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Plasmids Responsible for Horizontal Transfer of Naphthalene Catabolism Genes between Bacteria at a Coal Tar-Contaminated Site Are Homologous to pDTG1 from *Pseudomonas putida* NCIB 9816-4

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The presence of a highly conserved *nahAc* allele among phylogenetically diverse bacteria carrying naphthalene-catabolic plasmids provided evidence for in situ horizontal gene transfer at a coal tar-contaminated site (J. B. Herrick, K. G. Stuart-Keil, W. C. Ghiorse, and E. L. Madsen, Appl. Environ. Microbiol. 63:2330–2337, 1997). The objective of the present study was to identify and characterize the different-sized naphthalene-catabolic plasmids in order to determine the probable mechanism of horizontal transfer of the *nahAc* gene in situ. Filter matings between naphthalene-degrading bacterial isolates and their cured progeny revealed that the naphthalene-catabolic plasmids were self-transmissible. Limited interstrain transfer was also found. Analysis of the restriction fragment length polymorphism (RFLP) patterns indicated that catabolic plasmids from 12 site-derived isolates were closely related to each other and to the naphthalene-catabolic plasmid (pDTG1) of *Pseudomonas putida* NCIB 9816-4, which was isolated decades ago in Bangor, Wales. The similarity among all site-derived naphthalene-catabolic plasmids and pDTG1 was confirmed by using the entire pDTG1 plasmid as a probe in Southern hybridizations. Two distinct but similar naphthalene-catabolic plasmids were retrieved directly from the microbial community indigenous to the contaminated site in a filter mating by using a cured, rifampin-resistant site-derived isolate as the recipient. RFLP patterns and Southern hybridization showed that both of these newly retrieved plasmids, like the isolate-derived plasmids, were closely related to pDTG1. These data indicate that a pDTG1-like plasmid is the mobile genetic element responsible for transferring naphthalene-catabolic genes among bacteria in situ. The pervasiveness and persistence of this naphthalene-catabolic plasmid suggest that it may have played a role in the adaptation of this microbial community to the coal tar contamination at our study site.

Microbial adaptation to environmental change may be facilitated by transfer and sorting of genetic material between and within naturally occurring populations (35). Horizontal gene transfer (HGT) is a major mechanism for microorganisms to acquire new metabolic traits in new combinations. As such, HGT has important implications in the spread of antibiotic resistance (8, 42), in assessment of the risk of releasing genetically engineered organisms (17, 41, 69), and in the effectiveness of microbially mediated control of environmental contamination (36, 52, 65). The occurrence and impact of HGT in naturally occurring microbial communities have been documented by using a variety of complementary experimental procedures that fall into two general categories: retrospective and mechanistic.

In the retrospective approach, HGT is inferred after the discovery of consistent phylogenetic patterns of particular genes, operons, or plasmids which contrast with those of their hosts. One far-reaching example of the retrospective phylogenetic approach involves the functional and evolutionary relationships among oxygenase enzymes that have been established by examining sequence similarities and operon structures in homologous genes coding for aromatic hydrocarbon metabolism (23, 65, 66, 72). Narrower, often site-specific examination of recently isolated bacteria and their genes has also shown or suggested HGT. In this regard, Fulthorpe et al. (19) found that several genes involved in 2,4-dichlorophenoxyacetic acid (2,4-D) metabolism in 32 bacteria from diverse locations were arranged in mosaics and concluded that recombination had played a major role in the evolution of 2,4-D-catabolic bacteria. In a related study, when Ka et al. (30) examined 2,4-D-degrading bacteria from eight different field plots by using catabolic gene probes, they found that a large group of taxonomically diverse isolates shared the *tfdA*, *tfdB*, *tfdC*, and *tfdD* genes on mobile plasmids. A closer examination of the plasmids from *Alcaligenes paradoxus* 2811P and *Pseudomonas pickettii* 712 isolates revealed that they carried nearly identical 2,4-D-catabolic plasmids, indicating that HGT had occurred (31). HGT was also inferred when Matheson et al. (40) sequenced a chromosomally located *tfdA* gene in a *Burkholderia* sp. isolated in Michigan and found 99.5% sequence similarity to a chromosomal gene in a *Burkholderia* strain isolated in Oregon. Furthermore, Rosselló-Mora et al. (49) examined 11 strains of naphthalene-degrading bacteria from contaminated sites in Spain and, after finding that consistent hybridization patterns for the *nahA*, *nahG*, and *nahH* gene probes were

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TABLE 1. Naphthalene-degrading bacterial strains and plasmids used in this study

Strain abbreviation[a]	Strain identity[b]	Origin	Naphthalene plasmid	Plasmid size (kb)[c]	Reference(s)
PpG7	*Pseudomonas putida* PpG7	Berkeley, Calif.	NAH7	77	12
NCIB9816-4	*Pseudomonas putida* NCIB 9816-4	Bangor, Wales	pDTG1	81	15, 54
Cg1	*Pseudomonas putida* Cg1	Glens Falls, N.Y.	pCg1	88	26
Cg2	*Pseudomonas fluorescens* Cg2	Glens Falls, N.Y.	pCg2	76	26
Cg4	Gram-negative rod Cg4	Glens Falls, N.Y.	pCg4	70	26
Cg5	*Pseudomonas fluorescens* Cg5	Glens Falls, N.Y.	pCg5	78	26
Cg7	*Pseudomonas* strain Cg7	Glens Falls, N.Y.	pCg7a and b	78 and 96	26
Cg8	Gram-negative rod Cg8	Glens Falls, N.Y.	pCg8	76	26
Cg9	*Pseudomonas fluorescens* Cg9	Glens Falls, N.Y.	pCg9	76	26
Cg11	*Pseudomonas mendocina* Cg11	Glens Falls, N.Y.	pCg11	86	26
Cg12	*Pseudomonas* strain Cg12	Glens Falls, N.Y.	pCg12	76	26
Cg15	Gram-negative rod Cg15	Glens Falls, N.Y.	pCg15	86	26
Cg16	*Pseudomonas* strain Cg16	Glens Falls, N.Y.	pCg16	76	26
Cg21	Gram-negative rod Cg21	Glens Falls, N.Y.	pCg21	76	26

