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Identification and Catabolic Activity of Well-Derived Gasoline-Degrading Bacteria from a Contaminated Aquifer

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Approximately 300 gasoline-degrading bacteria were isolated from well water and core material from a shallow coastal aquifer contaminated with unleaded gasoline. Identification of 244 isolates revealed four genera: *Pseudomonas*, *Alcaligenes*, *Nocardia*, and *Micrococcus*, with pseudomonads making up 86.9% of bacteria identified. A total of 297 isolates was sorted into 111 catabolic groups on the basis of aerobic growth responses on 15 gasoline hydrocarbons. Each test hydrocarbon was degraded by at least one isolate. Toluene, *p*-xylene, ethylbenzene, and 1,2,4-trimethylbenzene were most frequently utilized as growth substrates, whereas cyclic and branched alkanes were least utilized. Most isolates were able to grow on 2 or 3 different hydrocarbons, and nearly 75% utilized toluene as a sole source of carbon and energy. Isolates were remarkably specific for hydrocarbon usage, often catabolizing only one of several closely related compounds. A subset of 220 isolates was sorted into 51 groups by polyacrylamide gel electrophoresis. *Pseudomonas aeruginosa* was partitioned into 16 protein-banding groups (i.e., subspecies) whose catabolic activities were largely restricted to substituted aromatics. Different members of subspecies groups defined by protein-banding pattern analysis often exhibited different growth responses on the same hydrocarbon, implying marked strain diversity. The catabolic activities of well-derived, gasoline-degrading bacteria associated with this contaminated aquifer are consonant with in situ adaptation at the site.

Gasoline is a complex mixture of refined petroleum hydrocarbons comprising more than 200 different compounds and stereoisomeric forms differing in their volatilities, solubilities, sorptive characteristics, and subsurface transport properties (2, 24). Some aromatic constituents of gasoline (e.g., benzene) are recognized human carcinogens whose maximum permissible concentrations in potable water supplies are federally regulated (9). Thus, gasoline hydrocarbons originating from underground leaks or surface spills pose a demonstrable threat to the public health and quality of potable groundwater resources. The low solubility of many gasoline constituents coupled with a tendency for hydrocarbons to strongly sorb to aquifer materials enhances the persistence of gasoline constituents in subsurface environments, especially if biotic processes are suppressed (2, 5, 7, 18).

Many gasoline hydrocarbons have been demonstrated to be directly biodegraded (1, 2, 4–6, 13, 22, 26) or cometabolized (2, 16, 17, 23) by native groundwater microflora given appropriate inorganic nutrient supplementation. Despite exploitation of gasoline-degrading microorganisms for purposes of in situ aquifer bioremediation (2, 5, 7, 8, 10, 11, 16, 17, 27–30), there is still very little known about the ecology, taxonomy, and catabolic diversity of gasoline-degrading bacteria associated with areas of subterranean contamination (1–3, 6, 12, 13). Introduction of gasoline hydrocarbons into a previously uncontaminated subsurface environment could be expected to perturb local selective conditions, thereby inducing adaptive responses in the size, structure, physiology, and genetics of indigenous microbial communities (13). The magnitude of and time required for such adaptive changes are, at best, poorly understood. Information concerning the temporal and spatial dynamics of microbial assemblages involved in gasoline hydrocarbon catabolism is critical for accurately evaluating and modeling in situ degradation rates and potentials (5, 7, 18).

A primary objective of the present investigation was to explore and quantify the taxonomic and physiologic diversity of an aerobic, gasoline-degrading bacterial community associated with a shallow coastal aquifer contaminated with unleaded gasoline. In order to accomplish this goal, gasoline-degrading bacteria isolated from observation well groundwater and aquifer cores from this study site were differentiated by three independent methods: (i) determination of growth responses on 15 different hydrocarbon substrates representing the major hydrocarbon categories found in unleaded gasoline, (ii) computer-programmed sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cellular proteins followed by dendrogram analysis, and (iii) identification of isolates by conventional biochemical and nutritional testing. Results obtained by these methods were compared with one another to determine the taxonomic and catabolic diversity of the sample population as well as the potential for microbially mediated degradation of different hydrocarbon contaminants at the site.

