EPA/600/A-94/198

STUDIES ON THE MICROBIAL ECOLOGY OF POLYCYCLIC AROMATIC HYDROCARBON BIODEGRADATION

J. G. Mueller, S. E. Lantz, R. Devereux, J. D. Berg, and P. H. Pritchard

ABSTRACT

Soils with known history of exposure to polycyclic aromatic hydrocarbons (PAHs) were collected from Norway, Germany, and the United States and screened for the presence of PAH-degrading bacteria. Purified PAH-degrading isolates were characterized by fatty acid profile analysis (GC-FAME), substrate utilization patterns (Biolog™ assays), 16S rRNA sequence comparisons, and total DNA:DNA hybridizations. Microbial respirometry and chemical analyses also were performed to define the PAH-biodegradation potential of these soils. These studies showed that all soils contaminated with PAHs harbored competent PAH-degrading bacteria that are biochemically similar and phylogenetically related. However, bioremediation strategies relying exclusively on indigenous PAH degraders should be closely evaluated for the ability to achieve site-specific cleanup standards in a timely manner.

INTRODUCTION

The use of specially selected microorganisms to enhance bioremediation efforts has proved effective in a number of applications, especially when combined with bioreactor systems (Mueller et al. 1993, Pritchard 1992). In our studies, the successful use of such isolates to remediate soil and water contaminated with organic wood preservatives (e.g., creosote, and pentachlorophenol [PCP]) has resulted in the opportunity to employ these technologies at similarly contaminated sites throughout the world.

Prior to distribution of these bioremediation strategies, concerns regarding the import of nonindigenous microorganisms needed to be addressed. Toward this end, we embarked on a program to ascertain whether (1) microorganisms similar to those used in our bioremediation strategy could be found in soils far removed from each other geographically; (2) previous exposure of soil microorganisms to PAH mixtures affected their PAH-degrading abilities; and (3) introduction of

specially selected inoculant strains would offer any advantages, in terms of operating performance, to bioremediation systems employing indigenous microbiota.

MATERIALS AND METHODS

Acquisition of Soil Samples

Eight samples of soil with a history of exposure to PAH mixtures, such as creosote or diesel fuel, were recovered from a variety of locations. Six of these samples were obtained from creosote-contaminated sites in Norway (long-term exposure), one was sent from a diesel-contaminated site in Germany (recent spill), and one was recovered from an abandoned wood-preserving facility (American Creosote Works [ACW]) in northwest Florida, USA (long-term exposure). Additionally, two soils with no known history of exposure to such chemicals were recovered from agricultural farmland in south-central Illinois, USA.

Soil Analyses

Soil texture, moisture, nutritional status (NH_4-N, NO_3-N, available phosphorus, total phosphorus), water-holding capacity, and pH were determined in accordance with standard methods for soil analyses (Page et al. 1982). Analytical methods for extraction and quantitative determination of pentachlorophenol and 41 creosote constituents by gas chromatography (GC) are described elsewhere (Mueller et al. 1989, 1991).

For microbiological analysis, triplicate 1.0-g samples (wet weight) were placed in 9.0-mL volumes of sterile phosphate buffer (25 mM KH_2PO_4, 25 mM K_2HPO_4, pH 7.1) and shaken vigorously for 15 min (350 rpm). Soil suspensions were allowed to settle for 1 min before serial dilution in the same buffer. Total heterotrophic plate counts were performed with each soil sample using a standard heterotrophic plate count medium (Luria-Bertani agar; Maniatis et al. 1982) and standard microbiological methods (duplicate samples plated in replicate) (Page et al. 1982). Phenanthrene (PHE)- and fluoranthene (FLA)-degrading bacteria were enumerated using an overlay technique (Kiyohara et al. 1981). These values were recorded as the number of colony-forming units (CFUs) on carbon-substrate-free mineral salts agar that cleared the hydrocarbon substrate after 12 to 14 days of incubation at 28°C.

Soil Enrichment with PAHs

Soils were enriched for PAH-degrading microorganisms by adding 5.0 mL of a 10% soil slurry (prepared in sterile mineral salts medium) to 250-mL Erlenmeyer flasks containing 45 mL of sterile mineral salts medium (Mueller et al. 1990). Either PHE or FLA previously had been added to each flask in sterile acetone (evaporated) for a PAH concentration of 500 mg/L. Soils were incubated in the dark with shaking (150 rpm) at 30°C for 14 days. Following 14 days of

aerobic incubation and enrichment, cultures were diluted 1:5 (vol/vol) with fresh mineral salts medium including PAHs at 500 mg/L. This transfer procedure was repeated two more times for a total of four enrichments over a 10-week period.

Screening Enrichment Cultures for PAH Degraders

After the second and fourth enrichments, liquid samples were removed from each vessel and screened for the presence of bacteria capable of using PHE and FLA as primary growth substrates. Once individual colonies were single-colony purified, they were transferred to utilizable-carbon-free agar and complex agar, then overlain with PHE or FLA as described by Kiyohara et al. (1981). Plates were incubated for 14 days prior to scoring for individual colonies exhibiting zones of clearing of the PAH substrate.

