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Spatial and Temporal Variation of Phenanthrene-Degrading Bacteria in Intertidal Sediments

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Phenanthrene-degrading bacteria were isolated from a 1-m^2 intertidal sediment site in Boston Harbor. Samples were taken six times over 2 years. A total of 432 bacteria were isolated and characterized by biochemical testing. When clustered on the basis of phenotypic characteristics, the isolates could be separated into 68 groups at a similarity level of approximately 70%. Several groups (a total of 200 isolates) corresponded to well-characterized species belonging the genera *Vibrio* and *Pseudomonas*. Only 51 of the 437 isolates (<11.7% of the total) hybridized to a DNA probe that encodes the upper pathway of naphthalene and phenanthrene degradation in *Pseudomonas putida* NCIB 9816. A cluster analysis indicated that the species composition of the phenanthrene-degrading community changed significantly from sampling date to sampling date. At one sampling time, 12 6-mm-diameter core subsamples were taken within the 1-m^2 site to determine the spatial variability of the degrading communities. An analysis of molecular variance, performed with the phenotypic characteristics, indicated that only 6% of the variation occurred among the 12 subsamples, suggesting that the subsamples were almost identical in composition. We concluded that the communities of phenanthrene-degrading bacteria in the sediments are very diverse, that the community structure undergoes significant change with time but does not vary significantly on a spatial scale of centimeters, and that the predominant genes that encode phenanthrene degradation in the communities are not well-characterized.

Polycyclic aromatic hydrocarbons (PAHs) are widespread pollutants in the marine environment. These hydrophobic compounds display a high affinity for organic matter and particles and accumulate in organic compound-rich marine sediments (21). An estimated 2.3×10^5 metric tons of PAHs enter aquatics systems every year (16). Urban estuaries in particular, such as Boston Harbor, contain elevated PAH concentrations in their sediments (26). High PAH levels are of public health concern because of the toxic, mutagenic, and carcinogenic properties of PAHs (7, 16). Therefore, bacteria present in contaminated marine sediments are of interest as agents of PAH bioremediation and as models of bacterial population dynamics.

Taxonomically diverse bacteria that are able to utilize low-molecular-weight PAHs, such as naphthalene, phenanthrene, and fluorene, as sources of carbon and energy have been isolated and characterized. For example, bacteria belonging to the genera *Pseudomonas* (6, 11, 22, 29), *Alcaligenes* (33), *Vibrio* (34), *Mycobacterium* (3, 4, 13), *Comamonas* (12), *Rhodococcus* (14), and *Cycloclasticus* (9) have been isolated from marine sediments and soils. Members of two other PAH-degrading genera (23) previously identified as members of the genera *Pseudomonas*, *Burkholderia*, and *Sphingomonas* are also likely to be isolated from marine waters. However, isolation of pure cultures, which is typically accomplished by enrichment methods, is not necessarily an indication of the importance of organisms as PAH degraders in situ. An understanding of the basic microbial ecology of PAH degraders is still lacking; one fundamental component is to characterize the spatial and temporal variability of the PAH-degrading communities.

We report here on the dynamics of communities or guilds of phenanthrene-degrading bacteria isolated from muddy intertidal sediments over a period of 2 years. One of the major objectives of this study was to determine the extent and scale of diversity of potential phenanthrene-degrading bacteria in moderately contaminated sediments. The isolates were phenotypically characterized and clustered to determine patterns of similarity. A second objective was to determine if the potential phenanthrene-degrading community changes significantly with time. Finally, we wished to determine what portion of the isolates contained the well-characterized genes encoding PAH catabolic pathways. These genes include a portion of the naphthalene dioxygenase gene, *nahAaAb*, isolated from *Pseudomonas putida* PpG7 (25, 37) and a gene cluster encoding the degradation of naphthalene, fluorene, and phenanthrene from *P. putida* NCIB 9816 (36).

MATERIALS AND METHODS

Study site. Savin Hill Cove is a small extensively intertidal embayment of Boston Harbor (27). It receives PAHs from a storm drain and a combined sewer overflow, as well as from atmospheric deposition and nonpoint source runoff. Its sediments have a mean silt-clay content of 87.5%, a mean total organic carbon content of 34 mg/g, and a mean C/N ratio of 10.4. The site is moderately contaminated with PAHs, and PAH-degrading bacteria are abundant in the sediments (19).