MATERIALS AND METHODS

Description of study site and sampling wells. The study site consisted of a shallow coastal aquifer underlying the U.S. Naval Weapons Station in Seal Beach, Calif. The aquifer was composed primarily of silty and sandy alluvial deposits and was contaminated with 20,000 to 40,000 liters of unleaded gasoline from an underground tank (R. Schroeder, U.S. Geological Survey, San Diego, Calif., personal communication). Approximately 30 polyvinylchloride-encased observation wells perforated the aquifer to a maximum depth of about 20 feet (ca. 6 m). The wells were placed in 1984 and 1985 by the U.S. Geological Survey to delineate the nature and extent of hydrocarbon contamination and plume migra-

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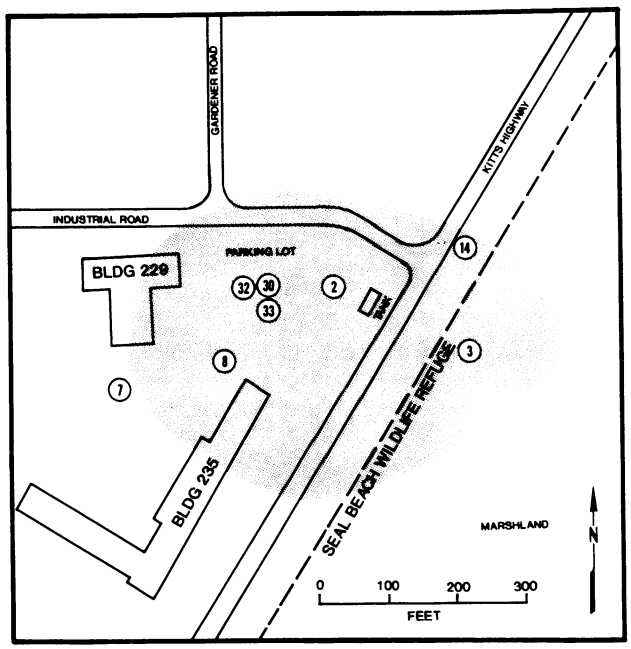


FIG. 1. Observation well locations used for groundwater sampling are indicated by circled numbers. Wells 7 and 14 were just within the plume boundary (stippled area). Plume origin is at tank. Hydraulic gradient is toward marsh.

tion (R. Schroeder, personal communication). Wells were bored without drilling fluids by using 5- to 15.2-cm-diameter hollow-stemmed augers; aseptic precautions were not taken to prevent contamination of the aquifer with surface microorganisms. Therefore, gasoline-degrading isolates obtained from groundwater samples in the present study were regarded as well-derived bacteria and not necessarily as true aquifer bacteria. Site characterization studies indicated that the groundwater is brackish (from seawater intrusion) and contains numerous gasoline hydrocarbons (unpublished data). Substituted aromatic hydrocarbons predominated at this site and were most concentrated in well 2 (Fig. 1) located approximately 10 m up-gradient from the spill origin. Concentrations of individual hydrocarbons typically ranged from 10 to 100 mg/liter in groundwater obtained from well 2 to less than 1.0 μg/liter in wells located along the periphery of the plume (e.g., well 7; unpublished data).

Origins of isolates used in study. Origins of the 309 gasoline-degrading bacteria used in this study are listed in Table 1. A total of 220 isolates were directly subcultured from groundwater samples (as described below) from the eight observation wells (Fig. 1). An additional 30 isolates were subcultured from an enrichment culture inoculated with groundwater from well 30, and 59 isolates were recovered by direct subculture from aquifer core material obtained near well 30 (see below). As noted above, the gasoline-degrading isolates were regarded as well-derived rather than native aquifer bacteria, since aseptic drilling practices were not employed when observation wells were placed in 1984 and 1985.

Core sampling. A total of 59 gasoline-degrading isolates were obtained from an aquifer core sample obtained on 30 July 1987 (Table 1). The core was recovered from a depth of approximately 2.75 m at a location about 2 m northeast of well 30. Coring was performed by using a 76.2-cm-diameter bucket auger which was disinfected with a pressurized steam

TABLE 1. Origins of gasoline-degrading bacteria used in this study

Well	No. of isolates recovered on (mo/day/yr):							
	4/29/86	5/31/86	7/22/86	9/16/86	1/16/87	5/12/87	7/30/87	9/3/87
2	3			23	17	2		
3	1	2	1					
7		2	6	1	3			
8								119
14					11			
30			1	22		59[a]		14
30							30[b]	
32					1			
33					1			

[a] Isolates recovered from a well 30 core sample (see Materials and Methods).

[b] Isolates recovered from laboratory enrichment culture (see Materials and Methods). Eighteen of these isolates were included in growth tests used to determine catabolic groups (see Fig. 2).

jet before and at intervals during drilling and also immediately prior to retrieval of the final core sample. The final core sample was extracted from the borehole, positioned above a sterile plastic container (diameter, 30 cm; 20-liter capacity), and abruptly released, completely plugging the container within about 0.1 s. The outermost 10 cm of the core was sheared away as the container was plugged. The container was sealed aseptically and transported to the laboratory for analysis.

