APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1996, p. 230–236 Vol. 62, No. 1
0099-2240/96/$04.00+0

Molecular Cloning of Novel Genes for Polycyclic Aromatic Hydrocarbon Degradation from *Comamonas testosteroni* GZ39

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Received 7 August 1995/Accepted 30 October 1995

Three strains of *Comamonas testosteroni* were isolated from river sediment for the ability to degrade phenanthrene; two of the strains also grew on naphthalene, and one strain also grew on anthracene. The homology of the genes for polycyclic aromatic hydrocarbon degradation in these strains to the classical genes (*nah*) for naphthalene degradation from *Pseudomonas putida* NCIB 9816-4 was determined. The three *C. testosteroni* strains showed no homology to the *nah* gene probe even under low-stringency conditions. The genes for naphthalene and phenanthrene degradation were cloned from one of the three *C. testosteroni* strains. Two cosmid clones expressing polycyclic aromatic hydrocarbon dioxygenase activity were identified from a library prepared with genomic DNA from *C. testosteroni* GZ39. The genes coding for the first two enzymes in the catabolic pathway, phenanthrene dioxygenase and *cis*-phenanthrene dihydrodiol dehydrogenase, were localized to a 5.4-kb *Nco*I-*Pst*I fragment by subcloning and gene expression experiments. Further subcloning and analysis revealed a novel organization of the genes, with the gene for *cis*-phenanthrene dihydrodiol dehydrogenase located between the genes for the individual phenanthrene dioxygenase components. A Southern blot with the cloned genes from *C. testosteroni* GZ39 confirmed that these genes are distinct from those found in *P. putida* NCIB 9816-4. Southern blots also demonstrated that *C. testosteroni* GZ38A possesses genes for phenanthrene degradation that are similar to those cloned from *C. testosteroni* GZ39. However, *C. testosteroni* GZ42 possesses genes for phenanthrene degradation that are not homologous to those cloned from *C. testosteroni* GZ39. This suggests that there are at least two different sets of genes for the degradation of phenanthrene among the three *C. testosteroni* strains.

The mechanisms by which bacteria degrade simple polycyclic aromatic compounds such as naphthalene, anthracene, and phenanthrene have been a topic of great interest over the last 30 years. The naphthalene degradation pathway has long been the paradigm for polycyclic aromatic hydrocarbon degradation. The catabolic pathway for naphthalene degradation was studied as early as 1964 by Davies and Evans (6), and recent investigations (9) have provided more detail on the individual enzymatic steps. Naphthalene degradation is initiated by the introduction of both atoms of molecular oxygen into the aromatic nucleus through the action of naphthalene dioxygenase (22). This multicomponent enzyme consists of a reductase (18), a ferredoxin (17), and an iron sulfur protein (10) and catalyzes the oxidation of naphthalene to *cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) (22). Subsequent enzymatic reactions by *cis*-naphthalene dihydrodiol dehydrogenase (35) and 1,2-dihydroxynaphthalene dioxygenase (9) result in the formation of 2-hydroxy-4-(2′-oxo-3,5-cyclohexadienyl)-buta-2,4-dienoate. Further reactions lead to the formation of salicylate and the eventual formation of tricarboxylic acid cycle intermediates (50). The enzymes involved in naphthalene degradation also have the ability to degrade phenanthrene and anthracene through similar catabolic steps (31, 37, 49, 53).

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The genes for the initial steps in the degradation of naphthalene have been cloned from many different *Pseudomonas* strains, and in many cases their nucleotide sequence has been determined. The initial substrate on which these different *Pseudomonas* strains were isolated includes naphthalene (29, 39–41), phenanthrene (28, 46), and dibenzothiophene (7). Subsequently, each of these strains was found to grow on naphthalene, and in many cases, it was shown that the initial enzymes had a broad substrate range (49, 53). Even though these sequences code for similar enzymes, the gene names were assigned on the basis of the substrate utilized, i.e., *nah* for naphthalene degradation (41), *ndo* for naphthalene dioxygenase (29), *dox* for dibenzothiopene oxidation (7), and *pah* for polycyclic aromatic hydrocarbon (phenanthrene) degradation (46). The gene designations are even more misleading when one considers that the sequenced regions are more than 90% identical to the analogous sequences from the well-studied *Pseudomonas putida* NCIB 9816-4 (7, 29, 41, 46).

Since the *nah*-like genes are available and seem to be widespread in the environment, they are utilized for detecting and monitoring polycyclic aromatic hydrocarbon-degrading microorganisms in the environment (20, 33, 38). However, the presence of a single, highly homologous group of genes for the degradation of simple polycyclic aromatic hydrocarbons does not reflect the true metabolic diversity and evolutionary potential of microorganisms. The present studies were undertaken to investigate the diversity of genes involved in phenanthrene degradation and to identify novel organisms and genes for polycyclic aromatic hydrocarbon degradation. A preliminary report of this work has been presented elsewhere (15).

