project (RDP) database (34) and the predicted *E. coli* 16S rRNA secondary structure described by Gutell et al. (19). These complete alignments were used for the simple similarity comparisons described below. Phylogeny reconstruction, on the other hand, depends on the assumption of strict positional homology (64), i.e., nucleotides at a given position in all compared sequences should be descended from a common ancestral nucleotide at that position. Positions of ambiguous alignment within helix 6 were therefore excluded from phylogenetic analyses.

Sequence comparison and phylogenetic analysis. Simple percent dissimilarities were used for pairwise comparisons of sequences and also as a measure of evolutionary distance for distance matrix-based phylogenetic analyses. Calculations of percent dissimilarity (d) were carried out by the formula $d = (1 - S) \cdot 100$, where S is the fractional sequence similarity, or number of aligned nucleotide positions containing identical residues divided by the total number of nucleotide positions (64). Percent dissimilarities, which do not correct for superimposed substitutions, are particularly appropriate when applied to closely related protein-coding sequences such as *nahAc* (64). However, evolutionary distances corrected for superimposed substitutions by the Jukes and Cantor (26), Kimura (30), and maximum likelihood (14) models were also determined for both the 16S rRNA and the *nahAc* genes by using the program DNADIST in PHYLIP, version 3.5c, for the Power Macintosh (15). These distances were compared to the uncorrected, dissimilarity-based distances and in all cases were found to be nearly identical.

Evolutionary distances, both uncorrected and corrected, were converted to dendrograms by the least-squares distance algorithm of Fitch and Margoliash (17) employed by the FITCH program in PHYLIP. Species input order was randomized, with 20 input orders examined, and the global branch-swapping option was employed. Output from FITCH was converted into unrooted dendrograms with the PHYLIP program DRAWTREE. The branching order of dendrograms derived from least-squares analysis was identical to that of dendrograms generated by maximum parsimony analysis (PAUP version 3.0s [63]) by the branch-and-bound algorithm.

Plasmid characterizations. Large plasmids were detected by the method of Anderson and McKay (2), with slight modifications. Sizing of plasmids was achieved by first linearizing the plasmids with a controlled S1 nuclease digestion and then subjecting the plasmids to pulsed-field gel electrophoresis (PFGE) (29). For this, a Bio-Rad clamped homogeneous electric field (CHEF) PFGE apparatus, model CHEF-DR II, was used. Gels (1.5% agarose in 0.5× Tris-borate-EDTA) were run at 200 V at 14°C. The pulse duration increased from 6 to 12 s during a 24-h run. A Mid Range I PFG (New England Biolabs, Beverly, Mass.)

marker served as the size standard. Complete separation of plasmid from chromosomal DNA was achieved via cell lysis in agarose plugs and application of plugs to CHEF PFGE (16, 29). Gels (1.0% agarose in 0.5× Tris-borate-EDTA) were run at 200 V at 15°C. Pulse duration increased from 15 to 50 s during a 20-h run. A lambda ladder (Bio-Rad) served as the size standard.

Southern hybridizations. Southern hybridizations were carried out with Boehringer Mannheim's Genius system according to the manufacturer's instructions. A *nahAc* hybridization probe for the Cg1 allele was generated via PCR from Cg1 cells with digoxigenin-labeled dUTP essentially as previously described (25). The 407-bp probe encompassed bp 326 to 733 of the *nahAc* gene and contained the 373-bp region previously sequenced in Cg1 (25, 57). Hybridizations were performed with a mismatch stringency of approximately 10 to 15% (54).