Collection of groundwater samples. Groundwater samples were obtained from observation wells by use of a manual bellows pump. The pump intake was connected by sterile tubing to a 1.27-cm-diameter polyvinylchloride sampling tube permanently installed in each observation well (to prevent cross-contamination of wells). A minimum of five residual well volumes of groundwater (30 to 40 liters) was pumped out of the well as a flush prior to sample collection. After flushing, a sterile 1-liter vacuum flask was placed between the polyvinylchloride sample tube and pump assembly by using sterile Tygon tubing and interconnectors. A 1-liter sample of groundwater was drawn into the flask, and this was dispensed aseptically into sterile plastic bottles (Nalgene). The samples were stored on ice and transported to the laboratory within 3 h of collection.

Isolation of gasoline-degrading bacteria. Gasoline-degrading bacteria (aerobic and facultative) were isolated by diluting groundwater samples in sterile hydrocarbon minimal medium (HCMM2) and spread-plating 0.1-ml aliquots onto HCMM2 agar plates. HCMM2 medium contained (per liter of distilled water) the following: 1.36 g of KH_2PO_4; 1.42 g of Na_2HPO_4; 0.5 g of KNO_3; 2.38 g of $(NH_4)_2SO_4$; 0.05 g of $MgSO_4 \cdot 7H_2O$; 0.01 g of $CaCl_2$; 2.86 mg of H_3BO_4; 1.54 mg of $MnSO_4 \cdot H_2O$; 3.53 mg of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$; 0.039 mg of $CuSO_4 \cdot 5H_2O$; 0.021 mg of $ZnCl_2$; 0.041 mg of $CoCl_2 \cdot 6H_2O$; and 0.025 mg of $Na_2MoO_4 \cdot 2H_2O$. The medium was adjusted to pH 7.2 with 1 N NaOH. Use of 0.8% (wt/vol) purified agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.) to prepare HCMM2 plates was essential in obtaining low background colony counts. Plates were prepared in duplicate, maintained at room temperature (about 23°C) in sealed plastic containers (Rubbermaid, Inc., Wooster, Ohio) for 2 weeks, and examined at intervals for colony formation. Hydrocarbon vapors for growth were supplied by an open vial of unleaded gasoline placed in the plastic containers; HCMM2 plates incubated in this manner are hereafter referred to as HCMM2-gasoline. Duplicate

control plates were initially incubated without gasoline in sealed plastic containers with a 1-cm layer of granular-activated carbon to adsorb trace organics in the container atmosphere. The use of granular-activated carbon was eventually abandoned when it was found to be ineffective at reducing background counts. Colony counts on control plates were typically less than 0.1 CFU/ml.

To isolate bacteria from aquifer core materials, 5.0 g of material was aseptically added to 100 ml of sterile HCMM2 broth amended with 0.1% (wt/vol) sodium pyrophosphate (3, 12). The resulting slurry was homogenized for approximately 30 s at high speed in a Waring blender equipped with a sterile, stainless steel microblender head. Aliquots were removed from the supernatant phase of the homogenized suspension for bacterial isolation on HCMM2-gasoline, as described above.

Some gasoline-degrading bacteria used in this study were obtained from an enrichment culture which had been inoculated with groundwater from well 30 (Table 1). The enrichment culture was prepared in a 100-ml sterile septum vial containing 50 ml of sterile HCMM2 broth supplemented with 0.1% (vol/vol) unleaded gasoline. Following inoculation with 5.0 ml of groundwater from well 30, the vial was sealed and incubated with shaking at ambient temperature (about 23°C). After 1 week, the vial was unsealed and gasoline-degrading bacteria were isolated as described above.

Catabolic screening of gasoline-degrading isolates. Gasoline-degrading bacteria were subcultured from initial isolation plates to fresh HCMM2-gasoline plates, twice checked for purity by restreaking on the same medium, and stored frozen on sterile Whatman filter disks at −120°C. Isolates were screened (in duplicate) for growth on each of 15 common gasoline hydrocarbons (Table 2). All test hydrocarbons were anhydrous and of spectrophotometric grade and were rated at >99.0%, with the exception of *o*-xylene and methylcyclopentane, which were rated at >97.0% and >98.0%, respectively. A Varian model 3400 gas chromatograph (GC) equipped with a flame ionization detector (FID; Varian, Inc., Sunnyvale, Calif.) and a 30-m DB624 megabore column (J. W. Scientific, Folsom, Calif.) was used to confirm reported purities of and identify contaminants in toluene, ethylbenzene, methylcyclopentane, and *o*-xylene (Table 2). Unstable or hygroscopic hydrocarbons (e.g., octane, ethylbenzene, *o*-xylene, and *p*-xylene) were stored under nitrogen for the duration of the study.