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *P. putida* NCIB 9816-4 containing plasmid pDTG1 was used as a representative of the classic *nah* class of organisms (6, 50). Plasmid pDTG112 contains two *Eco*RI fragments cloned from pDTG1 and contains all of the *nah* genes necessary for the conversion of naphthalene to salicylate (39, 40). *Escherichia coli* HB101 (*hsdS20 recA13 leuB6 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*) (4) was used as the host strain for the cosmid library construction, and *E. coli* DH5α [F^- ϕ80*dlacZ*Δ*M15* Δ(*lacZY-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1*; Gibco-BRL, Gaithersburg, Md.] was used as the recipient strain for all other cloning experiments. The cosmid cloning vector pHC79 (21) was obtained from Gibco-BRL, and the pGEM series of cloning vectors was obtained from Promega (Madison, Wis.).

Mineral salts basal (MSB) medium (43) was used for enrichment cultures, growth substrate tests, and metabolite accumulation studies. Solid minimal medium contained 2% Noble agar. Naphthalene, phenanthrene, and anthracene were added to liquid medium at a final concentration of 0.05%. Naphthalene was added in the vapor phase as crystals in the petri dish lid for solid medium. Phenanthrene and anthracene were added as an ethereal spray on the surfaces of solid medium as described by Kiyohara (27). All other growth substrates were added at a final concentration of 20 mM and with the sodium salts of acidic compounds when necessary. L broth (30) was used as complete medium. Solid L medium contained 2% agar. Ampicillin (100 μg/ml) or tetracycline (15 μg/ml) was added to the medium when required to select for plasmids. *P. putida* NCIB 9816-4 and bacterial strains isolated in this study were grown at 30°C. *E. coli* strains were grown at 37°C unless specified otherwise.

Enrichment and isolation of strains. Enrichment cultures were set up in MSB medium (100 ml per 500-ml Erlenmeyer flask) supplemented with phenanthrene as the sole source of carbon. Sediment samples from the Passaic River in New Jersey were the source of the inoculum for the enrichment cultures. The cultures were monitored for the presence of microorganisms and subcultured into fresh medium when growth was visible. After three such subcultures, the microorganisms were plated onto solid MSB medium, and phenanthrene was applied as an ethereal solution to the surface of the agar (27). Colonies that produced zones of clearing, as a result of degradation and utilization of phenanthrene, were subsequently purified on L medium. Strains were identified with the Biolog (Hayward, Calif.) identification system as recommended by the manufacturer.

Molecular techniques. Total genomic DNA was prepared by the method of Olsen et al. (34). Bacterial strains were grown either in L broth or in MSB medium supplemented with naphthalene or phenanthrene prior to cell lysis. Plasmid DNA was isolated by the alkaline-sodium dodecyl sulfate (alkaline-SDS) procedure of Birnboim and Doly (2). Plasmid DNA was transformed into competent *E. coli* DH5α by the procedure of Hanahan (19). Restriction digests and ligations of DNA samples were performed as recommended by the supplier (Gibco-BRL). Agarose gel electrophoresis was performed in 40 mM Tris–20 mM acetate–2 mM EDTA buffer. Transfer of DNA from agarose gels to Zeta-probe nylon membranes (Bio-Rad Laboratories, Rockville Center, N.Y.) was carried out with a vacuum blot apparatus as recommended by the supplier (LKB Instruments, Piscataway, N.J.). DNA restriction fragments to be used as probes in Southern blotting experiments were separated by gel electrophoresis and eluted from gel fragments by the procedure of Vogelstein and Gillespie (47). DNA fragments were labeled by the random priming method of Feinberg and Vogelstein (12). Southern hybridizations were performed as recommended by the nylon membrane supplier (Bio-Rad). Prehybridization and hybridization were performed at 37°C. Following hybridization, the nylon membrane was washed under low-stringency conditions (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% SDS) at room temperature.

The cosmid library of *Comamonas testosteroni* GZ39 genomic DNA was prepared with partially *Sau*3AI-digested DNA size fractionated by a 10 to 40% sucrose gradient (36). DNA fragments in the 25- to 45-kb size range were pooled and ligated to *Bam*HI-digested pHC79 as described previously (21). Packaging of the ligated DNA and transfection into *E. coli* HB101 were carried out by the procedure recommended by the packaging extract supplier (Promega). Colonies were tested for ampicillin resistance and tetracycline sensitivity, indicative of an inserted fragment. The library was replica plated onto L agar plates with ampicillin and screened for clones that turned purple or blue-grey as a result of the formation of indigo by dioxygenase activity expressed by the clones (11).