Colonies demonstrating PAH-clearing abilities were purified and transferred to 125-mL Erlenmeyer flasks containing 25 mL mineral salts broth plus 500 mg/L PHE or FLA as the sole carbon and energy source. Cultures were incubated for 5 to 7 days with shaking (150 rpm) at 30°C. Bacterial growth at the expense of the PAH substrates was measured by visual assessment of turbidity and by monitoring changes in absorbance at 550 nm. As a control, growth in carbon-free mineral salts broth also was monitored.

Microbiological Characterizations

Once the PAH-degrading ability of purified cultures had been validated, cultures were characterized by GC-FAME and the Biolog™ Microplate System™ (Microbe Inotech Laboratories, Inc., St. Louis, Missouri). The taxonomic relationships among these strains was analyzed by evaluating similarity measures from GC-FAME and substrate utilization patterns with principal component analysis (Jacobs 1990).

Phylogenetic relationships were determined by 16S ribosomal RNA sequence comparisons. Universal primers and the polymerase chain reaction were used to amplify 16S rRNA genes (Weisburg et al. 1991) from select PAH-degrading bacterial strains CRE7, CRE11, CRE12, *Pseudomonas paucimobilis* strain EPA505, and the type strain of *Pseudomonas paucimobilis* ATCC 29837. Reverse transcriptase sequencing with the rRNA template as the primer was used to generate 16S rRNA sequence information for select PAH-degrading strains (Lane et al. 1985). Total DNA:DNA hybridizations were performed between select PAH-degraders to define homology (Amann et al. 1992).

Manometric Respirometry Studies

Into 14 flasks (250-mL Erlenmeyer flasks), each containing 10 mL of a 33% soil slurry, was added a predetermined level of inorganic nutrients. Duplicate flasks of each slurry were amended with 500 mg/L naphthalene (NAH), PHE, or FLA; readily utilizable carbon (250 mg/L glucose + 250 mg/L glycerol); or 500 mg/L specification creosote no. 450 (American Wood-Preserver's Association).

Two flasks received no supplemental carbon (to discern the effect of nutrient amendment and aeration), and two more flasks served as killed cell controls (acidified to pH 2.0 with 1 N HCl plus 3.7% formaldehyde) for each soil tested.

Microbial respirometric responses (rate of liberation of CO_2 and the simultaneous consumption of O_2 were determined at 8-hr intervals over an 8-day incubation period (23°C, 100 rpm shaker speed) with a MicroOxymax respirometer (Columbus Instruments, Columbus, Ohio). At the end of each incubation period, slurries from nutrient-amended only, creosote-amended, and killed-cell (control) treatments were extracted and analyzed for the presence of creosote constituents as previously described (Mueller et al. 1991). These values were compared with those determined at time zero for each soil.

RESULTS AND DISCUSSION

Soil Analyses

The results of the physicochemical analyses used to characterize the soils are summarized in Table 1. In general, all measured soil parameters were within a range conducive to biological activity. However, contaminated soils had rather low levels of available nitrogen and, to a certain extent, available phosphorus (Bray P1).

Microbiological analysis of soils prior to enrichment with PAHs showed that all soils harbored culturable heterotrophic bacteria (Table 2). With the exception of the ACW 47 site, all soils with a history of exposure to PAHs also had a discernible number of PHE-degraders, and, with the exception of soils UN 1 and ACW 47, a relatively high number of FLA-degraders. Conversely, the "control soils" (SIU ARC, SIU BRC), with no known history of exposure to PAHs, had no detectable PHE- or FLA-degraders.

The presence of PCP at a relatively high concentration (>250 mg PCP/kg soil dry wt) in soil collected from the abandoned American Creosote Works site (sample ACW 47), in combination with creosote at an average concentration >500 mg creosote PAHs/kg soil dry wt), may represent the reason for the low initial numbers of PHE- and FLA-degraders (i.e., toxicity). In the case of the UN 1 soil sample, the only soil being recently impacted by diesel fuel (accidental highway spill) and not long-term creosote exposure (<1 month exposure at the time of sampling), the high number of PHE-degraders along with a rather low number of FLA-degraders may be the result of low-level exposure to PHE (e.g., diesel vapors) or adaptation to structurally related compounds (Bauer & Capone 1988).

PAH Enrichment Studies

Using PHE as a growth substrate, all soils except SIU ARC and SIU BRC produced turbid cultures within the first week of the first enrichment. All subsequent transfers produced turbid cultures within the first 2 to 3 days of incubation.

TABLE 1. Description and characterization of soils used for the isolation of PAH-degraders.