[a] Abbreviations referring to plasmid-cured strains have the suffix C added. Corresponding strains that are both cured and resistant to rifampin have the suffix CR added. After successful matings, the corresponding transconjugants were assigned a T as suffix.
[b] All Glens Falls isolates are identified by multiple substrate utilization patterns in the Biolog identification system (26).
[c] Determined by pulsed-field gel electrophoresis.

shared among four different genomovars of *P. stutzeri* hosts, concluded that HGT had occurred.

Mechanistic approaches to HGT have sought evidence for mobilization of particular genes between donor and recipient populations in experiments of controlled duration and location. The major variables in these experimental systems have been the gene(s) of interest (e.g., catabolic genes or those involved in resistance to heavy metals or antibiotics), the donors and recipients (defined or undefined), and the setting (laboratory or field). Permutations of these variables have included the use of defined donor and recipient bacteria added to laboratory-incubated environmental samples (3, 20, 34, 43, 68, 73); unknown donors from environmental samples and defined recipients incubated in the laboratory (59, 63, 64); defined donors and recipients added and retrieved from field sites (3, 39, 43, 71); and defined donors commingled with undefined, naturally occurring recipient microbial communities in laboratory (16, 24, 68) or field (44, 76) experiments.

Herrick et al. (26) recently reported in situ horizontal transfer of a naturally occurring naphthalene-catabolic gene (*nahAc*) between naturally occurring bacteria isolated from a coal tar waste-contaminated field site. Partial sequencing of the *nahAc* gene showed that identical alleles were shared among seven taxonomically diverse hosts (as determined by partial 16S rRNA gene sequences). Furthermore, large (70- to 88-kb) plasmids that hybridized to *nahAc* gene probes were shared by 12 naphthalene-degrading, site-derived isolates. Intracellular gene rearrangements were suggested because the plasmids were of different sizes and because the *nahAc* gene was located on the chromosome or on both the chromosome and plasmid of selected isolates. The present study was designed to further characterize the nature of the mobile genetic element and its mechanism of transfer between the contaminated-site-derived bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Naphthalene-degrading strains isolated from the sediment of the seep region of a coal tar-contaminated site are designated Cg- and have been described previously (26). The naphthalene catabolic plasmids found within these strains are designated pCg-. The archetypal naphthalene degraders *Pseudomonas putida* PpG7 (12) and *P. putida* NCIB 9816-4 (15) were included in the study for comparison. All strains and sizes of plasmids are listed in Table 1. All naphthalene-degrading strains were maintained on mineral salts basal medium (MSB) with naphthalene vapor as the sole carbon source (MSB-N) (60). Rifampin (Sigma) was added to the medium at 300 μg/ml from a filter-sterilized 20-mg/ml stock in methanol as needed. Any traces of methanol that may not have volatilized from the medium did not support detectable growth of sediment bacteria. Cycloheximide (Sigma) was added as needed to the medium at 100 μg/ml from a filter-sterilized 10-mg/ml stock. Stock solutions of cycloheximide were initially prepared in 50% ethanol, but it was determined after an initial screen that the ethanol was serving as a carbon source for sediment bacteria. Thereafter, cycloheximide stock solutions were prepared in water.

Curing. Naphthalene-degrading strains were grown in tubes containing 3 ml of Luria-Bertani (LB) broth (51) and subcultured (1% transfer) into fresh medium every other day. Sampling for cured colonies continued periodically for approximately 9 months, at which time naphthalene-negative colonies from all Cg strains were found. Curing was verified by the absence of large plasmids relative to the presence of the plasmid in the original parent strain.

ERIC REP-PCR fingerprinting. The identities of cured and parent strains were compared by using the following enterobacterial repetitive intergenic consensus (ERIC) sequence PCR primers: ERIC-1R, 5′-ATGTAAGCTCCTGGGGATTCAC-3′; ERIC-2, 5′-AAGTAAGTGACTGGGGTGAGCG-3′ (25, 70). The ERIC repetitive extragenic palindrome (REP)-PCR amplification procedure was as follows. A 10-μl volume of sterile water was added to each PCR tube and inoculated with cells on the tip of a sterile platinum inoculating needle. The cells were lysed by being heated to 95°C for 5 min. PCR cocktail (37.5 μl containing 50 mM KCl, 10 mM Tris [pH 8.8], 1.5 mM $MgCl_2$, 0.1 mg of bovine serum albumin per ml, 0.05% Tween 20, 2.0 μM each primer, and 1 U of *Taq* polymerase) and 2 drops of sterile light mineral oil were added to each tube. The tubes were heated to 80°C (for "hot start"), and 50 μM (final concentration) deoxynucleoside triphosphates were added to each tube. Cycling on a thermal cycler (MJ Research, Watertown, Mass.) was carried out as follows: 95°C for 7 min (1 cycle); 94°C for 1 min, 52°C for 1 min, and 65°C for 6 min (34 cycles); 65°C for 16 min (1 cycle).