Transformation of naphthalene and phenanthrene by *E. coli* carrying cloned genes. *E. coli* DH5α cells containing a clone for analysis was cultivated in MSB medium supplemented with glucose (20 mM), thiamine (1 mM), yeast extract (1 mg/ml), Casamino Acids (1 mg/ml), and ampicillin (100 μg/ml). In general, 2 ml of an overnight culture was used to inoculate 100 ml of fresh medium in a 500-ml Erlenmeyer flask. The flasks were incubated at 37°C with shaking until the cells reached an optical density of 0.7 at 600 nm. Cells were harvested by centrifugation, washed with a half volume of 50 mM $NaPO_4$ or KPO_4 buffer (pH 7.25), and resuspended in 25 ml of the same buffer supplemented with 20 mM of glucose. Naphthalene or phenanthrene was added to a final concentration of 1 mg/ml by use of a stock solution prepared in *N*,*N*-dimethylformamide (100 mg/ml). The cells were incubated at 30°C with shaking overnight. The cells and residual substrate were removed by centrifugation, and the supernatant was extracted three times with equal volumes of ethyl acetate. The extract was dried over anhydrous sodium sulfate and then evaporated to dryness under vacuum. The dried residues were dissolved in 2.0 ml of methanol and analyzed by high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). Hydroxyl groups of the *cis*-dihydrodiol compounds were derivatized with phenylboronic acid prior to GC-MS analysis to confirm the *cis*-relative stereochemistry (44). HPLC analysis was performed with a Beckman (Fullerton, Calif.) HPLC fitted with a reverse-phase 5-μm C_{18} column (4.6 mm by 25 cm) with diode array spectral detection by using a gradient of 0 to 100% methanol in water. GC-MS analysis was performed on a Hewlett-Packard 5890 series II gas chromatograph equipped with a Hewlett-Packard 5791 mass spectrometer. Samples were injected at 280°C onto a fused silica capillary column (0.25 mm by 60 m; Supelco, Bellefonte, Pa.), operated with helium as the carrier gas and a temperature gradient of 20°C/min from 70 to 320°C.

Chemicals. Naphthalene, phenanthrene, salicylate (*o*-hydroxybenzoate), *m*-hydroxybenzoate, *p*-hydroxybenzoate, phthalate, and gentisate were obtained from Aldrich Chemical Co. (Milwaukee, Wis.) and were of the highest quality obtainable. *cis*-1,2-Dihydroxy-1,2-dihydronaphthalene and 1,2-dihydroxynaphthalene were produced as described earlier (51) by the transformation of naphthalene by *E. coli* JM109 harboring pDTG601A or pDTG602, respectively. *Beijerinckia* sp. strain B8/36 was used to synthesize *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene from phenanthrene (23).

TABLE 1. Growth of bacteria from the Passaic River on various substrates

Substrate	Growth of[a]:					
	C. testosteroni			*P. putida*		
	GZ38A	GZ39	GZ42	GZ41	GZ44	GZ45-2
Phenanthrene	+	+	+	+	+	+
Naphthalene	−	+	+	+	+	+
Anthracene	+	−	−	−	+	−
Phthalate	+	+	+	−	−	−
Gentisate	−	+	+	−	−	−
o-Hydroxybenzoate	−	+	+	+	+	+
m-Hydroxybenzoate	−	+	+	−	−	−
p-Hydroxybenzoate	−	+	+	+	+	+
Benzoate	−	−	−	+	+	−
Glucose	−	−	−	+	+	+
Succinate	+	+	+	+	+	+

[a] +, growth of the organism detected on the substrate; −, no growth detected under the conditions used.

RESULTS

Strain isolation and characterization. Several bacterial strains that are capable of using phenanthrene as the sole source of carbon were obtained from Passaic River sediment. Six of these isolates, GZ38A, GZ39, GZ41, GZ42, GZ44, and GZ45-2, were selected for this study. The six strains were tested, and all were found to be gram-negative, oxidase-positive, catalase-positive, short rods. The six strains were further identified to the genus and species level through the use of the Biolog identification system. Strains GZ38A, GZ39, and GZ42 were identified as *C. testosteroni* with a similarity index above 0.8. The remaining strains, GZ41, GZ44, and GZ45-2, were identified as *P. putida* with a similarity index above 0.5. Strains GZ44 and GZ45-2 were classified as *P. putida* biotype B1, while strain GZ41 was classified as *P. putida* biotype A1. The ability of the six strains to grow on various carbon sources is shown in Table 1. Five of the six strains can utilize both phenanthrene and naphthalene, while the sixth strain, *C. testosteroni* GZ38A, can utilize phenanthrene but not naphthalene. *C. testosteroni* GZ38A and *P. putida* GZ44 are also able to utilize anthracene. All of the strains accumulate indigo when phenanthrene-induced cells are exposed to indole, suggesting the presence of aromatic dioxygenase activity (11). *C. testosteroni* GZ39 and GZ42 are very similar in their substrate range characteristics but are distinct with respect to colony morphologies and rate of clear zone formation on phenanthrene. *C. testosteroni* GZ39 grows rapidly on phenanthrene and forms clear zones in 2 days, whereas *C. testosteroni* GZ42 takes at