Origin/Location of Soil	Exposure History	Classification	Nutrient Analysis (mg/kg soil dry wt)				pH	Field Capacity (% wgt)
			NH_4-N	NO_3-N	Bray P1	Bray P2		
Norwegian Soils								
Rade 1	Creosote	Loamy sand	2	10	0	28	6.4	7.1
Rade 2	Creosote	Sand	4	6	6	13	5.9	7.4
Lillestrøm 1	Creosote	Sand	10	6	10	19	6.2	16.0
Lillestrøm 2	Creosote	Loam	3	5	16	28	7.2	37.0
Drammen	Creosote	Sand	1	6	12	25	7.4	8.1
Hommelvik	Creosote	Loamy sand	9	31	3	23	7.2	10.7
German Soil								
UN 1	Mixed PAHs	Sand	1	0	82	87	7.4	23.9
American Soils								
ACW 47	Creosote/PCP	Sand	3	1	20	20	6.7	14.9
SIU ARC	None known	Silt loam	20	13	34	47	6.8	29.2
SIU BRC	None known	Silt loam	0	9	35	48	6.4	20.3

TABLE 2. Enumeration of total aerobic, culturable heterotrophic, phenanthrene-, and fluoranthene-degrading bacteria in soils.

Soil[a]	Total Heterotrophs[b]	Phenanthrene Degraders[c]	Fluoranthene Degraders[c]
	log CFU/mL slurry[d]		
Rade 1	6.47	4.87	5.82
Rade 2	6.00	4.44	5.85
Lillestrøm 1	7.57	3.93	5.26
Lillestrøm 2	7.63	3.65	3.74
Drammen	6.18	4.13	5.16
Hommelvik	6.98	4.04	3.85
UN 1	6.43	5.49	<3.00
ACW 47	6.60	<3.00	<3.00
SIU ARC	7.00	<2.00	<2.00
SIU BRC	7.31	<2.00	<2.00

(a) See Table 1 for description of soils.
(b) Total heterotrophs on Luria-Bertani agar after 5 days incubation at 28°C.
(c) PAH-degraders based on the number of colonies to clear PAH substrates on minimal medium after 14 days incubation at 30°C.
(d) CFU = colony forming units.

Simultaneously, all undissolved PHE crystals were removed, and rapid changes in medium coloration were observed.

Using FLA as an enriching substrate, the observed growth responses were very similar to those recorded in the presence of PHE. Here, fluoranthene biodegradation was apparent within the first 10 days of enrichment for all soils except SIU ARC, SIU BRC, UN 1, and Hommelvik. Growth (as determined by visually apparent increases in turbidity, change in medium coloration, and disappearance of undissolved FLA crystals) with inocula from Hommelvik and UN 1 soil became evident within 21 days of enrichment. Following 40 days of incubation, biodegradation or solubilization of PHE and FLA in liquid medium inoculated with microorganisms recovered from the SIU ARC and SIU BRC soils was not observed.

The enrichment culture conditions used in these studies (e.g., excess inorganic nutrients, elevated temperatures, mixing, and aqueous solutions saturated with PAHs) are substantially different than expected environmental conditions. However, they closely resemble conditions associated with bioreactor operations that are widely used in the bioremediation industry (Berg et al., this volume; Mueller et al. 1993). Thus, while the relatively low number of PAH degraders from unexposed sites may not fully reflect the potential for long-term adaptation, the fact that non-PAH-history soils did not readily yield PHE or FLA degraders suggests that, in the event of recent contamination, bioremediation of PAHs on a short-term basis may require the use of inoculants.

Respirometric Analyses

The respiratory activity of indigenous microflora from all soils with a recorded history of exposure to PAHs was stimulated upon the addition of all organic carbon sources, as well as with the addition of inorganic nutrients alone. Using soil from the Hommelvik site as a typical example, increased respiratory responses were observed upon the addition of individual PAHs (NAH, PHE, or FLA) and creosote (Figure 1, top and middle panels). These responses were shown to be above and beyond that expected from the conversion of resident carbon, which includes creosote PAHs, due to the addition of inorganic nutrients and aeration. Hence, these chemicals represented utilizable carbon sources to the indigenous microflora. Conversely, both soils with no known exposure to PAHs (SIU soils) showed very limited response to the addition of PAHs or inorganic nutrients, but the indigenous soil microflora rapidly mineralized added glucose and glycerol (Figure 1, bottom panel).

In all cases, no activity was observed in the poisoned systems. Compared with analytical chemistry data, increases in respiratory activities generally were associated with accelerated biodegradation of monitored creosote constituents (data not shown).

Microbial Ecology and Bacterial Taxonomy

All soils with a history of PAH exposure, except ACW 47, harbored PHE- and FLA-degrading bacteria of various genera. Of the many isolates recovered, the degradative abilities of 13 PHE-degraders, 14 FLA-degraders, and 1 pentachlorophenol degrader (Resnick & Chapman 1990), isolated from myriad contaminated sites, were positively verified (Table 3, data not shown). These strains were processed for characterization and identification based on GC-FAME and Biolog™ assays (Table 4).