Isolates were toothpicked from stock cultures (grown on HCMM2-gasoline) onto fresh HCMM2 agar and exposed to single-hydrocarbon vapors in sealed chambers (e.g., HCMM2-benzene). Chambers consisted of 4-liter wide-mouthed glass jars fitted with Teflon-lined screw-cap lids. Each jar contained a hydrocarbon vapor generator consisting of a standard 20-ml glass liquid scintillation vial filled with cotton soaked in the appropriate hydrocarbon and capped with a Teflon-lined septum. The septum was pierced with a 22-gauge hypodermic needle to provide a gradual release of hydrocarbon vapor to the chamber atmosphere. Gas chromatographic analyses indicated that the amount of ethylbenzene which dissolved into sterile HCMM2 broth (in a petri dish placed within a chamber) increased linearly from 0.018 μM/ml to 0.08 μM/ml between 8 and 145 h (unpublished data).

All negative-control HCMM2 plates were placed in a single hydrocarbon-free chamber. Positive-control plates were prepared by toothpicking cells onto a final plate of R2A medium (Difco Laboratories, Inc., Detroit, Mich.) and HCMM2-gasoline medium at the end of each series of experimental HCMM2 plates. All plates were incubated at room temperature (about 23°C) for up to 2 weeks and examined at intervals for bacterial growth. Growth on a negative-control plate nullified use of that particular isolate in the study. Absence of growth on R2A or HCMM2-gasoline plates was interpreted as failure to transfer cells to the experimental hydrocarbon plates, and results were rejected for all plates in the series. Thus, failure to grow was not a result of insufficient transfer of viable cells. Positive growth was interpreted as biodegradation of the test hydrocarbon (not necessarily complete mineralization) coupled with cellular growth, and the isolate was considered to exhibit catabolic activity. Growth of *Pseudomonas putida* (strain SB2-091686-54g) from the study site in a continuous-flow, sand-filled column was correlated with removal of benzene, toluene, and ethylbenzene as determined by GC-FID analysis (unpublished data). These same hydrocarbons also served as sole growth substrates for this isolate in the vapor assay described above, indicating that growth on vapors could be used to infer actual catabolism of a hydrocarbon.

TABLE 2. Purities of test hydrocarbons used[a]

Test hydrocarbon	Purity (% by wt)
Simple aromatics (benzene)	>99.9
Substituted aromatics	
Toluene[b]	>99.9
o-Xylene[c]	>97.0
p-Xylene	>99.0
1,2,4-Trimethylbenzene	>99.0
Ethylbenzene[d]	>99.0
Polyaromatic (naphthalene)	>99.7
Linear alkanes	
Hexane	>99.0
Octane	>99.0
Decane	>99.0
Branched alkanes	
2,2,4-Trimethylpentane	>99.0
2-Methylbutane	>99.7
Cyclic alkanes	
Cyclohexane	>99.0
Cycloheptane	>99.0
Substituted cyclic alkane (methylcyclopentane[e])	>98.0

[a] All hydrocarbons were from Aldrich Chemical Co., Inc., Milwaukee, Wis., except hexane, which came from Sigma Chemical Co., Inc., St. Louis, Mo.