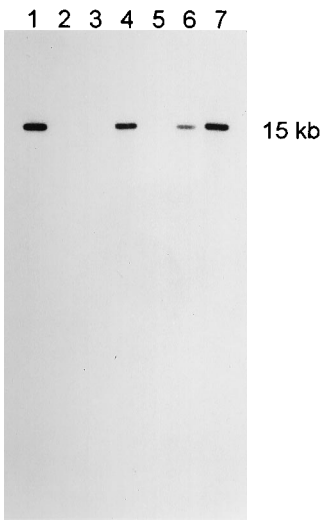

FIG. 1. Southern blot with the *nah* genes from *P. putida* NCIB 9816-4. *Eco*RI-digested genomic DNA was probed with a 15-kb DNA fragment from *P. putida* NCIB 9816-4 containing the genes for naphthalene degradation. Lanes: 1, *P. putida* NCIB 9816-4; 2, *C. testosteroni* GZ38A; 3, *C. testosteroni* GZ39; 4, *P. putida* GZ41; 5, *C. testosteroni* GZ42; 6, *P. putida* GZ44; 7, *P. putida* GZ45-2.

least 4 days to grow on phenanthrene. On the other hand, *C. testosteroni* GZ42 grows faster than *C. testosteroni* GZ39 on naphthalene. Growth of all of the strains on naphthalene and phenanthrene in liquid culture was visible within 24 to 48 h, except for *C. testosteroni* GZ38A, which took 5 to 7 days for visible growth on phenanthrene. Liquid cultures of all of the strains grown on phenanthrene turned yellow or brown during the late stationary phase of growth. On the basis of substrate range and growth characteristics, the six strains were regarded as distinct isolates.

Identification of strains with novel genes for phenanthrene degradation. The six isolated phenanthrene-degrading strains were screened for genetic homology to the classical *nah* genes. The *nah* gene probe utilized for this purpose is a 15-kb *Eco*RI fragment from pDTG112 originally cloned from *P. putida* NCIB 9816-4 (40). This 15-kb fragment contains all of the genes necessary for the conversion of naphthalene to salicylate (39–41). A Southern blot was performed on total genomic DNA from the six newly isolated strains digested with *Eco*RI and by use of the *nah* gene probe. The Southern blot was performed under low-stringency conditions as indicated in Materials and Methods to detect even low levels of homology. The results (Fig. 1) indicate that the *P. putida* strains, GZ41, GZ44, and GZ45-2, have a single hybridization band equal in size to the hybridization band in the positive control *P. putida* NCIB 9816-4. This indicates that the three newly isolated *P. putida* strains contain genes very similar to the *nah* genes found in *P. putida* NCIB 9816-4. However, a different result was obtained with the three *C. testosteroni* strains. Genomic DNA isolated from these three strains did not show any significant hybridization to the probe. Prolonged exposure of the blot to X-ray film showed two very faint hybridizing bands, 2.5 and 2.2 kb in size, for genomic DNA prepared from *C. testosteroni* GZ39 and GZ42. Since the Southern blots were performed with low-stringency conditions, lack of hybridization (or faint hybridization) indicates that there are no genes homologous to the *nah* gene probe in the three *C. testosteroni* strains.

Cloning of the genes for phenanthrene degradation from *C. testosteroni* GZ39. The Southern blots performed with the *nah* gene probe indicated that the *C. testosteroni* strains GZ38A, GZ39, and GZ42 do not contain genes highly homologous to the *P. putida* NCIB 9816-4 *nah* genes. To investigate this difference in more detail, the genes for phenanthrene degradation were cloned from *C. testosteroni* GZ39. A cosmid library of 1,800 independent clones was constructed with partially *Sau*3AI-digested total genomic DNA and the cosmid pHC79. The cosmid library (in *E. coli* HB101) was screened for colonies which turned blue on L agar as a result of the formation of indigo from indole, indicative of aromatic dioxygenase activity (11). Two cosmid clones showed the formation of indigo following incubation on L agar for 7 days. In addition, these two cosmid clones accumulated brown metabolites when exposed to naphthalene vapors and cleared phenanthrene on spray plates. A restriction map of the two clones, designated pGJZ1701 and pGJZ1702, is shown in Fig. 2. The two cosmid clones each contain an insert of approximately 40 kb and overlap each other by about 20 kb. Thus, the entire genomic region encompassed by the two clones is approximately 60 kb.