Amplification of *nahAc*. Cured strains were also tested for amplification of the *nahAc* gene with the following PCR primers: Cg1 upper, 5′-GCCCCAACGGTGAACTGC-3′ (this study, corresponding to positions 326 to 343 of *nahAc* [56]) and nahAc5, 5′-GGAGGTCATTTGCAAGCCTG-3′ (26). PCR amplification was carried out in a 50-μl volume with the thermal cycler and reagents as previously described (26) but with primer concentrations of 0.8 μM and a cycling regime of 95°C for 5 min (1 cycle); 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min (35 cycles); 72°C for 5 min (1 cycle).

Selection of rifampin-resistant recipient strains. Spontaneous rifampin-resistant cured strains were selected on LB plates containing a rifampin gradient (0 to 100 μg/ml) (13). Colonies able to grow at the highest rifampin concentration were picked and transferred onto LB plates containing successively higher concentrations of rifampin, up to 500 μg/ml. ERIC REP-PCR was performed as above to confirm that the identities of rifampin-resistant cured strains matched the original strains.

Conjugal filter matings. Recipient cells (10^9, grown overnight in LB broth amended with 200 μg of rifampin per ml) were harvested and washed with phosphate-buffered saline (PBS) (51). To the same tube, 10^9 donor cells (grown overnight in LB broth) were added and harvested. The cells were resuspended in 50 μl of PBS and placed on a sterile, 0.22-μm-pore-size, 25-mm-diameter Millipore filter on an LB plate. The plates were incubated overnight, agar side down, in the dark at 22°C. The filters were removed and vortexed in 1 ml of PBS for 30 s

to remove cells. Transconjugants were enumerated on MSB-N plates amended with rifampin. Controls containing donor cells only were enumerated on MSB-N plates, and recipient cells were enumerated on LB plates amended with rifampin. Both donor and recipient controls were also plated onto MSB-N plates amended with rifampin to account for spontaneous rifampin resistance and/or experimental errors.

Plasmid characterization and Southern hybridizations. Plasmids were isolated by a modification of the alkali lysis method of Anderson and McKay (2, 55). Because we had no success in isolating plasmids by other procedures attempted (32, 46), we describe our isolation protocol here. Cultures grown overnight (or until turbid) in 50 ml of LB broth at 23°C were harvested, washed with Tris-EDTA (TE, pH 8.0), and resuspended in 10 ml of 6.7% sucrose–50 mM Tris–20 mM EDTA (pH 8) in a 35-ml Oakridge tube. RNase A (15 μl of a 10-mg/ml solution) and 25 mg of dry lysozyme were added, and the contents of the tubes were gently mixed. After an incubation for 5 min at 37°C, 400 μl of 0.5 M disodium EDTA, 1.25 ml of 0.25 M EDTA-50 mM Tris (pH 8.0), and 1.0 ml of 20% sodium dodecyl sulfate–50 mM Tris–20 mM EDTA (pH 8) were all added, with gentle mixing between additions. The tubes were incubated at 37°C for 10 min (until lysis) and then incubated at 55°C for 30 min with proteinase K (100 μl of a 20-mg/ml solution). Freshly prepared 3 N NaOH (0.8 ml) was added, and the contents of the tubes were mixed gently for 10 min. Tris-HCl (pH 7.0) (1.3 ml of a 2 M solution) was then added, and the contents of the tubes were again mixed gently for 3 min. Sodium acetate (pH 5.2) (0.55 ml of a 3 M solution) and 5 M NaCl (1.65 ml) were added, and the contents of the tubes were mixed gently. A phenol-chloroform extraction was performed, and the aqueous phase was removed with a truncated pipette tip and reextracted again with chloroform to remove residual phenol. An equal volume of isopropanol (approximately 16 ml) was added to the separated aqueous phase, and DNA was precipitated overnight at −20°C. DNA was pelleted by centrifuging at 10,000 × *g* for 30 min. The air-dried pellet was resuspended in 200 μl of sterile water for direct plasmid detection by standard gel electrophoresis (15 μl on a 0.7% agarose gel) or in 1 ml of TE (pH 7.0) for further purification on a Qiagen anion-exchange column. For the latter procedure, 4 ml of QBT buffer (750 mM NaCl, 50 mM morpholinepropanesulfonic acid [MOPS; pH 7.0], 15% ethanol, 0.15% Triton X-100) was added, and the tubes were centrifuged at 12,000 × *g* for 5 min. The supernatant was applied to a Qiagen plasmid midi-tip and purified as specified by the manufacturer for purification of plasmid DNA prepared by other methods. Plasmid DNA (17.5 μl of the final 200-μl preparation) was digested with restriction enzymes overnight under the reaction conditions recommended by the supplier (Gibco). Restriction fragments were separated on a 0.7% agarose gel in Tris-acetate-EDTA at 3 to 4 V/cm. The gels were stained with SYBR Green (Molecular Probes, Eugene, Oreg.) for 40 min and visualized under 300-nm UV illumination. A probe for Southern hybridization was prepared by digesting pDTG1 with *Bam*HI and labeling by nick translation with digoxigenen-dUTP, as specified in the Genius system user's guide, version 3.0 (Boehringer Mannheim). Southern hybridization was performed with nylon membranes (Micron Separations Inc., Westboro, Mass.) at 65°C overnight, and the membranes were washed with 0.5× wash buffer at 65°C (Boehringer Mannheim).