Isolation of phenanthrene-degrading bacteria. Surface sediment grab samples, ranging in weight from 0.5 to 2 g (wet weight), were taken from Savin Hill Cove over a period of 2 years. Only the top 0.5 cm of the sediment, the aerobic layer, was sampled. Single grab samples were taken by hand on 21 May 1992, 8 June 1992, 23 June 1993, and 18 March 1994. To determine spatial variability, two 6-mm-diameter core samples were taken approximately 15 cm apart on 13 May 1993, and on 11 June 1994 12 6-mm-diameter core samples were taken randomly over an area of 0.9 m^2. The sediments were refrigerated at 4°C within 20 min of sampling and were processed within 1 h. Grab samples were placed in sterile 250-ml plastic containers with 50% headspace. The 6-mm-diameter core samples each had a 0.5-cm headspace.

Sediment samples were diluted in 1.5% Instant Ocean (Aquarium Systems, Mentor, Ohio) and were spread onto a modified medium of Anderson (1). This medium contained (per liter) 0.01 g of yeast extract, 0.01 g of peptone, 0.01 g of ferric chloride, 0.05 g of potassium phosphate, 15 g of agar, and either 500 ml of distilled water and 500 ml of filtered seawater or 1 liter of distilled water and 15 g

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TABLE 1. Bacterial type culture strains used in this study

Taxon	Strain[a]	Source
Pseudomonas fluorescens biotype A	ATCC 13525	Type strain
Pseudomonas fluorescens	ATCC 49838	Quality control for Micro-Scan products
Pseudomonas putida biotype A	ATCC 12633	Prefilter tanks, England
Pseudomonas putida	NCIMB 9816	Soil
Vibrio alginolyticus	ATCC 17749	Spoiled fish
Vibrio harveyi	ATCC 14126	Dead luminescing amphipod
Vibrio parahaemolyticus	ATCC 27969	Blue crab hemolymph
Vibrio parahaemolyticus	ATCC 17802	Shirasu food poisoning
Vibrio proteolyticus	ATCC 15338	Intestine of *Limnoria tripunctata*

[a] ATCC, American Type Culture Collection, Rockville, Md.; NCIMB, National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Great Britain.

of Instant Ocean. This method was used to enumerate bacteria that degraded phenanthrane as a sole carbon source or metabolized phenanthrene while they were growing on the peptone and yeast extract in the medium. The spread plates were incubated at 25°C for 2 to 3 days and then overlaid with phenanthrene (Aldrich Chemical Co., Milwaukee, Wis.) by spraying a 0.5% solution of phenanthrene in acetone with a chromatography sprayer onto the surfaces of the plates. The overlaid plates were incubated for 4 weeks, and colonies forming zones of clearing in the phenanthrene overlay were picked and streaked to determine purity. Isolates were stored at 4°C on dilute modified Luria-Bertani agar (containing [per liter of distilled water] 5 g of tryptone, 2.5 g of yeast extract, and 15 g of Instant Ocean, as well as 1.5% agar) and were preserved by freezing at −90°C in a solution containing 10% glycerol and 1.5% Instant Ocean.

Phenotypic testing. The isolates were characterized by determining biochemical characteristics in 24-well tissue culture plates as described by Hansen and Sorheim (15). The methyl red, Voges-Proskauer, chitinase, amylase, β-galactosidase, and urease tests were not performed. Additional tests were performed in the same manner to determine the ability to utilize arabinose, citrate, gluconate, glucose, malate, maltose, mannitol, mannose, and *N*-acetylglucosamine. The assimilation broth contained (per liter) 0.01 g of yeast extract, 2.0 g of ammonium sulfate, 0.05 g of potassium phosphate, 15 g of Instant Ocean, and 1.0 g of agar. After autoclaving, the carbon sources were filter sterilized and added aseptically to a final concentration of 1% (wt/vol). Inoculations were performed by suspending colony material in 1.5% Instant Ocean in a multiwell plate and using a multipoint inoculator consisting of 24 stainless steel dowels imbedded in a polycarbonate block. In addition, the following morphological characteristics were determined: colony morphology, colony pigment, cell morphology, and the presence of cell inclusions. Selected organisms were also characterized with an API NFT kit (Analytab Products, Montalieu-Vercieu, France). Several American Type Culture Collection cultures and one National Collection of Industrial Microorganisms culture were included in the battery of tests (Table 1).

Isolates were tentatively identified to either the genus or species level by comparing their phenotypic characteristics with those of American Type Culture Collection type cultures or by comparing biochemical test results, carbohydrate utilization patterns, and cell morphologies to those of species described in *Bergey's Manual of Systematic Bacteriology* (17).