RESULTS AND DISCUSSION

Sequence identity among *nahAc* genes of site bacteria. A group of 21 gram-negative naphthalene-degrading bacteria were isolated from the contaminated seep area of our study site. By PCR amplification, these were found to contain homologs to the *P. putida* G7 naphthalene dioxygenase gene *nahAc* (data not shown). A single gram-positive naphthalene-degrading bacterium was also isolated, and further characterization of this bacterium is in progress. Successful amplification implied that there was sequence conservation among these *nahAc* homologs, at least within the PCR primer binding regions. Because of the study site's 35-year history of contamination with naphthalene and other soluble coal tar constituents and its relatively large populations of naphthalene-degrading bacteria (45), we hypothesized that conditions may have been favorable for horizontal transfer and selection of genes encoding naphthalene catabolic enzymes. If horizontal transfer had indeed occurred, transferred naphthalene catabolic genes should be closely related. To determine precisely how similar the seep isolates' *nahAc* genes were, we sequenced a 373-bp portion of the *nahAc* gene from eight contaminated seep-derived strains (identified, whenever possible, by the Biolog system as *P. putida* Cg1, *P. fluorescens* Cg2, gram-negative rod Cg4, *P. fluorescens* Cg5, *Pseudomonas* sp. strain Cg7, *Pseudomonas mendocina* Cg11, gram-negative rod Cg15, and gram-negative rod Cg21). The eight strains from the seep were chosen because of their distinctive ERIC REP-PCR fingerprints (24, 72) and their phenotypic dissimilarity from one another as judged by cluster analysis of their Biolog carbon source utilization profiles (data not shown). For comparative purposes, the *nahAc* genes from *P. fluorescens* Nd9 and *P. fluorescens* N1 were also sequenced. These sequences were compared to one another and to the published sequences of the two archetypical strains (*P. putida* G7 and *P. putida* NCIB 9816-4).

Surprisingly, complete conservation of the nucleotide sequence was found in the *nahAc* fragments from a group of bacteria that included six of the eight seep isolates and strain Nd9 (Table 1). The *nahAc* allele of these seven isolates, which is referred to hereafter as the Cg1 allele, differed from the Cg2 allele by only a single base, a thymine substituted for an adenine at position 328 (Table 1). Moreover, the two closely related Cg1 and Cg2 alleles differed substantially from the archetypical *nahAc* allele of *P. putida* G7 (Table 1). The other archetypical *nahAc* allele, that of *P. putida* NCIB 9816-4, differed from the *P. putida* G7 allele in 17 of the 373 positions (4.6% dissimilarity) but was nearly identical to the Cg2 allele (a single-base-pair difference at position 1) (Table 1). The only site-derived *nahAc* allele which differed substantially from that of the other isolates came from isolate Cg7; its sequence was identical to that of strain N1 (Table 1). The deduced protein sequence for the Cg1, Cg2, and NCIB 9816-4 *nahAc* alleles differed from that of *P. putida* G7 in 5 of 124 amino acids, and

TABLE 1. Comparison of nucleotide sequence differences in partial *nahAc* alleles from naphthalene-mineralizing bacterial strains

Allele group[a]	Bacterial strain	Geographic origin[b]	Nucleotide position and corresponding base substitutions[c]																													
			1	9	30	39	60	96	111	120	132	145	162	183	186	189	192	198	204	222	234	266	285	300	328	330	333	346	363	366	368	372
PpG7	PpG7	CAB	T	T	C	G	C	G	C	T	T	C	G	A	G	C	C	A	A	C	C	C	A	G	T	G	A	G	C	A	T	G
Cg1	Cg1	NYS	C	•	T	T	T	•	•	C	C	•	•	G	A	T	G	G	C	•	•	•	•	•	A	•	G	T	T	G	C	A
	Cg4	NYS	C	•	T	T	T	•	•	C	C	•	•	G	A	T	G	G	C	•	•	•	•	•	A	•	G	T	T	G	C	A
	Cg5	NYS	C	•	T	T	T	•	•	C	C	•	•	G	A	T	G	G	C	•	•	•	•	•	A	•	G	T	T	G	C	A
	Cg11	NYS	C	•	T	T	T	•	•	C	C	•	•	G	A	T	G	G	C	•	•	•	•	•	A	•	G	T	T	G	C	A
	Cg15	NYS	C	•	T	T	T	•	•	C	C	•	•	G	A	T	G	G	C	•	•	•	•	•	A	•	G	T	T	G	C	A
	Cg21	NYS	C	•	T	T	T	•	•	C	C	•	•	G	A	T	G	G	C	•	•	•	•	•	A	•	G	T	T	G	C	A
	Nd9	NYU	C	•	T	T	T	•	•	C	C	•	•	G	A	T	G	G	C	•	•	•	•	•	A	•	G	T	T	G	C	A
Cg2	Cg2	NYS	C	•	T	T	T	•	•	C	C	•	•	G	A	T	G	G	C	•	•	•	•	•	•	•	G	T	T	G	C	A
NCIB 9816	NCIB 9816	BAW	•	•	T	T	T	•	•	C	C	•	•	G	A	T	G	G	C	•	•	•	•	•	•	•	G	T	T	G	C	A
N1	N1	WAR	C	C	•	•	•	A	G	•	•	T	C	G	A	•	•	•	•	A	T	T	T	C	A	A	C	T	•	•	•	•
	Cg7	NYS	C	C	•	•	•	A	G	•	•	T	C	G	A	•	•	•	•	A	T	T	T	C	A	A	C	T	•	•	•	•

[a] Allele group designations are derived from the first bacterial strain found to possess the respective gene sequence.