Exogenous isolation of plasmid from seep sediment. Recipient cells (5×10^8) were harvested and washed as above for conjugal filter matings. Donor cells were obtained from seep sediment as follows. A 100-ml volume of 0.1% $Na_4P_2O_7$ (pH 7.2) was added to 10 g of seep sediment (sampled 7 months before the experiment and stored at 4°C) and blended in a Waring blender for two 30-s intervals (4, 28). The soil was allowed to settle for 5 min, after which 0.5 ml of the supernatant was added to the microcentrifuge tube containing the recipient cells and centrifuged for 5 min. The pellet was resuspended in 50 μl of PBS, and the filter mating proceeded as above. Transconjugants were enumerated on MSB-N plates amended with rifampin and cycloheximide. Plates containing potential transconjugant colonies were incubated under a mixture of naphthalene and indole vapors for 2 days, after which the plates were examined for the presence of indigo colonies (14, 29). These indigo-positive colonies were tested for growth with and without naphthalene on MSB plates amended with rifampin and cycloheximide (prepared in water). Confirmation that transconjugant colonies arose from the recipient used was obtained by comparing the ERIC REP-PCR fingerprints. Detection of large plasmids and amplification of *nahAc* gene in indigo-positive, naphthalene-positive colonies were carried out as above.

RESULTS

Development of recipients for conjugation experiments. (i) Curing. All naphthalene-degrading bacteria were repeatedly transferred in LB broth for several months. During this time, 10 of the 12 seep strains (strains Cg1, Cg2, Cg4, Cg5, Cg8, Cg9, Cg11, Cg12, Cg16, and Cg21), as well as the archetypal naphthalene degraders NCIB9816-4 and PpG7, were cured of their large plasmids. Large supercoiled plasmids were detected in parent naphthalene-degrading strains but were absent in the plasmid isolations from the respective cured strains (data not shown). Confirmation that each of the cured strains was, in fact, derived from each parent strain was provided by comparing the ERIC REP-PCR fingerprints of the cured and original parent strains (data not shown). Loss of plasmid correlated with a loss in ability to grow with naphthalene as the sole carbon source. Concurrent loss of the *nahAc* gene was verified by lack of PCR amplification of a 407-bp fragment of *nahAc* in the cured strains, while the parent naphthalene-degrading strains all contained the amplifiable *nahAc* gene (data not shown). The remaining two seep-derived strains, Cg7 and Cg15, also lost the ability to grow on naphthalene during this curing regime. However, these strains retained smaller plasmids (data not shown) and are the subject of another study.

TABLE 2. Results of laboratory filter matings between plasmid-containing, naphthalene-degrading strains and cured, rifampin-resistant recipient strains[a]

Donor[b]	Recipient	No. of transconjugants per donor	Spontaneous resistance frequency[c]
PpG7	PpG7.CR	10^{-2}	10^{-6}
NCIB9816-4	NCIB9816-4.CR[d]	10^{-7}[e]	10^{-8}
NCIB9816-4	Cg21.CR	10^{-6}[e]	10^{-6}
Cg1	Cg1.CR	10^{-3}	10^{-8}
Cg1	NCIB9816-4.CR	10^{-8}[e]	10^{-8}
Cg2	Cg2.CR	10^{-4}	10^{-7}
Cg2	Cg12.CR	10^{-8}[e]	10^{-8}
Cg4	Cg4.CR[f]	10^{-4}	10^{-7}
Cg4	Cg2.CR	10^{-8}[e]	10^{-9}
Cg5	Cg5.CR[g]	10^{-6}[e]	10^{-8}
Cg8	Cg8.CR	10^{-4}	10^{-7}
Cg9	Cg9.CR[f]	10^{-6}	10^{-9}
Cg12	Cg12.CR	10^{-5}	10^{-8}
Cg16	Cg16.CR	10^{-4}	10^{-8}
Cg21	Cg21.CR	10^{-3}	10^{-7}
Cg21	Cg1.CR	10^{-5}	0
Cg21	Cg8.CR	10^{-5}	0
Cg21	NCIB9816-4.CR	10^{-6}	0

[a] All matings attempted once, unless otherwise noted.
[b] For strain designation, see Table 1.
[c] Number of spontaneous antibiotic-resistant colonies per donor.
[d] Mating attempted five times. pDTG1 was previously shown to be self-transmissible (10^{-6}/donor) (45).
[e] A mating was considered unsuccessful if there was less than a 10^3-fold difference between the number of transconjugants and the number of spontaneous antibiotic-resistant donors.
[f] One of three matings successful.
[g] Mating attempted four times.

(ii) Selection for rifampin resistance in cured strains. By successively growing spontaneous resistant mutants on increasing concentrations of rifampin, strains that were resistant to rifampin (MIC of greater than 500 μg/ml) were obtained (data not shown). Confirmation that each of the rifampin-resistant cured strains was derived from each parent strain was again provided by comparing the ERIC REP-PCR fingerprints of the rifampin-resistant cured and original parent strains (data not shown). These rifampin-resistant cured strains, designated Cg1.CR, Cg2.CR, Cg4.CR, Cg5.CR, Cg8.CR, Cg9.CR, Cg12.CR, Cg16.CR, Cg21.CR, PpG7.CR, and NCIB9816-4.CR, were then used as recipients in mating experiments. The REP-PCR pattern for the cured strain Cg11 did not match its parent; therefore, additional experiments with this bacterium were abandoned.