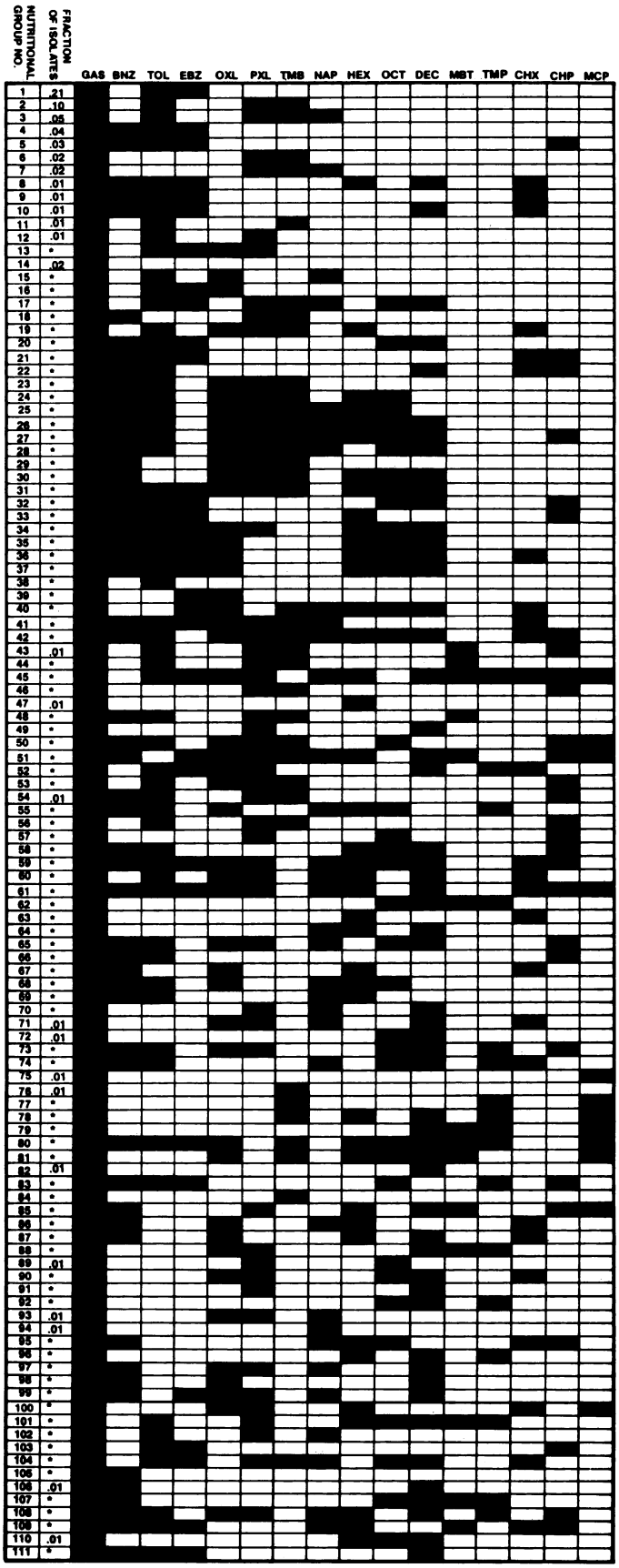
[b] Toluene purity was confirmed by GC-FID (this study; see Materials and Methods).

[c] *o*-Xylene contained 2.7% (wt/wt) toluene as confirmed by GC-FID (this study).

[d] Ethylbenzene contained 0.75% toluene and 0.33% *o*-xylene as confirmed by GC-FID (this study).

[e] Methylcyclopentane contained about 1.0% toluene as confirmed by GC-FID (this study).

Sample preparation for SDS-PAGE. Isolates were transferred from R2A medium to a defined radiolabeling (RL) medium and grown for 2 or 3 days at 28°C. RL medium contained the following reagents (all from Sigma): 0.1 g (each) of the amino acids arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, histidine, hydroxyproline, isoleucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine; 50 mg (each) of the nucleic


NUTRITIONAL GROUP NO.
FRACTION OF ISOLATES
GAS BNZ TOL EBZ OXL PXL TMB NAP HEX OCT DEC MBT TMP CHX CHP MCP
1 .21
2 .10
3 .05
4 .04
5 .03
6 .02
7 .02
8 .01
9 .01
10 .01
11 .01
12 .01
13 •
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acid bases adenosine, adenosine 2'- and 3'-monophosphate, cytidine 2'- and 3'-monophosphate, guanosine, guanosine 2'- and 3'-monophosphate, uridine, and uridine 2'- and 3'-monophosphate; 10.0 μg (each) of the vitamins *p*-aminobenzoic acid, D-biotin, folic acid, niacinamide, *p*-pantothenic acid, pyridoxal hydrochloride, pyridoxamine hydrochloride, pyridoxine hydrochloride, riboflavin hydrochloride, thiamine hydrochloride, and DL-6,8-thioctic acid; 1.0 g of glucose; 1.42 g of Na_2HPO_4; 1.36 g of KH_2PO_4; 5.0 mg of $MgSO_4 \cdot 7H_2O$; 2.8 mg of H_3BO_4; 1.8 mg of $MnCl_2 \cdot 4H_2O$; 1.36 mg of $FeCl_2$; 1.77 mg of NaK-tartrate; 26.9 μg of $CuCl_2 \cdot 2H_2O$; 20.8 μg of $ZnCl_2$; 40.4 μg of $CoCl_2 \cdot 2H_2O$; 25.2 μg of $Na_2MoO_4 \cdot 2H_2O$; 15.0 g of Bacto-Agar (Difco); and 1 liter of distilled water. The pH of RL medium was adjusted to 7.2, and vitamins were added after it was autoclaved as a filter-sterilized solution. Colonies were toothpicked from RL plates to 96-well microtiter plates, each well containing 100 μl of sterile RL medium supplemented with 20 μCi of [^{35}S]sulfate (specific activity, 25 to 40 Ci/mg; Amersham Corp., Arlington Heights, Ill.). Sulfate was employed as a label instead of [^{35}S]methionine, since many isolates failed to efficiently incorporate the latter. Microtiter plates were incubated at 28°C in sterile, sealed plastic bags (to prevent evaporation) until growth was visible (usually 2 days). Cells were rinsed from wells with 50 μl of sterile, deionized water and transferred to a 1.5-ml microcentrifuge tube, and proteins were solubilized by addition of an equal volume of double-strength lysis buffer containing 0.125 M Tris buffer (adjusted to pH 8.3 with 2.0 N HCl), 4.0% (wt/vol) SDS, 10.0% (vol/vol) 2-mercaptoethanol, and 0.005% (wt/vol) bromophenol blue (all from Sigma Chemical Co., St. Louis, Mo.). This suspension was mixed, boiled for 5 min, and then sonicated for 5 min with an equal volume of 0.15-mm zirconium oxide beads by using a Heat Systems, Inc., model 450 ultrasonic processor equipped with a cup horn.