Because the established databases for both GC-FAME and Biolog™ assays are focused predominantly on the identification of pathogenic microorganisms and those of clinical importance, many of the identifications made have low similarity coefficients (Table 4). Hence, most of the identifications were considered to be suggestive rather than conclusive. Principal component analyses using GC-FAME profiles of each of the PAH-degrading bacteria showed that many microorganisms isolated from U.S. soils were closely related to microorganisms recovered from other soils in the United States and Europe (Figure 2).

For example, strain CRE7 (PHE-degrader isolated from the ACW site at Pensacola, Florida, and used commercially in a bioremediation process) was found to be closely related to PHE-degrading strains N2P5 and N2P6 (Rade soil, Norway); strains N3P2 and N3P3 (Lillestrøm soil, Norway); and strains PJC 2288, 2289, and 2295 (PAH degraders also from the Pensacola site). Likewise, FLA-degrading bacteria very similar to strain EPA505 were recovered from Germany (strains G1F1 and G1F2) and geographically separate sites in the United States (strains PJC 2286, 2287). Gram-positive "mycobacterial" strains PJC 2282, PJC 2283, and FDA PYR-1 were found to be very closely related to each other, but, as expected, distinctly different from Gram-negative bacterial isolates.

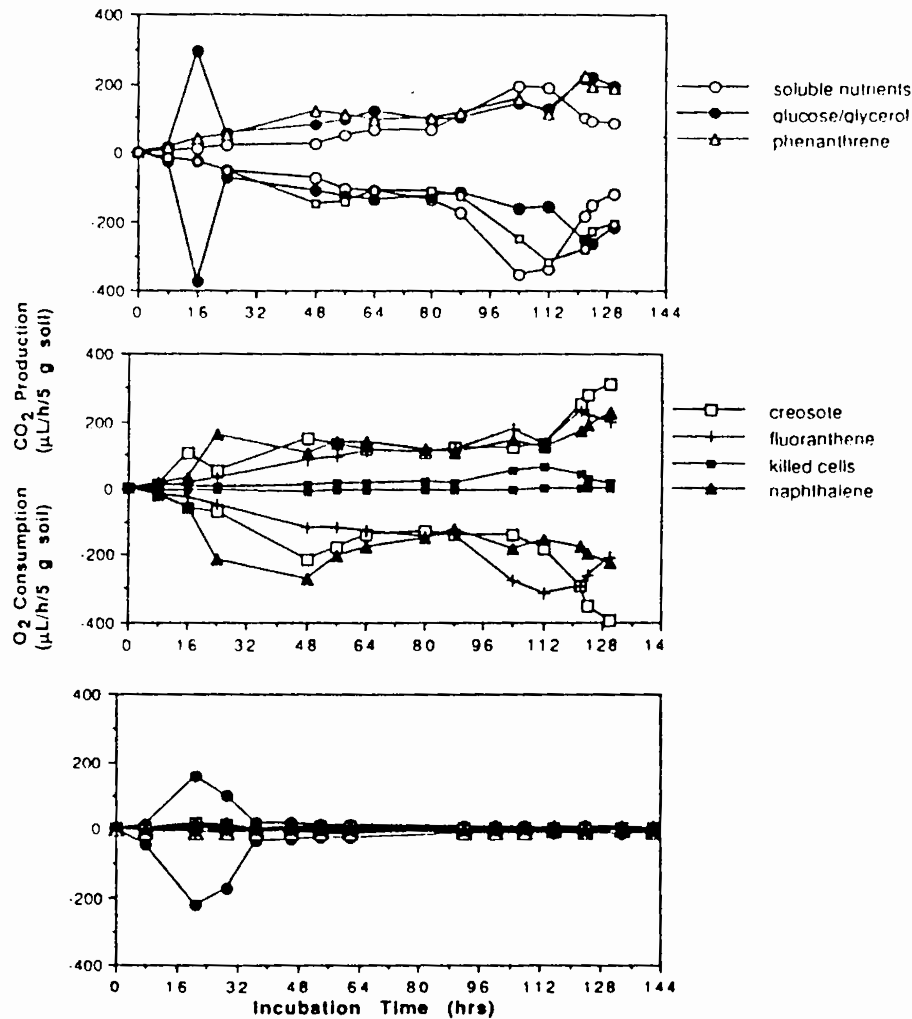

FIGURE 1. Respiratory response of indigenous microorganisms to organic and inorganic amendments. Respiratory activity of soil microorganisms present in Hommelvik soil contaminated with creosote (top and middle panels), and SIU ARC farmland soil with no known history of exposure to PAHs (bottom panel).