[b] Isolated originally from soil or sediment in CAB (Berkeley, Calif.), NYS (the contaminated seep area of our study site South Glens Falls, N.Y.), NYU (upgradient contaminated source area of our study site), BAW (Bangor, Wales), or WAR (Richland, Wash.).

[c] The sequence of *nahAc* from *P. putida* G7 is the reference sequence. Positions at which departures from the reference sequence were found are designated by numbers (e.g., 30 is the 30th nt). Numbering is relative to the 373-bp region of *nahAc* sequenced in this study. •, a nucleotide which is identical to that of *P. putida* G7 at the indicated position.

TABLE 2. Comparison of nucleotide sequence differences in partial 16S rRNA alleles from naphthalene-mineralizing bacterial strains[a]

Allele group	Strain	Geographic origin	Nucleotide position and corresponding base substitutions[b]																												
			48	49	51	53	54	55	56	57	58	63	64	65	66	67	68	69	70	71	72	73	74	96	124	129	130	131	132	137	139	252
PpG7	PpG7	CAB	A	T	—	G	A	A	G	A	G	C	T	C	T	T	C	G	A	T	—	T	C	G	C	T	T	T	C	G	A	G
Cg1	Cg1	NYS	•	•	•	A	G	•	•	•	•	•	•	•	•	C	T	•	•	•	•	•	•	•	•	•	C	•	•	•	G	•
	Cg5	NYS	•	•	•	A	G	n	•	•	•	•	•	•	•	C	T	•	•	•	•	•	•	•	•	•	C	•	•	•	G	•
CG4	Cg4	NYS	C	A	C	C	G	G	•	T	A	T	A	•	C	•	G	•	T	G	G	C	G	A	T	•	•	C	G	C	G	T
	Cg11	NYS	C	A	C	C	G	G	•	T	A	T	A	•	C	•	G	•	T	G	G	C	G	A	T	•	•	C	G	C	G	T
	Cg15	NYS	C	A	C	C	G	G	•	T	A	T	A	•	C	•	G	•	T	G	G	C	G	A	T	•	•	C	G	C	G	T
CG21	Cg21	NYS	T	A	•	•	•	G	A	•	•	•	•	T	C	•	•	T	T	G	•	A	G	•	T	•	•	C	G	C	G	•
	Nd9	NYU	T	A	•	•	•	G	A	•	•	•	•	T	C	•	•	T	T	G	•	A	G	•	T	•	•	C	G	C	G	•
Cg2	Cg2	NYS	T	A	•	•	•	G	A	•	•	•	•	T	C	•	•	T	T	G	•	A	G	•	T	C	•	C	G	C	G	•
NCIB 9816	NCIB 9816	BAW	•	•	•	A	G	•	•	•	•	•	•	•	•	C	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
N1	N1	WAR	•	•	•	A	G	G	•	•	•	•	•	•	C	C	T	•	•	•	•	•	•	•	•	•	C	•	•	•	G	•
CG7	Cg7	NYS	T	A	•	•	•	G	A	•	•	•	•	T	C	•	•	T	T	•	•	•	•	•	T	•	•	C	G	C	•	•

[a] Order of strains, designation of allele groups, and geographic origins are identical to those described in footnotes *a* and *b* of Table 1.

[b] The 16S rRNA allele of *P. putida* G7 is the reference sequence. Positions at which departures from the reference sequence were found are designated by numbers (e.g., 48 is the 48th nt). Numbering is relative to the 305-bp region of the 16S rRNA gene sequenced in this study. Nucleotide number 1 corresponds to nt 23 on the *E. coli* 16S rRNA gene sequence described by Brosius et al. (7). •, a nucleotide which is identical to that of *P. putida* G7 at the indicated position; n, a nucleotide position unresolved during sequencing; —, a gap inserted to align sequences.

the deduced amino acid sequence of the N1 allele differed from that in *P. putida* G7 by 4 amino acids (not shown).