SDS-PAGE method. Solubilized proteins were loaded onto 12% (wt/vol) polyacrylamide gels and subjected to electrophoresis for 20 min at 20 W and then at 60 W for approximately 2 h at 8°C by using a refrigerated, computer-controlled electrophoresis system (AMBIS, Inc., San Diego, Calif. [14]). Gels were dried and scanned for radioactivity incorporated into cellular proteins with an AMBIS, Mark II, computer-programmed beta radioactivity scanner. A detailed description of this system has been reported by Hook et al. (14) and Smith (25).

Scanning and analysis of SDS-PAGE gels. Scan data were automatically retrieved and stored on the hard disk of an IBM-PC/AT microcomputer as individual SDS-PAGE lane files. Lane files were adjusted and normalized according to ^{14}C-labeled molecular weight standards consisting of lysozyme (14,300 Da), β-lactoglobulin (18,400 Da), carbonic anhydrase (29,000 Da), ovalbumin (43,000 Da), bovine serum albumin (68,000 Da), phosphorylase *b* (97,400 Da), and H-chain myosin (200,000 Da) (Bethesda Research Laboratories, Gaithersburg, Md.). The specific activity of a 1.0-μl solution of each ^{14}C-methylated standard was 3,100 dpm/mg of protein. The length of each lane was adjusted and normalized by electronically clipping the ends of the lanes at two locations, one corresponding to phosphorylase *b* and the second corresponding to β-lactoglobulin. To further account for gel variations, lane files were normalized by using software that stretched or compressed banding profiles to a standard length and stored them as adjusted lane files. Adjusted lane files were transformed into Fourier space by using appropriate algorithms and were then searched for matches or compared with one another by using dendrogram software described elsewhere (14, 15, 19–21). Isolates were sorted into groups and assigned Fourier or Pearson correlation coefficients (14). A coefficient of 1.0 indicates an exact match between two banding patterns. In actual practice, experimental error, gel variations, and machine noise limited resolution to a correlation coefficient of about 0.93 for multiple runs of the same isolate (unpublished data). Previous studies indicated that microorganisms correlated above 0.90 are very closely related and are typically members of the same species or subspecies (14, 15, 19–21). Therefore, isolates with protein-banding profiles correlated at >0.90 were placed into the same group (referred to as an AMBIS group).

Bacterial identification by conventional methods. Conventional identification of gasoline-degrading bacteria was performed by morphological examinations, Gram- and flagellum-staining reactions, oxidative/fermentative analyses, and inoculation and reading of NFT biochemical strips obtained from Analytab Products, Inc., Plainview, N.Y. Strips were routinely incubated at 28°C.

RESULTS

Hydrocarbon degradation patterns of isolates. Catabolic activities of 297 isolates were evaluated by recording aerobic growth responses on the 15 different hydrocarbon substrates which were gradually introduced as vapors in sealed vessels (see Materials and Methods). Bacteria degrading the same hydrocarbon(s) were included in the same catabolic group. A total of 111 catabolic groups were delineated among the isolates screened (Fig. 2). The types of hydrocarbons catabolized by each group varied widely, but the majority of isolates favored the substituted monoaromatics as growth substrates, which is consistent with in situ adaptation to aromatic compounds prevalent in groundwater at this site (unpublished data).