Phylogenetic studies using 16S rRNA sequence comparisons showed that two PAH-degrading strains, isolated from creosote-contaminated sites in the USA (strains CRE7 and CRE11), were related to *Pseudomonas aeruginosa*. The 16S rRNA sequences between the PAH-degraders and *P. aeruginosa* were 92 to 93% similar. Correlations developed between 16S rRNA sequence similarity and %DNA relatedness (Amann et al. 1992, Devereux et al. 1990) suggest that, at 92% 16S rRNA

TABLE 3. Bacteria isolated from PAH-contaminated sites for their ability to degrade phenanthrene or fluoranthene.

Isolate Number	Culture Number	Source/Reference	Enrichment Substrate	Soil of Origin
1	EPA505	Mueller et al. 1990	Fluoranthene	Pensacola, Florida
2	PJC 2282	U.S. EPA, GBERL	Fluoranthene/pyrene	Pensacola, Florida
3	PJC 2283	U.S. EPA, GBERL	Fluoranthene/pyrene	Live Oaks, Florida
4	PJC 2285	U.S. EPA, GBERL	Fluoranthene	Live Oaks site, Florida
5	PJC 2286	U.S. EPA, GBERL	Fluoranthene	Live Oaks site, Florida
6	PJC 2287	U.S. EPA, GBERL	Fluoranthene	Pensacola, Florida
7	PJC 2288	U.S. EPA, GBERL	Phenanthrene	Pensacola, Florida
8	PJC 2289	U.S. EPA, GBERL	Phenanthrene	Live Oaks, Florida
9	PJC 2295	U.S. EPA, GBERL	Phenanthrene	Pensacola, Florida
10	CRE7	Mueller et al. 1989	Phenanthrene	Pensacola, Florida
11	CRE11	Mueller et al. 1989	Phenanthrene	Pensacola, Florida
12	CRE12	Mueller et al. 1989	Phenanthrene	Pensacola, Florida
13	AK Phen6	Mueller et al. 1992	Phenanthrene	Prince William Sound, Alaska
14	N1F1	This study	Fluoranthene	Rade, Norway
15	N2P5	This study	Phenanthrene	Rade, Norway
16	N2P6	This study	Phenanthrene	Rade, Norway
17	N3P2	This study	Phenanthrene	Lillestrøm, Norway
18	N3P3	This study	Phenanthrene	Lillestrøm, Norway
19	N3F1	This study	Fluoranthene	Lillestrøm, Norway
20	N3F2	This study	Fluoranthene	Lillestrøm, Norway
21	N4F4	This study	Fluoranthene	Lillestrøm, Norway
22	N5F4	This study	Fluoranthene	Drammen, Norway
23	N6F4	This study	Fluoranthene	Hommelvik, Norway
24	G1F1	This study	Fluoranthene	Germany
25	G1F2	This study	Fluoranthene	Germany
26	G1P1	This study	Phenanthrene	Germany
27	G2P2	This study	Phenanthrene	Germany
28	FDA PYR-1	Heitkamp & Cerniglia 1988	Pyrene	Port Aransas, Texas
29	SR3	Resnick & Champman 1990	Pentachlorophenol	northwest Florida

TABLE 4. Bacterial identifications based on GC-FAME and Biolog™ assays.

Culture Number	GC-FAME Identification (similarity coefficient)	Biolog™ Identification[a] (similarity coefficient)
EPA505	*Pseudomonas paucimobilis* (0.13)	No ID [insufficient growth 36 h]
PJC 2282	*Mycobacterium parafortuitum* (0.056)	*Corynebacterium jeikeium* (0.632)
PJC 2283	*Mycobacterium parafortuitum* (0.025)	No ID [*Corynebacterium variabilis* (0.425)]
PJC 2285	No ID [*Pediococcus halophilus* (NA)]	No ID [*Corynebacterium jeikeium* (0.361)]
PJC 2286	*Enterococcus faecium* (0.011)	No ID [*Micrococcus luteus* (0.396)]
PJC 2287	*Pseudomonas saccharophila* (0.441)	No ID [*Moraxella atlantae* (0.358)]
PJC 2288	<u>*Pseudomonas pseudomallei*</u> (0.192)	*Pseudomonas cepacia* (0.557)
PJC 2289	<u>*Pseudomonas pseudomallei*</u> (0.177)	*Pseudomonas cepacia* (0.602)
PJC 2295	*Pseudomonas cepacia* (0.059)	*Pseudomonas cepacia* (0.426)
CRE7	*Pseudomonas cepacia* (0.399)	No ID [*Pseudomonas cepacia* (0.401)]
CRE11	*Pseudomonas aeruginosa* (0.778)	*Pseudomonas aeruginosa* (0.779)
CRE12	<u>*Pseudomonas aeruginosa*</u> (0.366)	*Pseudomonas azelaica* (0.678)
AK PHEN6	No ID [*Pseudomonas saccharophila* (NA)	No ID [*Alteromonas haloplanktis* (0.393)]
N1F1	*Xanthomonas maltophilia* (0.35)	*Pseudomonas corrugata* (0.80)
N2P5	*Pseudomonas cepacia* (0.294)	*Pseudomonas phenazinium* (0.634)
N2P6	*Pseudomonas cepacia* (0.114)	*Pseudomonas gladioli* (0.515)
N3P2	*Pseudomonas cepacia* (0.379)	*Pseudomonas gladioli* (0.404)
N3P3	*Pseudomonas acidovorans* (0.165)	No ID [*Comamonas testosteroni* (0.407)]
N3F1	No ID [*Pseudomonas putida* biotype A]	*Xanthomonas maltophilia* (0.78)
N3F2	*Xanthomonas maltophilia* (0.41)	*Xanthomonas maltophilia* (0.73)
N4F4	*Xanthomonas maltophilia* (0.32)	*Comamonas acidovorans* (0.78)
N5F4	<u>*Pseudomonas putida* biotype B</u> (0.54)	No ID [*Alcaligenes paradoxus*]
N6F4	*Alcaligenes paradoxus* biotype II (0.34)	*Xanthomonas maltophilia* (0.78)
G1F1	No ID [*Pseudomonas delafieldii* (NA)]	*Brucella abortus* biovar 2 (0.79)
G1F2	No ID [*Pseudomonas saccharophila* (NA)]	No ID [*Brucella abortus* biovar 2 (NA)]
G1P1	*Pseudomonas saccharophila* (0.11)	No ID [insufficient growth 36 h]
G1P2	*Acinetobacter calcoaceticus* (0.40)	*Acinetobacter genospecies* 13 (0.65)
FDA PYR-1	*Mycobacterium parafortuitum* (0.059)	*Corynebacterium variabilis* (0.59)
SR3	*Pseudomonas saccharophila* (0.603)	No ID [poor growth]