Comparative divergence of 16S rRNA and *nahAc* genes. If lateral spread of the *nah* gene has occurred between these naphthalene-degrading isolates, one would expect the lineage of the transferred gene to be phylogenetically less diverged (i.e., more closely related) than that of the host cells. (This assumes, as expected, that the mutation rate of the 16S rRNA gene is lower than that of *nahAc*.) Moreover, the phylogeny of the cells and transferred gene would not be expected to be congruent. Conversely, lack of horizontal gene transfer would be indicated if the lineage of the biodegradation gene is diverged to an extent equal to that of the host cells or is more phylogenetically diverged than that of the host cells. Therefore, we examined 16S rRNA gene sequences from the 12 strains listed in Table 1 in order to compare the relative divergence of this gene to that of *nahAc*. The 16S rRNA gene was selected based on the following characteristics of the gene as outlined by Woese (75): (i) there is no evidence of horizontal transfer of the 16S rRNA gene or portions thereof (although some recent reports suggest otherwise [12, 58]); (ii) it is highly conserved, with significant sequence identity retained even across domain and kingdom boundaries; and (iii) its phylogeny is considered representative of the phylogeny of the organism possessing it. Thus, sequence divergence in this highly conserved chromosomally located gene, coupled with sequence identity in the *nahAc* gene, would constitute strong evidence for relatively recent in situ horizontal transfer of *nahAc* between bacteria indigenous to the field study site.

With the 16S rRNA gene sequence as a criterion, the 12 strains listed in Table 2 formed 8 individual or group alleles, rather than the 5 alleles listed in Table 1. The increase in allelic diversity occurred primarily within the Cg1 *nahAc* group. The 16S rRNA sequences of the seven members of this group (all of which were field site-derived strains) fell into three distinctive allelic groups (Table 2). Within each of the three 16S rRNA groups (designated Cg1, Cg4, and Cg21 in Table 2), there was complete sequence identity; yet the three 16S rRNA alleles displayed as much as 7.9% dissimilarity from one another. Because there is generally little or no 16S rRNA gene sequence variation within species of bacteria (75), it is evident that the different 16S allelic groups are not closely related and may represent different species or even genera. This indicates that bacteria in 16S rRNA allele groups Cg1, Cg4, and Cg21 were evolutionarily far more divergent than were the *nahAc* genes they were carrying. The most likely explanation for these contrasting gene phylogenies is in situ horizontal gene transfer between naphthalene-degrading bacteria prior to their isolation from the contaminated field site.

An alternative hypothesis for explaining the uniformity observed in the *nahAc* gene in these field isolates is that natural selection acted to maintain an ancestral *nahAc* DNA sequence present in the phylogenetically diverse clonal lineages represented by the populations which we sampled. One can envision selective pressure against alternative amino acids due to possible detrimental effects on the function of the naphthalene dioxygenase enzyme. However, the selective constraints on nucleotide substitution at synonymous (silent) sites are generally weak to nonexistent, although codon bias can exert some influence on the evolution of synonymous sites (23). Synonymous sites in protein-coding genes such as *nahAc* are generally considered to be relatively neutral with regard to selection and, therefore, should easily accumulate substitutions even with selective pressure acting to maintain an adapted protein sequence. Because no synonymous site substitution was observed among the bacteria possessing the Cg1 *nahAc* allele, selective maintenance of an ancestral protein from among these bacteria is very unlikely.

Another hypothesis alternative to in situ gene transfer is that genetic exchange occurred between bacteria native to our sediment samples after their removal from the site (77). However, we feel that this possibility is unlikely because enrichment conditions were intentionally avoided by keeping the samples on ice from the moment they were obtained and by completing soil dilution and plating in less than 24 h from the time of sampling. Plate or dilution matings were also unlikely, because dilutions were completed rapidly and only individual, dispersed colonies were selected from dilution plates. In addition, as is shown later in this article, identical *nahAc* alleles were not found on identical plasmids, suggesting that extremely recent conjugation events had probably not occurred.