A total of 17 catabolic groups included degradation of the multiply branched alkane 2,2,4-trimethylpentane, and 12 groups degraded the branched cyclic alkane methylcyclopentane. Group 14 was unusual, since its members failed to grow on any of the 15 test hydrocarbons, suggesting that these isolates required one or more gasoline hydrocarbons other than the 15 evaluated. Each of the 15 test hydrocarbons was biodegraded by at least one of the 297 isolates, suggesting that the primary hydrocarbon categories of gasoline could be aerobically biodegraded in situ given appropriate conditions.

Several catabolic groups (e.g., groups 38, 47, 75, 84, 94, and 105) included degradation of a single test hydrocarbon. Such groups were not the most frequently observed, as might be suspected because of their simplicity, suggesting that maintenance of multiple catabolic abilities might provide some selective advantage. Catabolic groups which included degradation of five or more test hydrocarbons were likewise infrequently observed (e.g., groups 42, 45, 51, 61,

FIG. 2. Catabolic groups defined in this study. A filled box indicates that a hydrocarbon supported the growth of all members of a group. Asterisks denote a frequency of <0.01 (n = 297). BNZ, Benzene; TOL, toluene; OXL, *o*-xylene; PXL, *p*-xylene; TMB, 1,2,4-trimethylbenzene; EBZ, ethylbenzene; NAP, naphthalene; HEX, hexane; OCT, octane; DEC, decane; TMP, 2,2,4-trimethylpentane; MBT, 2-methylbutane; CHX, cyclohexane; CHP, cycloheptane; MCP, methylcyclopentane; GAS, regular-grade unleaded gasoline.

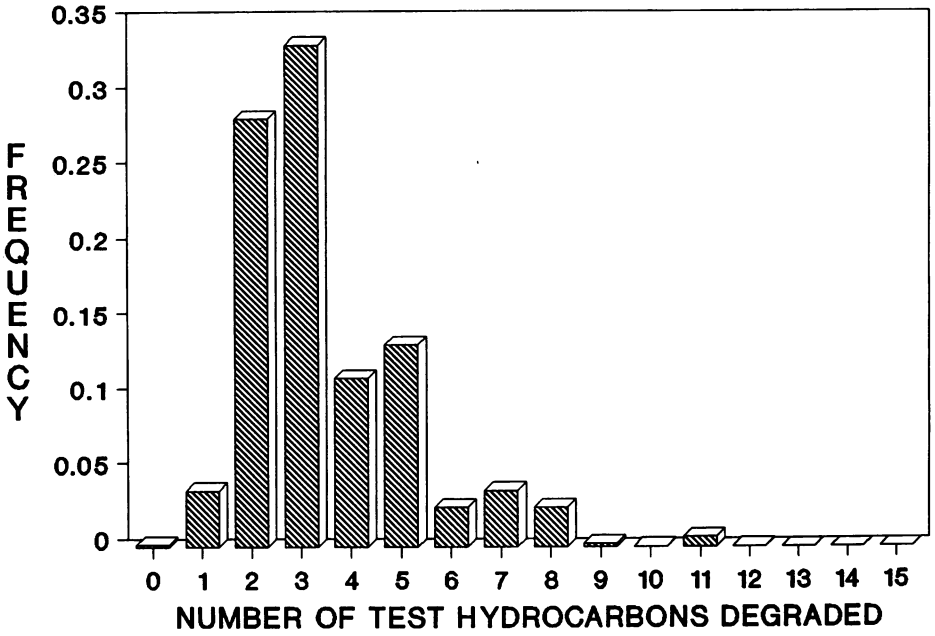


FIG. 3. Histogram showing frequency of Seal Beach gasoline-degrading isolates capable of growth on different numbers of the 15 test hydrocarbons used in this study (n = 297 isolates).

and 80). The frequency of occurrence of bacteria able to utilize different numbers of test hydrocarbons is plotted in Fig. 3. Inspection of these data indicates that most isolates were able to grow on 2 or 3 different test hydrocarbons, suggesting that retention of other numbers of catabolic activities might not offer selective advantage at this site.