Self-transmissibility of naphthalene plasmids. Filter matings between eight of the nine naphthalene-degrading seep isolates and their respective cured, rifampin-resistant progeny yielded transconjugant colonies which were able to grow on MSB-N amended with rifampin (Table 2). Conjugation was

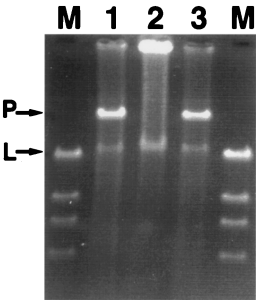

FIG. 1. Gel electrophoresis profiles for donor, recipient, and transconjugant bacteria. Data, shown here for strain Cg12, were obtained for all successful matings listed in Table 2. Partially purified plasmids were separated on a 0.7% agarose gel. P, Covalently closed supercoiled plasmid; L, linear genomic DNA; M, lambda DNA cut with *Hin*dIII. Lanes: 1, Cg12 (donor); 2, Cg12.CR (recipient); 3, Cg12.T (transconjugant).

considered successful when the number of transconjugant colonies was at least 3 orders of magnitude greater than the number of spontaneous resistant donor colonies on the same selective medium. Thus, of the 11 intrastrain matings performed, 9 were successful (all but NCIB9816-4 and Cg5). Intrastrain matings for Cg5 and for NCIB9816-4 were attempted four and five times, respectively, before being considered unsuccessful. One donor, Cg21, was able to transfer the naphthalene-catabolic plasmid to three other hosts (Table 2). No transconjugants were found in other interstrain matings tested (Table 2). The presence of a new plasmid in the transconjugants was verified by alkali lysis and gel electrophoresis. Figure 1 shows plasmid profiles for the donor, recipient, and transconjugant for strain Cg12. Analogous profiles were obtained for all successful matings. Data in Table 2 and Fig. 1 clearly demonstrate that the naphthalene-catabolic plasmids in bacteria isolated from our contaminated study site were self-transmissible.

Analysis of RFLP patterns. To characterize the self-transmissible naphthalene plasmids, we isolated them from each of their hosts by a modification of the alkali lysis method of Anderson and McKay (2, 55) and then purified them on a Qiagen anion-exchange column. Digestion of the naphthalene catabolic plasmids with restriction enzymes *Bam*HI, *Hin*dIII, *Sma*I, and *Xho*I revealed a pattern of restriction fragment lengths common to plasmids from all of our site-derived bacteria and pDTG1 (data not shown for *Bam*HI, *Sma*I, and *Xho*I, but see the description of Southern hybridization of pDTG1 to *Hin*dIII-digested site-derived plasmids in Fig. 2 [below]). This common pattern was unlike the restriction fragment length polymorphism (RFLP) pattern for the canonical naphthalene catabolic plasmid, NAH7. Table 3 lists the sizes of the restriction fragments common to all seep-derived naphthalene-catabolic plasmids and pDTG1, as determined by comparing the distance of band migration to the migration of a size standard (lambda DNA digested with *Hin*dIII). RFLP patterns generated for NAH7 (*Sma*I and *Hin*dIII) and pDTG1 (*Bam*HI, *Hin*dIII, and *Xho*I) agreed well with previously published results (53, 54, 74). Some of the plasmids (pDTG1, pCg1, pCg4, pCg5, pCg7, pCg11, and pCg15) contained one or two restriction fragments that were slightly altered (shifted, missing, or additional) from the RFLP pattern shared among the naphthalene plasmids (Table 3; Fig. 2 [see below]). These alterations in restriction fragment lengths correlated well with differences in the overall size of the plasmids (Table 1).

Southern hybridization of pDTG1 to naphthalene-catabolic plasmids from field-site derived bacteria. To confirm similarities between our site-derived naphthalene-catabolic plasmids and pDTG1, the entire pDTG1 plasmid was labeled and used as a probe in Southern hybridizations to three of the four restriction digests described above. All of the restriction fragments found in the common plasmid pattern hybridized to pDTG1 with the same high intensity (Fig. 2, lanes 3 to 14; here an *Hin*dIII digestion is shown); pDTG1 self-hybridization is shown in Fig. 2, lane 2. Hybridization of the entire pDTG1 to NAH7 showed variable intensity (lane 1). In strain Cg7, an additional large restriction fragment of approximately 21 kb was also found (largest band in lane 7). This is consistent with our previous finding that strain Cg7 contains two closely related naphthalene-catabolic plasmids that differ in size by approximately 20 kb (Table 1) (26). Besides the two fragment sizes that differ in *Hin*dIII digests of the naphthalene-catabolic plasmids (Table 3), other variations in the banding patterns in Fig. 2 include a possible shift in the 4.5-kb fragment to 5.5 kb in pDTG1 (lane 2), a deleted 9.8-kb band in pCg4 (lane 5), and a possible shift in the 13.2-kb fragment to an approximately 11-kb fragment in strain Cg7 (lane 7). When our site-derived

TABLE 3. Sizes of the restriction fragments common to all naphthalene-catabolic plasmids from site-derived isolates and *P. putida* NCIB9816-4[a]