(a) Closest species identification listed in brackets [ ] when no identification is made. Underscore indicates a match to the clinical database of Microbe Inotech Laboratories, St. Louis, MO.

sequence similarity and ca 50% DNA relatedness, the PAH-degrading strains are related to *P. aeruginosa* at the genus/species level where greater than 20% DNA relatedness indicates a genus-level relationship.

Likewise, results of DNA:DNA hybridization studies showed that strains isolated from Norwegian and U.S. sites also were somewhat related at the genus level. For example, the U.S. isolate strain CRE7 demonstrated 56% and 36% DNA:DNA homology with the Norwegian isolate N2P5 and the German isolate G1P2, respectively, all being isolated for their ability to utilize PHE as a sole carbon source. These relationships follow the GC-FAME principal component analyses presented in Figure 2. Further, the U.S. strain EPA505 demonstrated 24 and 35% DNA:DNA homology with the ATCC type strain of *Pseudomonas paucimobilis* and the Norwegian strain N2P5, respectively.

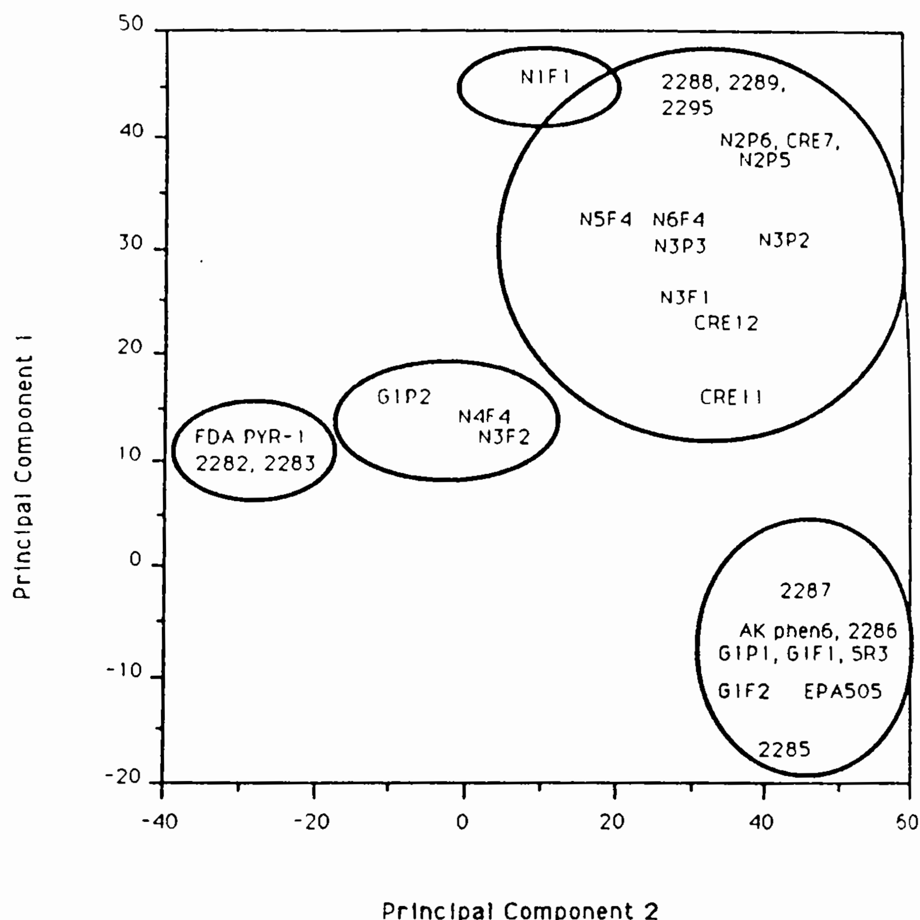

FIGURE 2. Principal component analysis with GC-FAME data from PAH-degrading bacteria isolated from geographically distant PAH-contaminated sites.