Phylogenies of 16S rRNA and *nahAc* genes. Comparison of phylogenetic trees for the *nahAc* and 16S rRNA genes (Fig. 1A and B) graphically illustrates that the Cg1 *nahAc* allele was distributed throughout bacteria in the three major 16S rRNA branches of the strains examined. Thus, the phylogenies of the

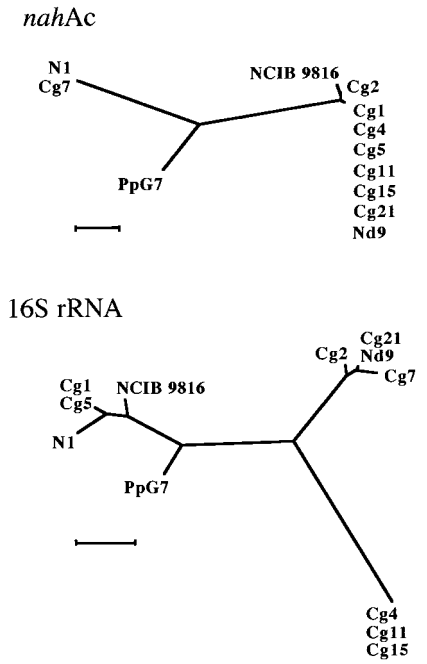

FIG. 1. Phylogenetic trees of *nahAc* (A) and 16S rRNA (B) gene sequences (Tables 1 and 2) for bacteria examined in this study. Phylogenetic analysis was done with the least-squares distance algorithm described by Fitch and Margoliash (17) and was based on a matrix of percent dissimilarity uncorrected for superimposed substitutions (see Materials and Methods). Horizontal bars represent a distance equivalent to approximately 1% dissimilarity.

nahAc and 16S rRNA genes are not congruent. For the seven strains that share our contaminated field site as their geographic origin, past in situ horizontal gene transfer is evident. The data also suggest that transfer of a naphthalene biodegradation gene, possibly coupled with transport of cells, has occurred not only within our study site but also over larger geographic distances. The Cg1 *nahAc* allele was shared by six strains found in the seep area and one strain (Nd9) linked to the seep via 400 m of flowing groundwater. Moreover, the Cg1 *nahAc* allele is phylogenetically much more closely related to that of the type strain *P. putida* NCIB 9816-4 (originally isolated from soil collected in Bangor, Wales) than to the allele in *P. putida* G7 that was isolated from soil in central California. Equally striking is the lack of phylogenetic congruence found for strains N1 and Cg7: an identical *nahAc* allele (which differs substantially from the Cg1 allele) was distributed between divergent bacterial lineages. However, strains N1 and Cg7 originated in geographically disparate locations (Washington and New York states, respectively), a distance of more than 3,600 km. Such a combination of 16S rRNA and *nahAc* genes is difficult to interpret without invoking long-distance dispersal mechanisms for genes and/or microorganisms. A similar result was seen by Matheson et al. (46) in which a *tfdA* allele in a Michigan isolate showed 99.5% similarity to a *tfdA* allele in a phylogenetically distinct bacterium from Oregon.

Genomic localization of *nahAc* and its implications. Because naphthalene catabolic genes are generally found on self-transmissible plasmids (56), it is reasonable to hypothesize that plasmid transfer, whether by conjugation or plasmid transformation (51), has played a role in *nah* gene exchange. With an alkaline lysis protocol and by standard gel electrophoresis, plasmids comparable in size to the NAH7 plasmid of PpG7 (11) were detected in all nine site-derived naphthalene-degrading bacteria whose partial *nahAc* sequences are shown in Table 1. Large plasmids were also found in four additional gram-negative isolates (designated strains Cg8, Cg9, Cg12, and Cg16) from the seep area of the coal tar waste site (data not shown). To accurately estimate plasmid sizes, PFGE in combination with Southern hybridization was performed with partially purified, S1 nuclease-linearized plasmid preparations from all 13 seep-derived bacteria and with the archetypical strains NCIB 9816-4 and PpG7. The 407-bp *nahAc* probe derived from strain Cg1 hybridized to all *nahAc* homologs under the conditions chosen, and the plasmids were found to vary in size from 70 to 88 kb (Fig. 2). Not shown in Fig. 2 are the plasmid preparations from strains PpG7 and Cg12, which were determined to be 77 and 75 kb, respectively. The large plasmid band in Fig. 2, lane 7, is the supercoiled version of the 76-kb plasmid of strain Cg8, resulting from incomplete linearization with S1 nuclease. Two faint hybridization signals in lane 6 suggest that strain Cg7 may possess two *nahAc*-containing plasmids.