Size of fragment (kb) with restriction enzyme:				
*Sma*I	*Bam*HI	*Xho*I	*Hin*dIII	
>23.0[b]	>23.0[b]	>23.0[b]	16.1[c]	3.3
11.6	23.0	10.9	13.2	2.5
7.2	20.0	6.0	11.2	2.0[d]
5.8	9.3	5.2	9.8	1.6
5.0	5.5	3.9	8.6	1.3
3.4	4.0	2.4	7.8	1.2
1.9		1.9	6.4[c]	1.0
1.5			4.5	0.9
1.4			3.4	0.8

[a] See the text for minor variations from this pattern for certain plasmids.
[b] Resolution of standard agarose gels (0.7%) did not allow accurate sizing of fragments greater than 23 kb.
[c] Plasmids contain either the 16.1-kb fragment (Cg1, Cg4, Cg5, Cg7, Cg11, Cg15) or the 6.4-kb fragment (PpNCIB9816-4, Cg2, Cg8, Cg9, Cg12, Cg15, Cg21).
[d] Doublet.

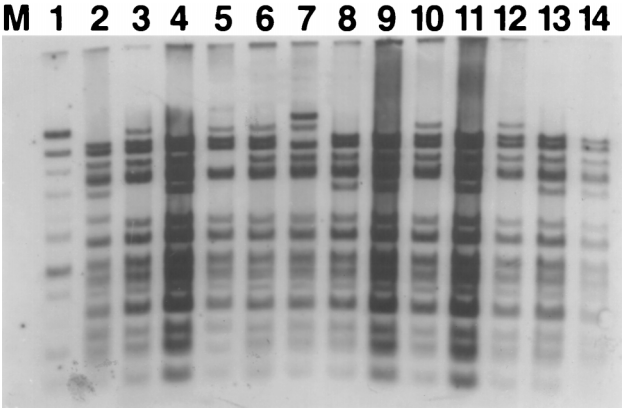

FIG. 2. Southern hybridization of pDTG1 to naphthalene-catabolic plasmids residing in bacteria isolated from coal tar waste-contaminated sediments. The plasmids were digested with *Hin*dIII and electrophoresed on a 0.7% agarose gel. M, lambda DNA cut with *Hin*dIII. Digested plasmids from each strain were isolated and loaded into the following lanes: 1, PpG7; 2, NCIB9816-4; 3, Cg1; 4, Cg2; 5, Cg4; 6, Cg5; 7, Cg7; 8, Cg8; 9, Cg9; 10, Cg11; 11, Cg12; 12, Cg15; 13, Cg16; 14, Cg21.

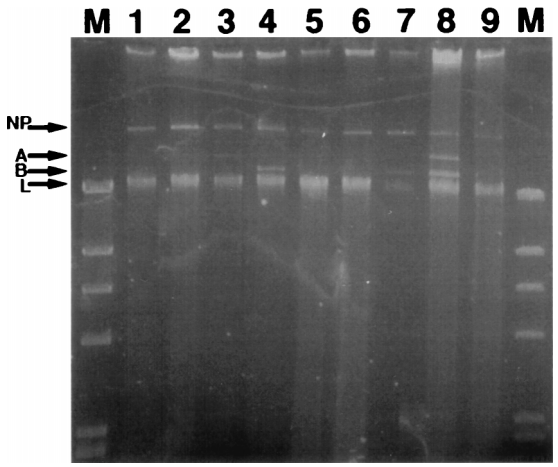

FIG. 3. Plasmids retrieved from eight transconjugant colonies after filter matings between indigenous sediment microorganisms and recipient strain Cg9.CR. Undigested, covalently closed supercoiled plasmid extractions were run on 0.7% agarose gel. NP, naphthalene-catabolic plasmid; A, uncharacterized plasmid; B, another uncharacterized plasmid; L, linear genomic DNA. Plasmids were isolated from strain Cg9 (original) (lane 1) and transconjugant colonies (lanes 2 to 9).

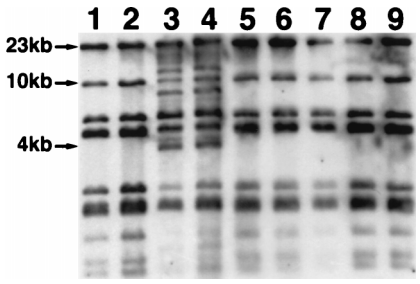

FIG. 4. Southern hybridization of pDTG1 to naphthalene-catabolic plasmids retrieved from sediment microorganisms after filter matings with recipient strain Cg9.CR. The plasmids were cut with *Xho*I and run on a 0.7% agarose gel. Plasmids were isolated from parent strain Cg9 (lane 1) and transconjugant colonies (lanes 2 to 9).

naphthalene plasmids were digested (*Bam*HI and *Sma*I) and probed with pDTG1, high-intensity hybridization signals again confirmed a pattern of restriction fragments common to all our site-derived naphthalene plasmids (Table 3 and data not shown).