CONCLUSIONS

All soils with a history of PAH exposure yielded microbial populations competent for the degradation of the PAHs phenanthrene and fluoranthene. According to GC-FAME, Biolog™, 16S rRNA sequence similarity, and DNA:DNA homology, many of these PAH-degrading microorganisms appeared to be closely related phenotypically and phylogenetically to similar types of organisms isolated from soils at geographically distant sites in the United States. The technique employed to enrich PAH-degrading bacteria thus appeared to select similar types of microorganisms that are indigenous to contaminated soils at each site. Admittedly, this does not necessarily include all types of microorganisms that may play a role in the biodegradation/bioremediation of PAHs (e.g., fungi).

Given that PAH-degraders appeared to be indigenous to geographically diverse PAH-contaminated soils, then if the stimulatory effect of controlled nutriation, mixing, and aeration on the activity of the indigenous microflora results in acceptable rates and extents of biodegradation of targeted chemicals, then, on a site-specific basis, it may be possible to rely solely on the activity of such microorganisms to facilitate site remediation (see Berg et al., this volume). Despite the phylogenetic similarities among these organisms, however, their catabolic abilities would seem to be the most important consideration from a bioremediation perspective. Thus, in the event that indigenous microorganisms do not perform favorably, then utilization of nonindigenous microbes in optimized bioremediation systems could be advantageous for cost-efficient, effective bioremediation. Based on the results of these studies, the export/import of the nonindigenous bacteria used in these studies to augment bioremediation efforts would not seem to represent the introduction of exotic biota, and thus would pose no discernible ecological risk.

ACKNOWLEDGMENTS

We thank Myke'lle Hertsgaard and Barbara Artlet (Technical Resources, Inc., Gulf Breeze, Florida), Sheree Enfinger (U.S. Environmental Protection Agency, Environmental Research Laboratory, Gulf Breeze, Florida), and Stephanie Willis (University of New Hampshire) for technical assistance; Brian Klubek (SIU-Carbondale, Illinois) for soil analyses; Bruce Hemming (Microbe Inotech Laboratories, St. Louis, Missouri) for help in the interpretation of GC-FAME and Biolog™ results; and Peter Chapman (U.S. EPA, ERL, Gulf Breeze, Florida) and Carl Cerniglia (U.S. Food and Drug Administration, National Center for Toxicological Research, Jefferson, Arkansas) for donating PAH-degrading strains for comparative analyses. Soil from Germany was provided by Wolfgang Fabig (Umweltshutz Nord, Germany).

Financial support for these studies was provided by the Norwegian State Railway (NSB) and the U.S. EPA (Gulf Breeze). These studies were performed as part of a Cooperative Research and Development Agreement between the Gulf Breeze Environmental Research Laboratory and SBP Technologies, Inc. (Atlanta, Georgia) as defined under the Federal Technology Transfer Act, 1986 (contract no. FTTA-003).

REFERENCES

Amann, R. I., C. Lin, R. Key, L. Montgomery, and D. A. Stahl. 1992. "Diversity among *Fibrobacter* isolates: Towards a phylogenetic classification." *Syst. Appl. Microbiol.* 15:23-31.

Bauer, J. E., and D. G. Capone. 1988. "Effects of co-occurring aromatic hydrocarbons on the degradation of individual polycyclic aromatic hydrocarbons in marine sediment slurries." *Appl. Environ. Microbiol.* 54:1649-1655.

Berg, J. D., B. Nesgard, R. Gundersen, A. Lorentsen, and T. E. Bennett. 1994. "Washing and slurry phase biotreatment of creosote-contaminated soil." In R. E. Hinchee, A. Leeson,

L. Semprini, and S. K. Ong (Eds.), *Bioremediation of Chlorinated and Polycyclic Aromatic Hydrocarbon Compounds.* Lewis Publishers, Ann Arbor, MI.

Devereux, R., S. H. He, C. L. Doyle, S. Orkland, D. A. Stahl, J. LeGall, and W. B. Whitman. 1990. "Diversity and origin of *Desulfovibrio* species: Phylogenetic definition of a family." *J. Bacteriol. 172*:3609-3619.

Heitkamp, M. A., and C. E. Cerniglia. 1988. "Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field." *Appl. Environ. Microbiol. 54*:1612-1614.