Colony hybridizations. Two gene probes, both derived from *P. putida* NCIB 9816 (6), were used in this work. The 16-kb pY3-E16 probe contains the gene cluster encoding the upper pathway for the catabolism of phenanthrene, naphthalene, and fluorene (36). The initial genes of the 16-kb probe make up the smaller, 2.4-kb probe (pY3-2.4). The sequence of the 2.4-kb probe is nearly identical to the sequence of the *nahAaAb* genes of the NAH7 degradation pathway, which encode reductase$_{nap}$ and ferredoxin$_{nap}$, respectively (28). Colony hybridizations were performed by using digoxigenin-labeled probes prepared with the Genius System (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Chemiluminescent detection was performed with a Lumi-Phos 530 apparatus (Boehringer Mannheim). The colonies were applied directly to the hybridization membrane (Magnagraph nylon transfer membrane; MSI, Westboro, Mass.) by using wooden applicator sticks; otherwise, all procedures were the procedures suggested by the manufacturer. *P. putida* NCIB 9816 and PpG7 were used as positive controls (8). Two pseudomonad strains that do not degrade phenanthrene were used as negative controls. All hybridizations were incubated at 65°C.

Data analysis. The Levels of relatedness among the bacteria were determined from the phenotypic data by using Jaccard's similarity index (30). The relationships among the degradative communities present in the 18 samples were analyzed by using the chord normalized expected species shared index (CNESS) (32). Phenograms were constructed by using unweighted pair group mean average (UPGMA) linkage. Indices and clustering were determined by using NTSYS-pc and COMPAH95 (24). COMPAH95 is available at http://www.es.umb.edu/edwebp.htm.

The variation in the composition of the phenanthrene-degrading community among samples was determined by applying the analysis of molecular variance (AMOVA) analytical model (10). This model delineates the extent of genetic or (in this case) phenotypic differentiation within and among populations. It was originally designed to study molecular variation in a single species. Information on DNA genotypes was incorporated into an analysis of variance format derived from a matrix of squared distances for all pairs of genotypes. Estimates of variance components at different levels of hierarchical subdivision were determined. The significance was tested by using a permutational approach. This approach was easily applied to ecological work (in this case using phenotypic characteristics rather than genotypic characteristics) in order to determine the variation within and among the degrading populations from each sampling date. The analysis was performed by using Winamova 1.04, a DOS-based Windows program available through anonymous ftp from acasun1.unige.ch.

RESULTS

A total of 432 phenanthrene-degrading bacteria were isolated from 18 samples that were taken on six dates in May 1992, June 1992, May 1993, June 1993, March 1994, and June 1994. These isolates were characterized to determine the presence of 35 characteristics. Levels of phenotypic similarity between strains were calculated by using Jaccard's index, and clustering was performed by using the UPGMA method. At a similarity level of 70%, 83 distinct taxonomic units were identified (Fig. 1). Twenty-seven of the taxa contained 3 to 66 isolates. The remaining 56 taxa contained only one or two members. Based on the placement of standard strains (Table 1), several taxa were tentatively identified. Taxa 1 and 2 consisted of aerobic gram-negative rods which were motile and produced a fluorescent pigment. Taxon 1 contained both *Pseudomonas fluorescens* and *P. putida* Savin Hill Cove isolates, and taxon 2 consisted of the *P. fluorescens* and *P. putida* type strains and one sediment isolate. Taxa 6 through 9 consisted of *Vibrio* spp. strains. These isolates were gram-negative, fermentative, motile rods, and at least 63% of them were capable of swarming behavior on agar plates, were arginine dehydrogenase negative, were lysine and ornithine decarboxylase and gelatinase positive, and were capable of utilizing sucrose. These bacteria were identified as *Vibrio alginolyticus* strains. All of the American Type Culture Collection *Vibrio* cultures belonged to taxon 8. Taxon 10 contained five unusual, pleomorphic degraders. These isolates were gram variable and formed star-shaped clusters when they were grown in cultures that were shaken and dense tangled mats when they were grown statically. Taxa 11 and 12 resembled *Burkholderia cepacia*. Taxa 15 and 16 were made up of *Sphingomonas* spp. Taxa 19 through 21 consisted of *Flavobacter*-like gram-negative, nonmotile, yellow-pigmented bacteria. These bacteria were oxidase positive and negative for all of the rest of the tests except the nitrite reduction and mannitol utilization tests. Finally, taxa 25 through 27 consisted of pigmented acid-fast bacteria identified as *Mycobacterium* spp.