Exogenous isolation of plasmids from seep sediment. Microorganisms indigenous to sediment from our Glens Falls, N.Y., study site were obtained by homogenizing the sediment in a blender with 0.1% $Na_4P_2O_7$. These cells from the sediment homogenate were used as donors in filter matings with all nine of the cured, rifampin-resistant, seep-derived recipient strains. Microorganisms in aliquots of sediment homogenate plated onto transconjugant-selective medium (no added recipient bacteria) failed to grow, indicating that the rifampin effectively inhibited native naphthalene-degrading bacteria. Potential transconjugants were screened in indole vapor to detect the activity of a dioxygenase gene in transforming indole to indigo (14, 29). Only when Cg9.CR served as a recipient were any indigo-colored putative transconjugant colonies observed. These 12 indigo colonies, all derived from Cg9.CR (as verified by ERIC REP-PCR [data not shown]), were picked and restreaked onto fresh transconjugant-selective medium, after which only 8 transconjugant colonies continued to grow. All the transconjugant strains were found to contain the *nahAc* gene by PCR amplification (data not shown). A large plasmid comparable in size to pCg9 (corresponding to band NP in Fig. 3, lane 1) was found in all of these transconjugant strains (lanes 2 through 9). Furthermore, additional smaller plasmids, bands A and B, were found either singly (lanes 3, 4, and 7) or together (lane 8) in four of the transconjugants. After Southern transfer, the plasmids in the gel shown in Fig. 3 were hybridized to the pDTG1 probe. This revealed that only the largest plasmid band found in each transconjugant (corresponding to band NP in Fig. 3, lanes 1 to 9) gave a positive hybridization signal (data not shown). Once confident that the uncharacterized plasmids carried by transconjugant strains would not hybridize to pDTG1, we digested total plasmid extractions from the eight transconjugant strains with *Xho*I, separated the fragments by electrophoresis, and performed Southern analysis with the pDTG1 probe (Fig. 4). All eight exogenously isolated plasmids (Fig. 4, lanes 2 to 9) shared the same pattern of nine restriction fragments found in pCg9 (lane 1). Transconjugant naphthalene-catabolic plasmids in lanes 3 and 4 (Fig. 4) contained additional restriction fragments of approximately 12, 8.5, and 4 kb. Longer exposure of the film shown in Fig. 4 revealed that the low-molecular-weight bands in lanes 1, 2, 4, 5, 6, 8, and 9 were also present in lanes 3 and 7 (data not shown). In addition to the *Xho*I restriction fragments common to all site-derived plasmids and pDTG1, pCg5 and pCg7 each have one *Xho*I restriction fragment of approximately 12 and 7 kb, respectively (data not shown). However, none of the naphthalene-catabolic plasmids previously isolated from our site-derived strains have the same *Xho*I RFLP pattern seen in the transconjugant naphthalene-catabolic plasmids in lanes 3 and 4 (Fig. 4). Thus, in addition to retrieving the same naphthalene-catabolic plasmid previously isolated from our site-derived strains, our exogenous plasmid isolation procedure has retrieved a novel variant of this plasmid. Data in Fig. 4 clearly show that the microorganisms indigenous to our coal tar-contaminated site possess the self-transmissible naphthalene-catabolic plasmid previously found in site-derived isolates and that its transmission to site-derived recipient strains can be rapidly induced under laboratory conditions.

DISCUSSION

The presence of a highly conserved *nahAc* allele among diverse bacteria carrying naphthalene-catabolic plasmids provided evidence for in situ horizontal gene transfer at a coal tar-contaminated site (26). Because the plasmids differed slightly in size (Table 1) and an isolate from another region of the same contaminated field site carried the same *nahAc* allele (the Cg1 allele [26]) on its chromosome, the mechanism of HGT was uncertain. Among the possible mechanisms were the following: (i) individual genes may have been recruited and transferred between replicons on gene cassettes or transposons (1, 64, 67); (ii) genes may have been transferred between bacteria via conjugation if the genes were located on self-transmissible conjugative transposons or plasmids (41, 50); and (iii) portions of the genome not otherwise organized on a mobile element may have been transferred via the mechanisms of transformation or transduction (38).

This study determined that all of the seep-derived naphthalene catabolic plasmids that we tested were self-transmissible (Table 2). This is consistent with previous reports that all other naphthalene-catabolic plasmids examined to date from pseudomonad strains are also self-transmissible (75). In addition, we found limited interstrain transfer of the naphthalene-catabolic plasmid among our seep-derived isolates (Fig. 1; Ta-

ble 2). RFLP analysis of these plasmids indicated that they are all closely related to each other and to the naphthalene-catabolic plasmid (pDTG1) of *P. putida* NCIB 9816-4, which was isolated decades ago in Bangor, Wales (Fig. 2; Table 3). Similarity among all site-derived naphthalene-catabolic plasmids and pDTG1 was confirmed by using the entire pDTG1 as a probe in Southern hybridizations (Fig. 2). The same naphthalene-catabolic plasmid and a related variant were retrieved directly from the microbial community indigenous to the contaminated-site sediment by using a cured, rifampin-resistant seep isolate as a recipient in laboratory filter matings (Fig. 3 and 4). This entire plasmid is therefore a mobile genetic element responsible for transferring naphthalene-catabolic genes among bacteria in situ at this site, most probably via conjugation.

To date, all of the naphthalene-catabolic plasmids examined with respect to incompatibility fall into the IncP9 or IncP7 groups (5, 75) and have been isolated from fluorescent *Pseudomonas* spp. (rRNA group I). Plasmids from these two incompatibility groups are not considered to have a broad host range (5, 47). The incompatibility group of pDTG1 has not been determined, but another naphthalene-catabolic plasmid from *P. putida* NCIB 9816-3 belongs to the IncP9 incompatibility group (75).