Jacobs, D. 1990. "SAS/GRAPH software and numerical taxonomy." *In Proceedings of the 15th Annual Users Group Conference*, pp. 1413-1418. SAS Institute, Inc., Cary, NC.

Kiyohara, H., K. Nagao, and K. Yaga. 1981. "Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates." *Appl. Environ. Microbiol.* 43:454-457.

Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace. 1985. "Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses." *Proc. Natl. Acad. Sci. USA 82*:6955-6959.

Maniatis, T., E. F. Frisch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual*, p. 68. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mueller, J. G., P. J. Chapman, B. O. Blattmann, and P. H. Pritchard. 1990. "Isolation and characterization of a fluoranthene-utilizing strain of *Pseudomonas paucimobilis.*" *Appl. Environ. Microbiol. 56*:1079-1086.

Mueller, J. G., P. J. Chapman, and P. H. Pritchard. 1989. "Action of a fluoranthene-utilizing bacterial community on polycyclic aromatic hydrocarbon components of creosote." *Appl. Environ. Microbiol. 55*:3085-3090.

Mueller, J. G., S. E. Lantz, B. O. Blattmann, and P. J. Chapman. 1991. "Bench-scale evaluation of alternative biological treatment processes for the remediation of pentachlorophenol- and creosote-contaminated materials: Solid-phase bioremediation." *Environ. Sci. Technol. 25*:1045-1055.

Mueller, J. G., S. E. Lantz, R. J. Colvin, D. Ross, D. P. Middaugh, and P. H. Pritchard. 1993. "Strategy using bioreactors and specially-selected microorganisms for bioremediation of ground water contaminated with creosote and pentachlorophenol." *Environ. Sci. Technol.* (April issue).

Mueller, J. G., S. M. Resnick, M. E. Shelton, and P. H. Pritchard. 1992. "Effect of inoculation on the biodegradation of weathered Prudhoe Bay crude oil." *J. Indust. Microbiol. 10*:95-105.

Page, A. L., R. H. Miller, and D. R. Keeney. 1982. *Methods of Soils Analysis:* Part 2, *Chemical and Microbiological Properties*, 2nd ed. American Society of Agronomy, Madison, WI, 1159 pp.

Pritchard, P. H. 1992. "Use of inoculation in bioremediation." *Curr. Opinions in Biotechnol.* 3:232-243.

Resnick, S. M., and P. J. Chapman. 1990. "Isolation and characterization of a pentachlorophenol-degrading, Gram-negative bacterium." *Abstr. Ann. Meet. Am. Soc. Microbiol.*, p. 300.

Weisburg, W. G., S. M. Barnes, D. A. Pelleteir, and D. J. Lane. 1991. "16S ribosomal DNA amplification for phylogenetic study." *J. Bacteriol. 173*:697-703.

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.: EPA/600/A-94/198

2.

3. REC

5. REPORT DATE

6. PERFORMING ORGANIZATION CODE

4. TITLE AND SUBTITLE
STUDIES ON THE MICROBIAL ECOLOGY OF PAH BIODEGRADATION

7. AUTHOR(S)
J.G. Mueller[1], S. E. Lantz[1], R. Devereux[2], J.D. Berg[3], and P.H. Pritchard

8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
[1]SPB Technologies, Inc., Gulf Breeze, FL;
[3]Aquateam, Oslo, Norway

10. PROGRAM ELEMENT NO.

11. CONTRACT/GRANT NO.

12. SPONSORING AGENCY NAME AND ADDRESS
[2]U.S. ENVIRONMENTAL PROTECTION AGENCY
ENVIRONMENTAL RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
GULF BREEZE, FLORIDA 32561-5299

13. TYPE OF REPORT AND PERIOD COVERED

14. SPONSORING AGENCY CODE

15. SUPPLEMENTARY NOTES
In: Bioremediation of Chlorinated and Polycyclic Aromatic Hydrocarbon Compounds. Robert E. Hinchee, et al. (ed.), Lewis Publishers, Boca Raton, FL. p. 218-230. 1994

16. ABSTRACT

Soils with known history of exposure to polycyclic aromatic hydrocarbons (PAHs) were collected from Norway, Germany and the United States and screened for the presence of PAH-degrading bacteria. Purified PAH-degrading isolates were characterized by fatty acid profile analysis (GC-FAME), substrate utilization patterns (Biolog assays), 16S rRNA sequence comparisons, and total DNA:DNA hybridizations. Microbial respirometry and chemical analyses were also performed to define the PAH-biodegradation potential of these soils. These studies showed that all soils contaminated with PAHs developed competent PAH-degrading bacteria that are biochemically similar and phylogenetically related. However, bioremediation strategies relying exclusively on indigenous PAH degraders should be closely evaluated for the ability to achieve site-specific clean up standards in a timely manner.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES 12
	20. SECURITY CLASS (This page)	22. PRICE