Figure 2 shows the change in the composition of the phenanthrene-degrading community with sampling date. The predominant group of phenanthrene degraders varied with time. Flu-

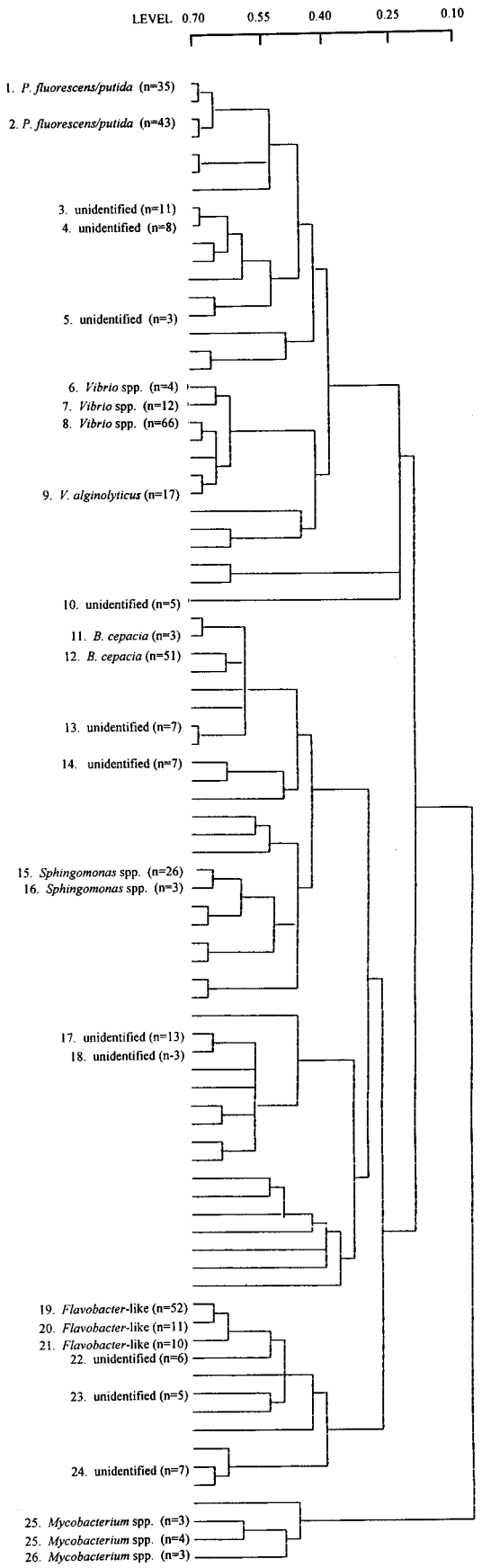

FIG. 1. Phenotypic similarities between isolates. Levels of similarity were calculated by using Jaccard's index, and clustering was by the UPGMA method. At a similarity level of 70%, 83 taxonomic units were present. Taxonomic units containing three or more strains are numbered, and the presumptive identity of each, if known, is indicated.

orescent pseudomonads (*P. putida* and *P. fluorescens*) predominated in May 1992, comprising 64% of all degraders that were isolated. In June 1992, fluorescent pseudomonads and *Flavobacter*-like spp. together accounted for 42% of the total, but unidentified bacteria comprised another 42%. In May 1993, 71% of the sample consisted of *Flavobacter*-like species. In June 1993, *Vibrio* spp. were the most numerous organisms (88% of the degraders isolated). In the March 1994 sample, 46% of the isolates were *Flavobacter*-like species, while the rest were mostly unidentified. On the last sample date, in June 1994, all of the groups were present, and no single group predominated.

None of the colonies hybridized strongly to either gene probe, compared to the control reactions. However, 11 of the March and June 1994 isolates (2.5% of the total isolates) hybridized weakly to the *nahAaAb* gene probe, which encodes the naphthalene dioxygenase. A greater proportion (11.7%) hybridized weakly to the 16-kb gene cluster, indicating that there was some homology with the upper-pathway genes other than the dioxygenase gene. In all, only 55 of 437 isolates (12.8%) hybridized to the *nahAaAb* probe or the 16-kb probe encoding the upper pathway of phenanthrene degradation (Table 2).

A cluster analysis was performed to determine the relatedness of samples with respect to the taxonomic structure. The resulting dendrogram, based on the CNESS index, reveals that all 12 samples taken at one time in June 1994 formed one distinct group, that the two samples taken at the same time in May 1993 also formed a distinct group, and that the two samples taken a month apart in 1992 formed a third group (Fig. 3). The organisms in the June 1993 sample, which consisted mostly of *Vibrio* spp., were not closely related to the other groups.