The exogenous plasmid isolation technique, in which plasmids are directly captured into an appropriate recipient bacterium without the prior cultivation of the plasmids' original host, has been successfully applied in the isolation of mercury resistance plasmids (7, 18, 37, 48, 59), 2,4-D-catabolic plasmids (63), and mobilizing plasmids (27, 61, 68). In our study, two closely related naphthalene-catabolic plasmids, as well as at least two other uncharacterized plasmids, were retrieved in the eight transconjugant colonies we obtained by the exogenous isolation technique (Fig. 3 and 4). Like the isolate-derived plasmids, these newly retrieved naphthalene-catabolic plasmids were both closely related to pDTG1 (Fig. 4). Other exogenous plasmid isolation experiments have retrieved a group of plasmids which varied in RFLP patterns but were still related to each other (37, 59, 63, 68). Different researchers, using this plasmid capture technique, have also reported a predominance of one plasmid type from a single isolation experiment (18, 27, 48). Although all eight exogenously isolated naphthalene-catabolic plasmids examined in the present study were obtained from a single filter mating experiment, subsequent exogenous plasmid isolations from seep sediment with the same recipients under different incubation conditions also obtained only naphthalene-catabolic plasmids closely related to pDTG1 (data not shown). Thus, after examining two types of plasmid sources (naphthalene-degrading isolates and plasmid capture from the sediment community), we have been able to isolate only variants of pDTG1 from our site. In contrast, when *Alcaligenes eutrophus* strains deficient in 2,4-D degradation were used to recover 2,4-D-degradative plasmids from contaminated soil, plasmids representing two incompatibility groups were isolated (63).

Unlike the present study, in which similarity to a previously examined plasmid was found, all of the mercury-resistant plasmids exogenously isolated by Dahlberg et al. (7) differed from previously described mercury-resistant plasmids, as gauged by hybridization to inc/rep probes. A unique aspect of this marine study (7) was that plasmid recovery was successful when a mating medium (artificial seawater) was used without addition of high concentrations of nutrients.

It should be noted that the success of exogenous plasmid isolation experiments is dependent on an appropriate recipient bacterium to act as a genetic sink for capture of transmissible plasmids. Naphthalene-catabolic plasmids whose host range did not include our recipient bacteria could not have been retrieved. It is possible that microorganisms native to our study site contain a greater diversity of naphthalene-catabolic genes (and the mobile genetic elements on which they may reside) than we are detecting. In support of this idea, a gram-positive (putatively *Arthrobacter* sp.) naphthalene degrader has been isolated from contaminated seep sediment (25). Based on negative results from both PCR amplification and hybridization experiments, the *nahAc* gene in this gram-positive bacterium shows no homology to the *nahAc* of *P. putida* PpG7, which differs by 5% from the Cg1 allele of *nahAc* (25). The use of recipient bacteria from genera other than the rRNA type I *Pseudomonas* used here (such as the gram-positive isolate) may allow the exogenous isolation of different naphthalene-catabolic plasmids from our site.

The results of our exogenous plasmid isolation experiment indicate the long-term persistence of this naphthalene-catabolic plasmid in our coal tar-contaminated site. The seep-derived strains which carried the plasmid were originally isolated from a sediment sample taken from the field in July 1992, whereas the exogenous plasmid isolation procedure was performed on sediment which had been sampled from the study site more than 4 years later. Other reports support in situ persistence of plasmids. In a study of exogenously isolated mercury-resistant plasmids, Lilley et al. (37) isolated and characterized 79 plasmids from the phytosphere of field-grown sugar beets. Three of the five types of plasmids (grouped on the basis of RFLP patterns) persisted over the course of 3 consecutive years, despite the absence of mercury contamination (37). Top et al. (63) also noted that one of the 2,4-D-catabolic plasmids isolated in their study had persisted in the treated soil for at least 3 years.

Despite the above-discussed methodological limitations, we have gathered evidence for in situ persistence and horizontal transfer of a single naphthalene-catabolic plasmid type in our coal tar-contaminated field site. This suggests that the coal tar contamination has applied selective pressure for the proliferation of this plasmid. This selective pressure could act by at least three different mechanisms. The coal tar contamination may have acted specifically to induce the transfer of pDTG1. Such substrate-specific active transfer has been found to be the case for transfer of tetracycline resistance determinants (50), as well as for conjugal transfer of opine-catabolizing Ti plasmids of *Agrobacterium tumefaciens* (21). However, other studies examining the transfer of plasmids encoding resistance to mercury (33), other heavy metals (9), or naphthalene catabolism (11) have failed to find any evidence for increased transfer of the mobile genetic element in the presence of their respective substrates.

Alternatively, carbon input associated with the coal tar contamination could have caused an increase in the total amount of conjugation among certain bacterial populations. Because conjugation is dependent on cell density (57), population growth would enhance conjugation rates simply because of greater cell-to-cell contact. In support of this, some researchers have observed a general increase in the number of plasmid-containing bacteria in environments contaminated with organic pollutants compared with that in pristine sites (6, 36, 61). The coal tar contamination in our study site may have increased the specific growth rate of native microorganisms. Enhanced growth rate has been correlated with an increase in the plasmid transfer rate (58); for example, soils amended with nutrients have shown increased plasmid transfer relative to unamended soils (22, 34, 41, 62).

However, we speculate that a third mechanism, selection for

growth of transconjugants containing pDTG1, may also be operating at our field site. Rather than increasing the actual plasmid transfer frequency, the selection may be acting on the particular catabolic genes which reside on pDTG1, possibly because the encoded enzymes have a kinetic or affinity advantage over other catabolic alleles at the ambient naphthalene concentrations. It has been noted that under strong selective pressure of high substrate levels, one type of catabolic gene can predominate (often in diverse bacterial hosts) or exclude other catabolic alleles (10, 30, 45).

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