To locate the genes for phenanthrene degradation on the cosmid clones, several subclones were constructed. Since two cosmid clones were originally obtained, the overlapping region must contain at least the genes for the initial aromatic dioxygenase. Initial subclones were constructed (Fig. 3) and screened for the ability to produce indigo from indole in *E. coli*. A 28-kb *Xho*I-*Xba*I fragment (pGJZ1703) cloned from pGJZ1702 shows aromatic dioxygenase activity when cloned into *E. coli*. Clones in *E. coli* containing either a 15-kb *Bgl*II fragment (pGJZ1704) or a 14-kb *Bam*HI fragment (pJGZ1705) do not show aromatic dioxygenase activity. Deletion of one *Hpa*I fragment from pGJZ1703 resulted in a plasmid (pGJZ1706) which retained the ability to produce indigo. However, deletion of the two adjacent *Hpa*I fragments from pGJZ1703 resulted in a clone which has no activity. The subclones made thus far suggest that the initial genes for phenanthrene degradation are contained by the region on the right end of pGJZ1703. Thus, a smaller clone was constructed by cloning a 5.8-kb *Eco*RI-

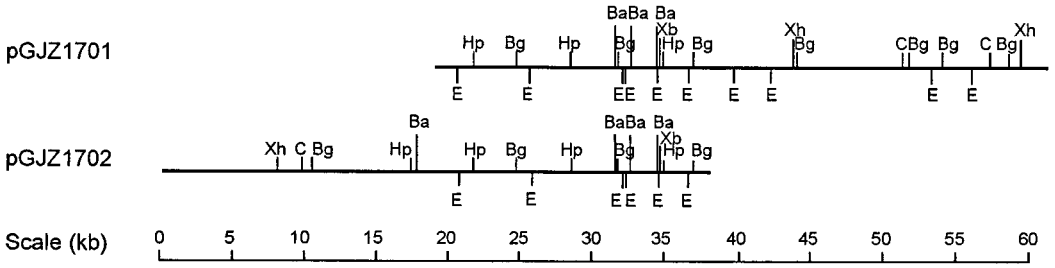

FIG. 2. Restriction map of the cloned region from *C. testosteroni* GZ39 containing the genes for phenanthrene degradation. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; Hp, *Hpa*I; Xb, *Xba*I; Xh, *Xho*I.

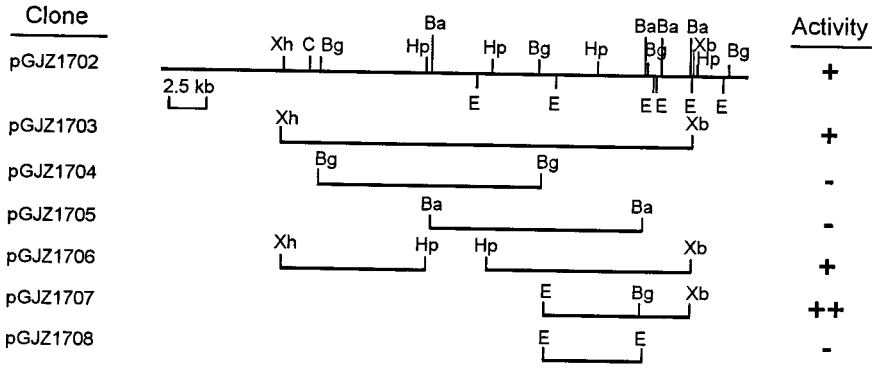

FIG. 3. Localization of the genes for phenanthrene degradation on the cosmid clone pGJZ1702. A plus in the activity column designates that the clone expresses phenanthrene- and naphthalene-degradative enzymes and has the ability to convert indole to indigo. The cloning vector pGEM11Zf(−) was used for pGJZ1703, pGJZ1706, and pGJZ1707, while pGEM7Zf(−) was used for pGJZ1704, pGJZ1705, and pGJZ1706. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; Hp, *Hpa*I; Xb, *Xba*I; Xh, *Xho*I.

*Bgl*II fragment with a 3.1-kb *Bgl*II-*Xba*I fragment from pGJZ1703. The resulting clone, pGJZ1707, showed aromatic dioxygenase activity. A smaller subclone (pGJZ1708) containing the 6.0-kb *Eco*RI fragment from the left side of pGJZ1707 does not show any aromatic dioxygenase activity.