An AMOVA analysis was performed by using the binary data matrix based on phenotypic characteristics. The analysis required a lower triangular distance-squared matrix; therefore,

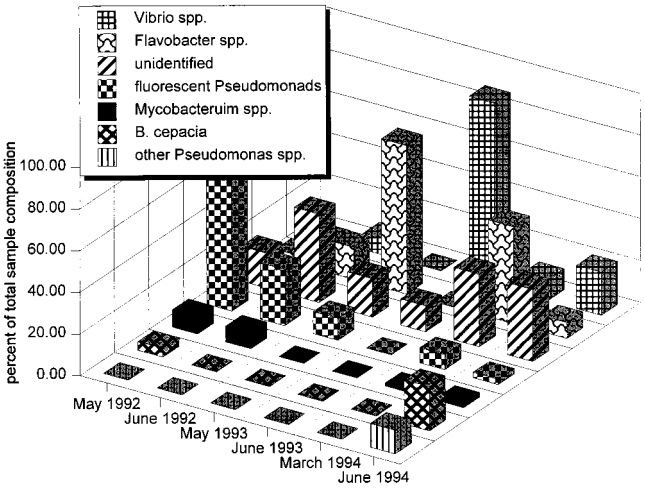

FIG. 2. Relative changes in species compositions of degrader communities.

TABLE 2. Colony hybridization of phenanthrene-degrading isolates to DNA probes constructed from genes encoding PAH-catabolic pathways

Sample date	Total no. of isolates	No. of isolates weakly hybridizing to:	
		nahAaAb probe[b]	pY3-E16[b]
May 1992	25	0	3 (12)[c]
June 1992	26	0	6 (23)
May 1993	38	0	10 (26)
June 1993	41	0	0
March 1994	70	8 (11)	4 (6)
June 1994	237	3 (1)	28 (12)

[a] The *nahAaAb* probe is a 2.5-kb DNA probe encoding reductase$_{nap}$ and ferredoxin$_{nap}$ of the naphthalene dioxygenase gene.
[b] pY3-E16 is a 16-kb DNA probe encoding the upper pathway for phenanthrene, naphthalene, and fluorene degradation and includes *nahAaAb*.
[c] The values in parentheses are percentages.

(1 − Jaccard's coefficient)^2 was used. The analysis was based on the data for the six sampling dates, which formed six test groups. Four of the groups consisted of one PAH-degrading community, one group (May 1993) consisted of two communities, and the June 1994 group consisted of 12 communities (total number of communities, 18). The design of the analysis is consistent with the design of a nested analysis of variance. The results of the AMOVA analysis indicate that 20.91% of the phenotypic variation was temporal and only 4.7% of the variation was spatial. The preponderance of the variation (74.3%) was attributed to differences among individuals in each community (Table 3). The phenotypic differences are significant ($P < 0.002$).

TABLE 3. Analysis of variance of phenotypic distances for all populations

Source of variation	Degrees of freedom	Variance	% of total variance	*P* value
Among sample dates	5	0.0498	21.06	<0.001
Among populations, within dates	12	0.0110	4.67	<0.001
Within populations	417	0.1755	74.27	<0.001

DISCUSSION

Our results indicate that the natural communities of phenanthrene-degrading bacteria in an intertidal sediment site are taxonomically diverse. This paradigm supports the emerging model of high microbial diversity in aquatic environments (2) and soils (31). We tentatively identified members of several genera, including the genera, *Pseudomonas*, *Burkholderia*, *Sphingomonas*, *Flavobacter*, *Vibrio*, and *Mycobacterium*. Many of these taxa occurred concurrently as a community of PAH-degrading bacteria in a single 0.5-g (wet weight) sediment sample. In addition, 140 isolates, or 32% of the total isolates, remained unidentified. While the ability to degrade phenanthrene is known to be spread widely across genera, this is the first time that such diversity has been reported from a single site. This high diversity is most likely a result of picking the phenanthrene degraders from primary spread plates. An alternative method, enrichment culturing, is typically used to isolate PAH-degrading bacteria, but enrichment selects for only the most rapidly growing strains under laboratory conditions. Even in this study, the diversity of PAH degraders was probably