In order to determine the locations (plasmid versus chromosome) of *nahAc* in the isolates, total genomic DNA from each strain was separated by PFGE (Fig. 3A) and was subsequently Southern hybridized with the *nahAc* probe (Fig. 3B). Beside the positive and negative controls shown in lanes 1 to 5, hybridization to the plasmid band was observed only for seep isolates Cg1, Cg5, Cg9, Cg11, Cg12, and Cg21 (Fig. 3B, lanes 6 to 10 and 12). In seep strain Cg7, hybridization to the chromosomal band as well as to both of its plasmids was observed (Fig. 3B, lane 11). Hybridization occurred with only the chromosomal DNA in strains Nd9 (isolated from the coal tar source area of the site) and N1 (isolated from Washington state; Fig. 3B, lanes 13 and 14, respectively). The *nahAc* probe annealed only to the plasmid and not to the chromosome of seep isolates Cg2, Cg4, Cg8, Cg15, and Cg16 (data not shown).

The occurrence of *nahAc* on chromosomes and on different-sized plasmids of naphthalene-degrading bacteria native to our contaminated study site suggests a variety of possible gene

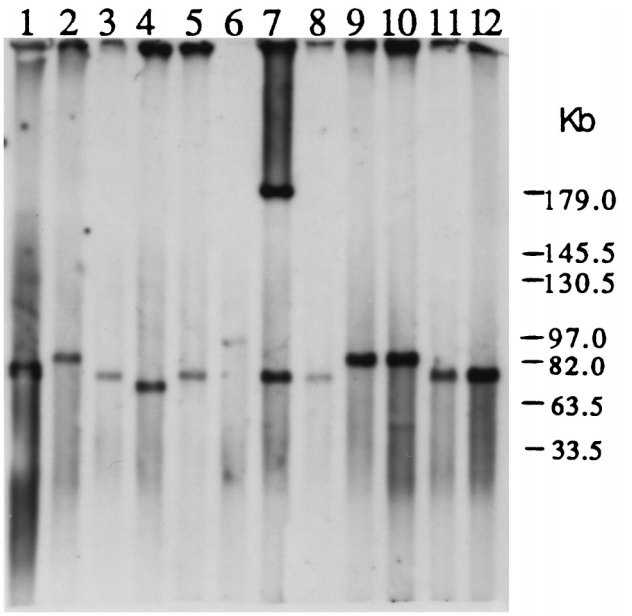

FIG. 2. Southern hybridization to *nahAc* of partially purified plasmids from naphthalene-degrading bacteria separated by CHEF-PFGE. The sizes from molecular markers are indicated in kilobases on the side. Plasmids were from the following organisms: lane 1, NCIB 9816-4; lane 2, Cg1; lane 3, Cg2; lane 4, Cg4; lane 5, Cg5; lane 6, Cg7; lane 7, Cg8; lane 8, Cg9; lane 9, Cg11; lane 10, Cg15; lane 11, Cg16; and lane 12, Cg21.