A more extensive restriction map of pGJZ1707 was constructed and is shown in Fig. 4. This restriction map was used to facilitate subcloning experiments to precisely locate the individual genes involved in the initial steps of phenanthrene degradation on pGJZ1707. Each of the subclones constructed was analyzed for the ability to oxidize phenanthrene and naphthalene to hydroxylated products as described in Materials and Methods. *E. coli* containing pGJZ1707 is able to transform naphthalene and phenanthrene to 1,2-dihydroxynaphthalene and 3,4-dihydroxyphenanthrene, respectively. In addition, *cis*-dihydrodiols of naphthalene and phenanthrene were detected in the respective culture supernatants. A subclone (pGJZ1709) derived from pGJZ1707 through deletion of the internal 2.0-kb *Nco*I fragment retained the ability to transform naphthalene and phenanthrene to the respective dihydroxylated compounds. Subclones of this region containing either a 6.0-kb *Eco*RI fragment (pGJZ1708), a 5.5-kb *Bgl*II fragment (pGJZ1710), or a 4.6-kb *Pst*I fragment (pGJZ1711) did not show any ability to transform naphthalene or phenanthrene to oxidized products. However, subcloning of a *Nco*I-*Bgl*II fragment together with a *Bgl*II-*Pst*I fragment resulted in a clone (pGJZ1712) which had the ability to transform both naphthalene and phenanthrene to the respective dihydroxylated products. To locate the genes responsible for the aromatic dioxygenase and *cis*-dihydrodiol dehydrogenase activities, internal deletions of pGJZ1712 were constructed. An internal 4.3-kb *Nsi*I fragment was subcloned (pGJZ1713) and subsequently shown not to have the capability to transform either naphthalene or phenanthrene. Removal of an internal *Sal*I fragment (pGJZ1714) also resulted in the loss of the ability to transform naphthalene and phenanthrene. However, removal of an internal *Pvu*I fragment (pGJZ1715) resulted in the formation of a clone capable of transforming naphthalene and phenanthrene only to the respective *cis*-dihydrodiol compounds. These data suggest that the gene for the *cis*-dihydrodiol dehydrogenase is located around the *Pvu*I cutting sites. To locate the genes for the aromatic dioxygenase activity, subclones containing either the left *Nco*I-*Pvu*I fragment (pGJZ1716) or the right *Pvu*I-*Pst*I fragment (pGJZ1717) of pGJZ1715 were constructed. Neither of these two subclones had the ability to transform either naphthalene or phenanthrene to a *cis*-dihydrodiol compound. These data suggest that the genes for the individual components of the initial aromatic dioxygenase are not contiguous and flank the gene for the *cis*-dihydrodiol dehydrogenase.

Homology studies with the newly cloned genes. The initial hybridization studies presented above indicated that the genes for phenanthrene degradation found in *C. testosteroni* GZ39 are different from the classical *nah* genes. To confirm this hypothesis, a second Southern blot was performed with the cloned fragment present in pGJZ1712 as a probe. This cloned fragment contains the genes from *C. testosteroni* GZ39 that code for the conversion of phenanthrene to 3,4-dihydroxyphenanthrene. Genomic DNA from *P. putida* NCIB 9816-4 and *C. testosteroni* GZ38A, GZ39, and GZ42 were digested with *Eco*RI and used in the Southern blot experiments. The results (Fig. 5) show that the probe does not hybridize with genomic DNA from *P. putida* NCIB 9816-4, confirming that the cloned genes from *C. testosteroni* GZ39 are different from the classical *nah* genes. The probe does hybridize to genomic DNA from the source strain *C. testosteroni* GZ39 and also hybridizes to genomic DNA from *C. testosteroni* GZ38A. A 2.4-kb hybridizing band is common to both *C. testosteroni* GZ39 and GZ38A. However, the 6.0-kb hybridizing band for *C. testosteroni* GZ39 is replaced by an 11.0-kb hybridizing band for *C. testosteroni* GZ38A. Since a 5.0-kb *Eco*RI fragment is adjacent to the 6.0-kb *Eco*RI fragment in pGJZ1702 (Fig. 2), it is possible that the 11.0-kb hybridizing band is due to a loss of the *Eco*RI site between these two fragments in *C. testosteroni* GZ38A. This hypothesis is borne out by Southern blots with pGJZ1702 as a probe which show that *C. testosteroni* GZ38A is missing both the 5.0- and 6.0-kb *Eco*RI fragments that are found in *C. testosteroni* GZ39 (data not shown). Interestingly, genomic DNA from *C. testosteroni* GZ42 does not hybridize to the probe. These Southern blot data confirm that the genes for phenanthrene degradation cloned from *C. testosteroni* GZ39 are different from the *nah* genes found in *P. putida* NCIB 9816-4. The data also indicate that the genes for phenanthrene degradation cloned from *C. testosteroni* GZ39 are different from the genes for phenanthrene degradation in *C. testosteroni* GZ42.

DISCUSSION

Three *C. testosteroni* strains that have the ability to degrade phenanthrene were isolated in the present work. Genomic DNA prepared from these three strains does not show hybridization to the *nah* genes cloned from *P. putida* NCIB 9816-4.