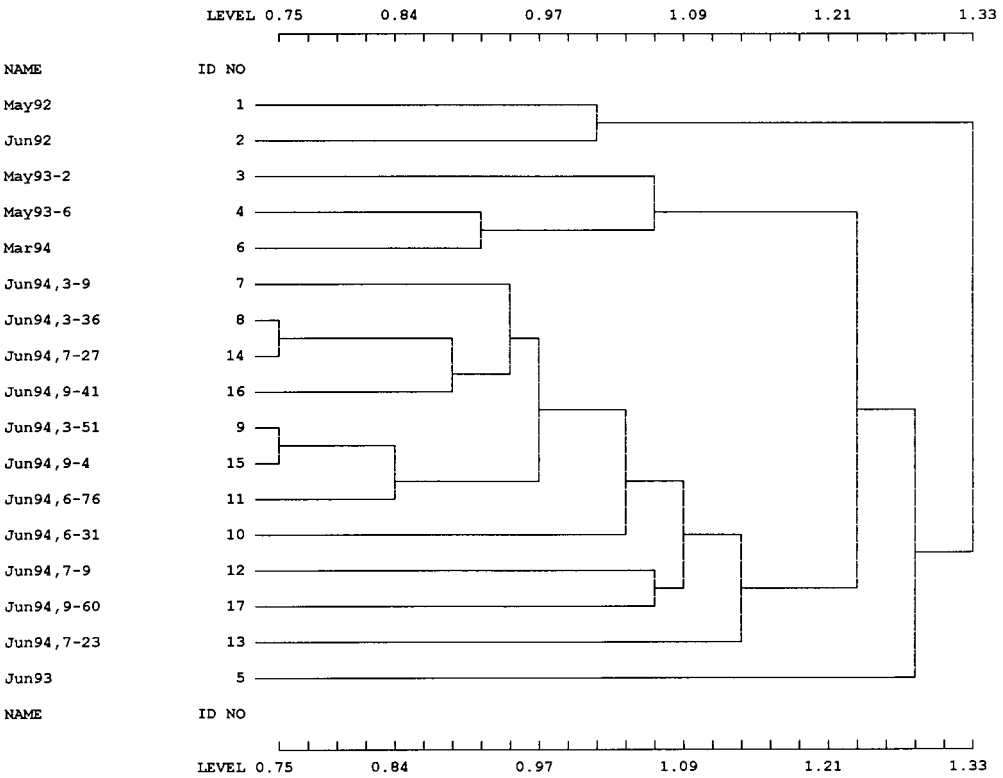

FIG. 3. Dendrogram showing the relationships among the phenanthrene-degrading communities. The analysis was performed by determining how many isolates belonging to each of the 83 taxa were present in each community. Communities are identified by sampling date and subsample number.

underestimated, since our methods were dependent on cell growth.

The spatial variation in the intertidal site among the communities of PAH degraders was low compared to the temporal variation. A separate AMOVA addressing the phenotypic variation between and among the isolates from 12 replicate core samples taken simultaneously showed that only 5% of the phenotypic variation was among the communities (Table 3). This indicates that there was little spatial variation in the site, whose area was approximately 1 m^2. In contrast to the small spatial variation, the structure of the PAH-degrading communities changed significantly with time. In some communities, one taxon predominated. For example, 88% of the June 1993 sample was comprised of *Vibrio* spp., and 71% of the March 1994 sample contained *Flavobacter*-like spp. The pooled AMOVA results indicate that the majority (74.27%) of the phenotypic variation was found within each of the 18 communities examined. Only 4.67% of the total variation was present among the 12 June 1994 and 2 May 1993 communities. A substantial portion (21%) of the change in phenotypes was temporal. Thus, while each community itself was very diverse, there was a distinguishable change in the taxonomic composition of the communities at each sampling time.

All of the identified genera detected in Savin Hill Cove sediments have been previously isolated as phenanthrene degraders from aquatic sediments (11, 34). García-Valdés et al. (11) meticulously identified the naphthalene degraders *Pseudomonas aeruginosa* and *P. putida* from coastal sediments. Many of the phenanthrene-degrading bacteria isolated in our study were identified as pseudomonads or pseudomonad-like bacteria. Thus, pseudomonads appear to be as important in coastal ecosystems as they are in soils (5). We also isolated typical coastal bacteria (i.e., *Vibrio* spp.) that are capable of phenanthrene degradation. West et al. (34) have previously described *Vibrio* isolates as phenanthrene degraders in coastal sediments.

The DNA-DNA hybridization results indicate that naphthalene dioxygenase and the *P. putida* NCIB 9816 phenanthrene- and naphthalene-degradative genes play only a minor role in the Savin Hill Cove intertidal site. This confirms the results of a previous survey of naphthalene-degrading isolates from soils, freshwater, and marine sediments (20). In that report, only 28.8% of the marine naphthalene degraders hybridized to a *nahABCD* probe. It is not unexpected that these genes are not predominant in marine isolates, since they were isolated from a soil pseudomonad. Some of the isolates did hybridize weakly with the 16-kb probe but not with the *nahAaAb* probe, which suggests that there is some homology with at least a portion of the upper-pathway genes. The upper pathway includes genes that encode dehydrogenases, oxygenases, and a ring fission dioxygenase. These genes are not homologous with the archetypical dioxygenase genes of *P. putida* NCIB 9816.

While none of the isolates in this study displayed strong homology to the *nahAaAb* and 16-kb probes, it was possible to isolate strongly hybridizing phenanthrene-degrading bacteria from Savin Hill Cove intertidal sediments with naphthalene enrichment cultures (unpublished results). This suggests that the genes which we examined are present only as a minor fraction of the PAH-degradative genes in the community. Ultimately, the relative roles of various PAH-degrading bacteria and their pathways in nature should be examined with nonculture methods, but such an analysis must await isolation and genetic study of the predominant PAH degraders. Recently, Goyal and Zylstra (12) have cloned novel genes for phenanthrene oxidation in *Comamonas testosteroni*.