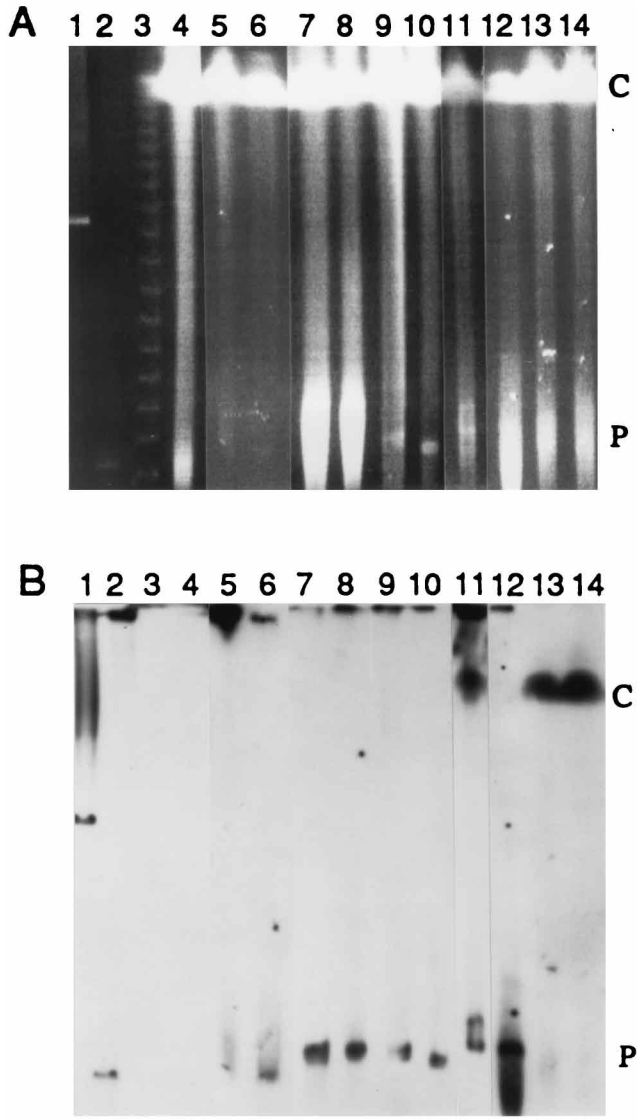

FIG. 3. CHEF-PFGE of total DNA (unless otherwise noted) extracted from naphthalene-degrading bacteria. The DNA was linearized with S1 nuclease unless otherwise noted. C, chromosomal DNA; P, plasmid DNA. Ethidium bromide-stained agarose gel (A) and corresponding Southern hybridization with *nahAc* probe (B) are shown. Lanes: 1, partially purified NAH7 plasmid from *P. putida* G7 (not linearized with S1 nuclease); 2, partially purified NAH7 plasmid from *P. putida* G7; 3, molecular weight size marker (lambda DNA concatamers of 48.5 kb); 4, *P. mendocina* (negative control); 5, strain NCIB 9816-4; 6, Cg1; 7, Cg21; 8, Cg9; 9, Cg11; 10, Cg12; 11, Cg7; 12, Cg5; 13, N1; and 14, Nd9.

transfer mechanisms. A single mobile plasmid may have been transferred between many hosts and subsequently undergone insertions or deletions (events known to occur in NAH plasmids [36]). In addition, transposons, conjugative transposons (53), and/or plasmid integration into the chromosome (27) may have operated in positioning *nahAc* on the chromosome of strain Nd9 (Fig. 3B) and on both plasmids and the chromosome of strain Cg7 (Fig. 3B). The results of ongoing curing and mating experiments (data not shown) indicate that the plasmids are self-transmissible. Work aimed at determining the extent of similarity among these plasmids by restriction fragment length polymorphism analysis, incompatibility grouping, and other molecular characterization procedures is also in progress.

The evidence presented here suggests that horizontal transfer of biodegradation genes may play a role in the adaptation of bacterial populations to organic contaminant compounds. Such compounds may provide selective pressure for genetic adaptation in field sites, because in many habitats carbon is the limiting factor for growth and metabolism of heterotrophic microorganisms (4). Moreover, increased plasmid transfer in nutrient-enriched environmental samples has previously been observed (32, 51, 61). Thus, if carbon limitation prevailed in the study site before its contamination with coal tar waste approximately 35 years ago, then the introduction of a new, exploitable resource (naphthalene and other coal tar-derived carbon sources) may have created conditions favorable for gene selection, transfer, and proliferation. The possible metabolic advantage conferred by the particular *nahAc* allele that predominates in the field site isolates examined here has yet to be determined. Catabolic genes for toluene (69), chlorobenzene (71), chlorobenzoate (76), 2,4,5-trichlorophenoxyacetic acid (21), and biphenyl (59) are associated with transposable elements. Particularly germane to the present study is that the *nah* catabolic genes of plasmid NAH7 of *P. putida* G7 (11) have been found on a defective class II transposon (68). Thus, intracellular rearrangements of catabolic genes (from plasmid to chromosome, chromosome to plasmid, or plasmid to plasmid) may contribute significantly to the patterns of gene distribution found at our study site and elsewhere. We recognize that this study has relied upon cultivation-based procedures; therefore, the results may provide only a glimpse of the full range of genetic exchange processes operating in the study site. In fact, when *nahAc* genes were amplified directly from soil-derived DNA, polymorphisms unlike those reported here were found (25). Understanding the extent, frequency, and mechanisms of gene transfer between microorganisms in contaminated field sites may advance knowledge of the evolution of natural bacterial populations and also improve strategies for manipulating these populations to enhance microbial detoxification of pollutant compounds in situ.

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