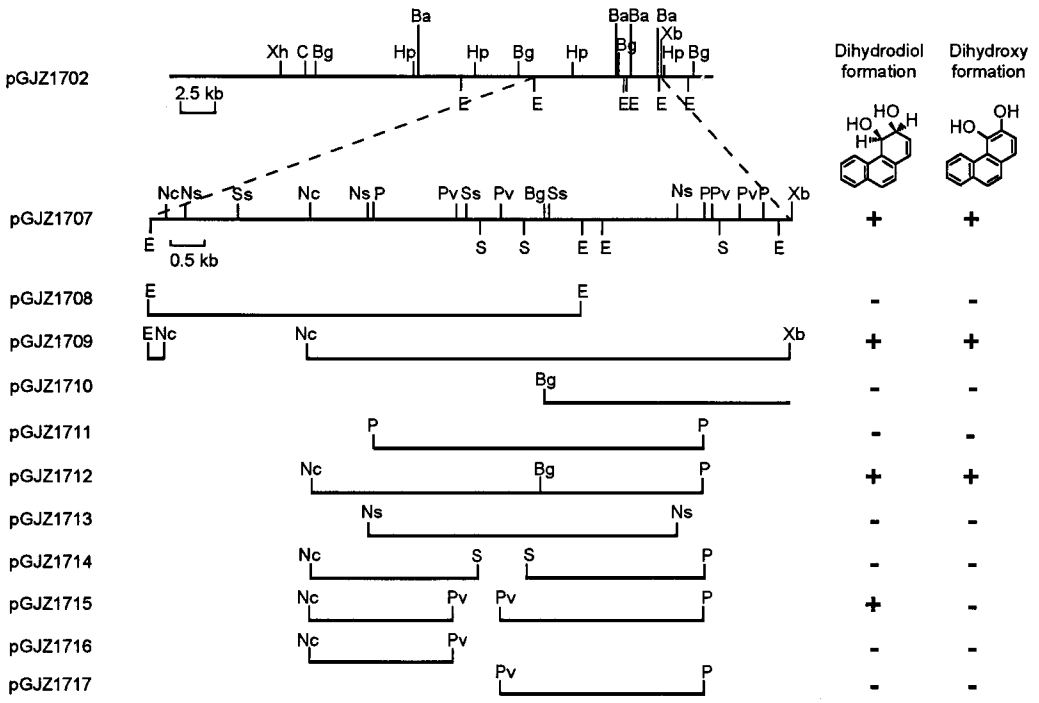

FIG. 4. Restriction map of the cloned region in pGJZ1707 and localization of the genes for phenanthrene dioxygenase and phenanthrene *cis*-dihydrodiol dehydrogenase. The columns labeled dihydrodiol formation and dihydroxy formation indicate whether the clone is able to transform phenanthrene to *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene and/or 3,4-dihydroxyphenanthrene. The cloning vector pGEM11Zf(−) was used for plasmid pGJZ1709, pGEM7Zf(−) was used for pGJZ1710 and pGJZ1713, and pGEM5Zf(−) was used for the other plasmids. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; Nc, *Nco*I; Ns, *Nsi*I; P, *Pst*I; Pv, *Pvu*I; S, *Sal*I; Ss, *Sst*I; Xb, *Xba*I; Xh, *Xho*I.

This indicates that the three *C. testosteroni* strains isolated possess novel genes for phenanthrene and naphthalene degradation. The genes for phenanthrene degradation were cloned from *C. testosteroni* GZ39. Two different cosmid clones representing a total cloned region of 60 kb were isolated. The genes for the initial steps in phenanthrene degradation were located on the cosmid clone through subcloning and metabolite accumulation assays. The genes for the initial dioxygenase and a *cis*-dihydrodiol dehydrogenase were located to a 5.4-kb *Nco*I-*Pst*I (partial) fragment. Genes for aromatic dioxygenases and *cis*-dihydrodiol dehydrogenases are often adjacent to each other and are coded for by approximately 4.5 kb of DNA (7, 41, 45, 46, 52). Thus, the 5.4-kb *Nco*I-*Pst*I (partial) fragment present in pGJZ1712 is sufficient to contain the genes for the initial dioxygenase and the *cis*-dihydrodiol dehydrogenase. Removal of a 0.8-kb *Pvu*I fragment from pGJZ1712 results in a clone (pGJZ1715) which lacks the *cis*-dihydrodiol dehydrogenase activity but retains the aromatic dioxygenase activity. These data indicate that the gene for the *cis*-dihydrodiol dehydrogenase is either entirely contained by the 0.8-kb *Pvu*I fragment or that the gene for the *cis*-dihydrodiol dehydrogenase is cleaved by one or both of the *Pvu*I cutting sites. The initial 5.4-kb *Nco*I-*Pst*I (partial) fragment is only slightly larger than that necessary to contain all of the genes for the aromatic dioxygenase and the *cis*-dihydrodiol dehydrogenase. It is entirely possible that the genes for the aromatic dioxygenase are present on both sides of the genes for the *cis*-dihydrodiol dehydrogenase. This hypothesis is borne out by the fact that clones containing either the left *Nco*I-*Pvu*I fragment (pGJZ1716) or the right *Pvu*I-*Pst*I fragment (pGJZ1717) do not by themselves display aromatic dioxygenase activity. The two fragments together in the same clone (pGJZ1715) do display aromatic dioxygenase activity. Thus, the gene order must be such that at least one gene for the aromatic dioxygenase is present on either side of the gene for the *cis*-dihydrodiol dehydrogenase. This arrangement is different from that seen in many other operons for aromatic hydrocarbon degradation, where the genes for the initial dioxygenase are clus-