Our results are a major step in understanding the ecology of PAH-degrading bacteria in estuarine sediments. The work presented here indicates that the phenanthrene-degrading bacterial community in Savin Hill Cove intertidal sediments is a dynamic system. One source of diversity is the microadaptation of bacteria to the microhabitats on sediment particles (35). Environmental factors, such as temperature, salinity, predation, and organic loading, can also affect the composition of a microbial community (18). These are significant considerations in understanding the fate of PAHs in the system and for developing bioremediation protocols. Our results also show that a single snapshot of a natural community of degraders is not sufficient to characterize a degrading community.

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REFERENCES

1. **Anderson, J. I. W.** 1962. Studies on micrococci isolated from the North Sea. J. Appl. Bacteriol. **25:**362–368.
2. **Barns, S. M., R. E. Fundyga, M. W. Jeffries, and N. R. Pace.** 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc. Natl. Acad. Sci. USA **91:**1609–1613.
3. **Boldrin, B., A. Tiehm, and C. Fritzsche.** 1993. Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a *Mycobacterium* sp. Appl. Environ. Microbiol. **59:**1927–1930.
4. **Burback, B. L., and J. J. Perry.** 1993. Biodegradation and biotransformation of groundwater pollutant mixtures by *Mycobacterium vaccae*. Appl. Environ. Microbiol. **59:**1025–1029.
5. **Cerniglia, C. E., and M. A. Heitkamp.** 1989. Microbial degradation of polycyclic aromatic hydrocarbons (PAH) in the aquatic environment, p. 41–68. *In* U. Varanasi (ed.), Metabolism of polycyclic aromatic hydrocarbons (PAH) in the aquatic environment. CRC Press, Boca Raton, Fla.
6. **Davies, J. I., and W. C. Evans.** 1964. Oxidative metabolism of naphthalene by soil pseudomonads. Biochem. J. **91:**251–261.
7. **Djomo, J. E., V. Ferrier, L. Gauthier, C. Zollmoreux, and J. Marty.** 1995. Amphibian micronucleus test in vivo: evaluation of the genotoxicity of some major polycyclic aromatic hydrocarbons found in a crude oil. Mutagenesis. **10:**223–226.
8. **Dunn, N. W., and I. C. Gunsalus.** 1973. Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. J. Bacteriol. **114:** 974–979.
9. **Dyksterhouse, S. E., J. P. Gray, R. P. Herwig, J. C. Lara, and J. T. Staley.** 1995. *Cycloclasticus pugetii* gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium from marine sediments. Int. J. Sys. Bacteriol. **45:**116–123.
10. **Excoffier, L., P. E. Smouse, and J. M. Quattro.** 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics **131:**479–491.
11. **García-Valdés, E., E. Cózar, R. Rotger, J. LaLucat, and J. Ursing.** 1988. New naphthalene-degrading marine *Pseudomonas* strains. Appl. Environ. Microbiol. **54:**2478–2485.
12. **Goyal, A. K., and G. J. Zylstra.** 1996. Molecular cloning of novel genes for polycyclic aromatic hydrocarbon degradation from *Comamonas testosteroni* GZ39. Appl. Environ. Microbiol. **62:**230–236.
13. **Grifoll, M., M. Casellas, J. M. Bayona, and A. M. Solanas.** 1992. Isolation and characterization of a fluorene-degrading bacterium—identification of ring oxidation and ring fission products. Appl. Environ. Microbiol. **58:**2910–2917.
14. **Grund, E., B. Denecke, and R. Eichenlaub.** 1992. *Rhodococcus* sp. strain B4, isolated from a soil sample contaminated with polycyclic aromatic hydrocarbons, grows with naphthalene as the sole source of carbon and energy. Appl. Environ. Microbiol. **58:**1874–1877.
15. **Hansen, G. H., and R. Sorheim.** 1991. Improved method for phenotypical characterization of marine bacteria. J. Microbiol. Methods **13:**231–241.
16. **Kennish, M. J.** 1992. Ecology of estuaries: anthropogenic effects. CRC Press, Inc., Boca Raton, Fla.
17. **Krieg, N. R., and Holt, J. G. (ed.).** 1984. Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore, Md.
18. **Lee, S., and J. A. Fuhrman.** 1991. Spatial and temporal variation of natural bacterioplankton assemblages studied by total genomic DNA cross-hybridization. Limnol. Oceanogr. **36:**1277–1287.
19. **MacGillivray, A. R., and M. P. Shiaris.** 1993. Biotransformation of polycyclic aromatic hydrocarbons by yeasts isolated from coastal sediments. Appl. Environ. Microbiol. **59:**1613–1618.