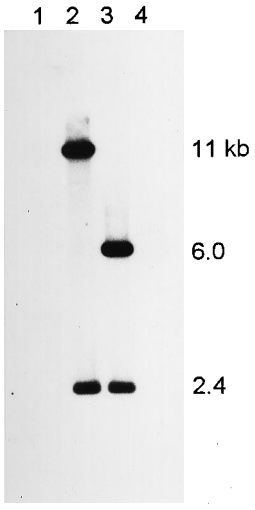

FIG. 5. Southern blot with the genes for phenanthrene degradation cloned from *C. testosteroni* GZ39. *Eco*RI-digested genomic DNA was probed with the cloned fragment from pGJZ1712 containing the genes for phenanthrene dioxygenase and phenanthrene *cis*-dihydrodiol dehydrogenase from *C. testosteroni* GZ39. Lanes: 1, *P. putida* NCIB 9816-4; 2, *C. testosteroni* GZ38A; 3, *C. testosteroni* GZ39; 4, *C. testosteroni* GZ42.

tered and are followed by the gene for the *cis*-dihydrodiol dehydrogenase (7, 41, 45, 46, 52).

The genes for phenanthrene degradation in *C. testosteroni* GZ38A are similar to those cloned from *C. testosteroni* GZ39 as illustrated by the Southern blotting data (Fig. 4). However, the two strains show a restriction fragment polymorphism with respect to one another, with *C. testosteroni* GZ39 possessing two *Eco*RI fragments (5.0 and 6.0 kb) that are homologous to a single *Eco*RI fragment (11.0 kb) found in *C. testosteroni* GZ38A (data not shown). These results are even more interesting when one considers that *C. testosteroni* GZ38A utilizes phenanthrene but not naphthalene as a carbon source (as opposed to *C. testosteroni* GZ39, which utilizes both). *C. testosteroni* GZ38A also does not utilize the known *nah* pathway intermediates salicylate and gentisate as carbon sources. However, *C. testosteroni* GZ38A does utilize phthalate as a carbon source, which has been shown to be an intermediate in the degradation of phenanthrene (25, 26). One hypothesis is that *C. testosteroni* GZ38A degrades phenanthrene through the phthalate pathway (25, 26) and is not capable of degrading naphthalene since it lacks a gentisate or a salicylate pathway (50).

The Southern blot with the genes cloned from *C. testosteroni* GZ39 as a probe (Fig. 5) confirms the conclusion that this strain possesses novel genes for phenanthrene degradation distinct from those found in *P. putida* NCIB 9816-4. The two Southern blots (Fig. 1 and 5) also show that the genes for phenanthrene degradation in *C. testosteroni* GZ42 are different from both the *nah* genes from *P. putida* NCIB 9816-4 and those cloned from *C. testosteroni* GZ39. These facts demonstrate that at least three nonhomologous families of genes responsible for phenanthrene degradation can be found in the same environmental sample.

Several different species of bacteria have the ability to degrade phenanthrene or naphthalene (3, 5, 13, 14, 16, 24, 32, 48). However, the majority of the strains isolated belong to the genus *Pseudomonas* (5). Almost all of our knowledge on the genetics of polycyclic aromatic hydrocarbon degradation comes from molecular studies on these *Pseudomonas* strains. For instance, the genes for polycyclic aromatic hydrocarbon degradation from at least five different *Pseudomonas* strains have been cloned and their nucleotide sequence has been determined (7, 8, 29, 41, 46). A comparison of the published nucleotide sequences indicates that the genes from the five sources are greater than 90% identical. Indeed, the present work illustrates that *P. putida* strains that contain genes for naphthalene and phenanthrene degradation that are homologous to analogous genes cloned by other investigators can readily be isolated from the Passaic River in New Jersey (Fig. 1). However, the present work also illustrates that naphthalene- and phenanthrene-degrading microorganisms possessing a different set of genes for the same catabolic ability can be isolated from the same environment. Other investigators have also reported the existence of microorganisms that degrade naphthalene and phenanthrene and that do not hybridize to the classical *nah* genes (1, 13, 42). However, to our knowledge, this is the first report on the cloning of such nonhomologous genes for naphthalene and phenanthrene degradation.

ACKNOWLEDGMENTS

This work was supported by a grant from the Electric Power Research Institute. G.J.Z. is the recipient of a Young Investigator Award from the National Science Foundation.

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