20. **MacGillivray, A. R., and M. P. Shiaris.** 1995. Microbial ecology of polycyclic aromatic hydrocarbon (PAH) degradation in coastal sediments, p. 125–147. *In* G. R. Chaudhry (ed.), Biological degradation and bioremediation of toxic chemicals. Timber Press/Discorides Press, Portland, Oreg.
21. **Means, J. C., J. J. Hassett, S. G. Wood, and W. L. Banwart.** 1980. Sorption properties of polynuclear aromatic hydrocarbons by sediments and soils. Environ. Sci. Technol. **14:**1524–1528.
22. **Mueller, J. G., P. J. Chapman, B. O. Blattmann, and P. H. Pritchard.** 1990. Isolation and characterization of a fluoranthrene-utilizing strain of *Pseudomonas paucimobilis*. Appl. Environ. Microbiol. **56:**1079–1086.
23. **Mueller, J. G., R. Devereux, D. L. Santavy, S. E. Lantz, S. G. Willis, and P. H. Pritchard.** 1997. Phylogenetic and physiological comparisons of PAH-degrading bacteria from geographically diverse soils. Antonie Leeuwenhoek In. J. Gen. Mol. Microbiol. **71:**329–343.
24. **Rohlf, F. J.** 1993. NTSYS-pc: Numerical taxonomy and multivariate analysis system, 1.8 ed. Exeter Software, Stony Brook, N.Y.
25. **Schell, M. A.** 1983. Cloning and expression in *Escherichia coli* of the naphthalene degradation genes from plasmid NAH7. J. Bacteriol. **153:**822–829.
26. **Shiaris, M. P., and D. Jambard-Sweet.** 1986. Distribution of polycyclic aromatic hydrocarbons in surficial sediments of Boston Harbor, Massachusetts, USA. Mar. Pollut. Bull. **17:**469–472.
27. **Shiaris, M. P., A. C. Rex, G. W. Pettibone, K. Keay, P. McManus, M. A. Rex, J. Ebersole, and E. Gallagher.** 1987. Distribution of indicator bacteria and *Vibrio parahaemolyticus* in sewage-polluted intertidal sediments. Appl. Environ. Microbiol. **53:**1756–1761.
28. **Simon, M. J., T. D. Osslund, R. Saunders, B. D. Ensley, S. Suggs, A. Harcourt, W. C. Suen, D. L. Cruden, D. T. Gibson, and G. J. Zylstra.** 1993. Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains PpG7 and NCIB-9816-4. Gene **127:**31–37.
29. **Sisler, F. D., and D. E. ZoBell.** 1947. Microbial utilization of carcinogenic hydrocarbons. Science **106:**521–522.
30. **Sneath, P. H. A., and R. Sokal.** 1973. Numerical taxonomy: the principles and practice of numerical classification. W. H. Freeman, San Francisco, Calif.
31. **Torsvik, V., K. Salte, R. Sorheim, and J. Goksoyr.** 1990. Comparison of phenotypic diversity and DNA hgeterogeneity in a population of soil bacteria. Appl. Environ. Microbiol. **56:**776–781.
32. **Trueblood, D. D., E. D. Gallagher, and D. M. Gould.** 1994. 3 Stages of seasonal succession on the Savin Hill Cove mudflat, Boston Harbor. Limnol. Oceanogr. **39:**1440–1454.
33. **Weissenfels, W. D., M. Beyer, and J. Klein.** 1990. Degradation of phenanthrene, fluorene, and fluoranthrene by pure bacterial cultures. Appl. Microbiol. Biotechnol. **32:**479–484.
34. **West, P. A., G. C. Okpokwasili, P. R. Brayton, D. J. Grimes, and R. R. Colwell.** 1984. Numerical taxonomy of phenanthrene-degrading bacteria isolated from the Chesapeake Bay. Appl. Microbiol. Biotechnol. **48:**988–993.
35. **Wise, M. G., L. J. Shimkets, and J. V. Mcarthur.** 1995. Genetic structure of a lotic population of *Burkholderia* (*Pseudomonas*) *cepacia*. Appl. Environ. Microbiol. **61:**1791–1798.
36. **Yang, Y. J., R. F. Chen, and M. P. Shiaris.** 1994. Metabolism of naphthalene, fluorene, and phenanthrene—preliminary characterization of a cloned gene cluster from *Pseudomonas putida* NCIB 9816. J. Bacteriol. **176:**2158–2164.
37. **Yen, K.-M., and C. M. Serdar.** 1988. Genetics of naphthalene metabolism in pseudomonads. Crit. Rev. Microbiol. **15:**247–268.