The frequency of occurrence of the 111 catabolic groups defined above is shown in Fig. 4. Given the 15 test hydrocarbons utilized in this study, there are 2^{15} (or 32,768) different possible catabolic groups. Since each group is unique, the probability of observing any one group should be equal to that of observing any other, resulting in a completely random distribution (assuming group independence and absence of selection). However, a random distribution was not observed, since groups 1 to 10 were the most common, representing greater than 57% of the 297 isolates analyzed (Fig. 4). Eight of these groups included degradation of toluene, which was the most frequently utilized test hydrocarbon. Departure of observed group frequency data from an expected random distribution evidently reflects

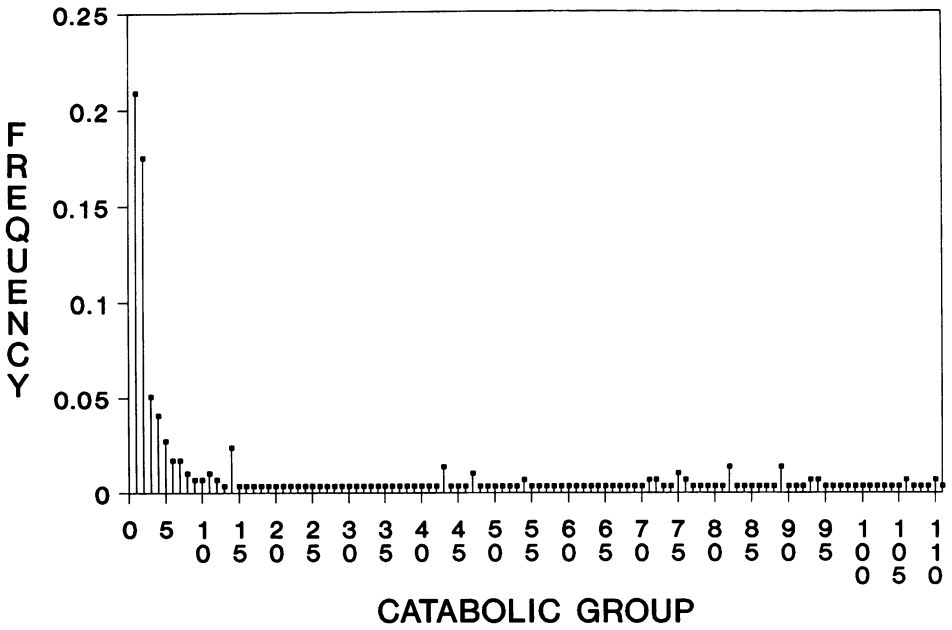


FIG. 4. Histogram showing frequency distribution of the 111 different catabolic groups defined in this study (n = 297 isolates). Hydrocarbons serving as growth substrates in each catabolic group are shown in Fig. 2.

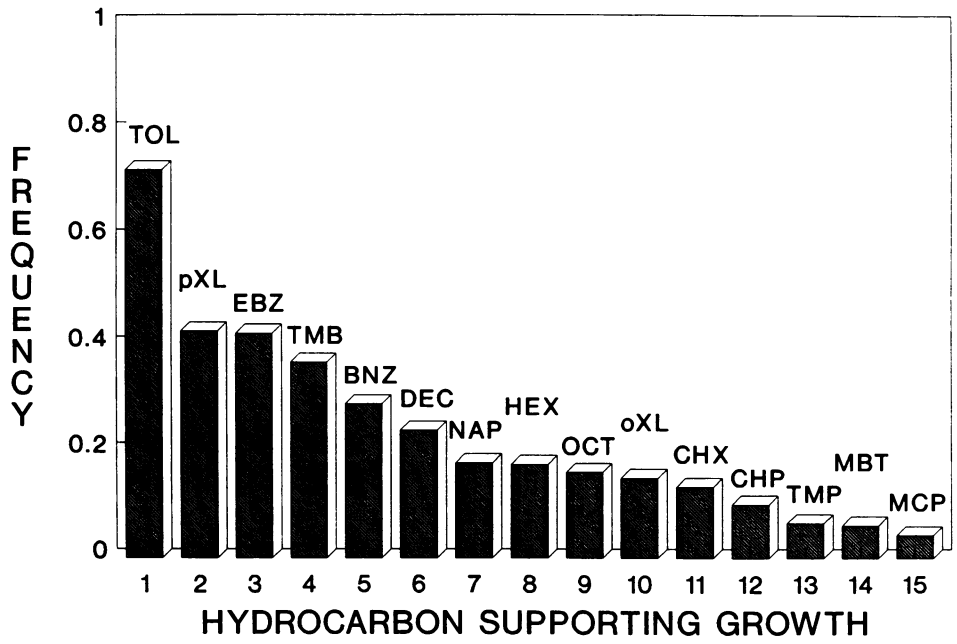


FIG. 5. Frequency of specific test hydrocarbon degradation (n = 297 isolates). Abbreviations for hydrocarbons are given in the legend to Fig. 2. oXL, *o*-Xylene; pXL, *p*-xylene.

existence of biologically favored combinations of hydrocarbon catabolic pathways.

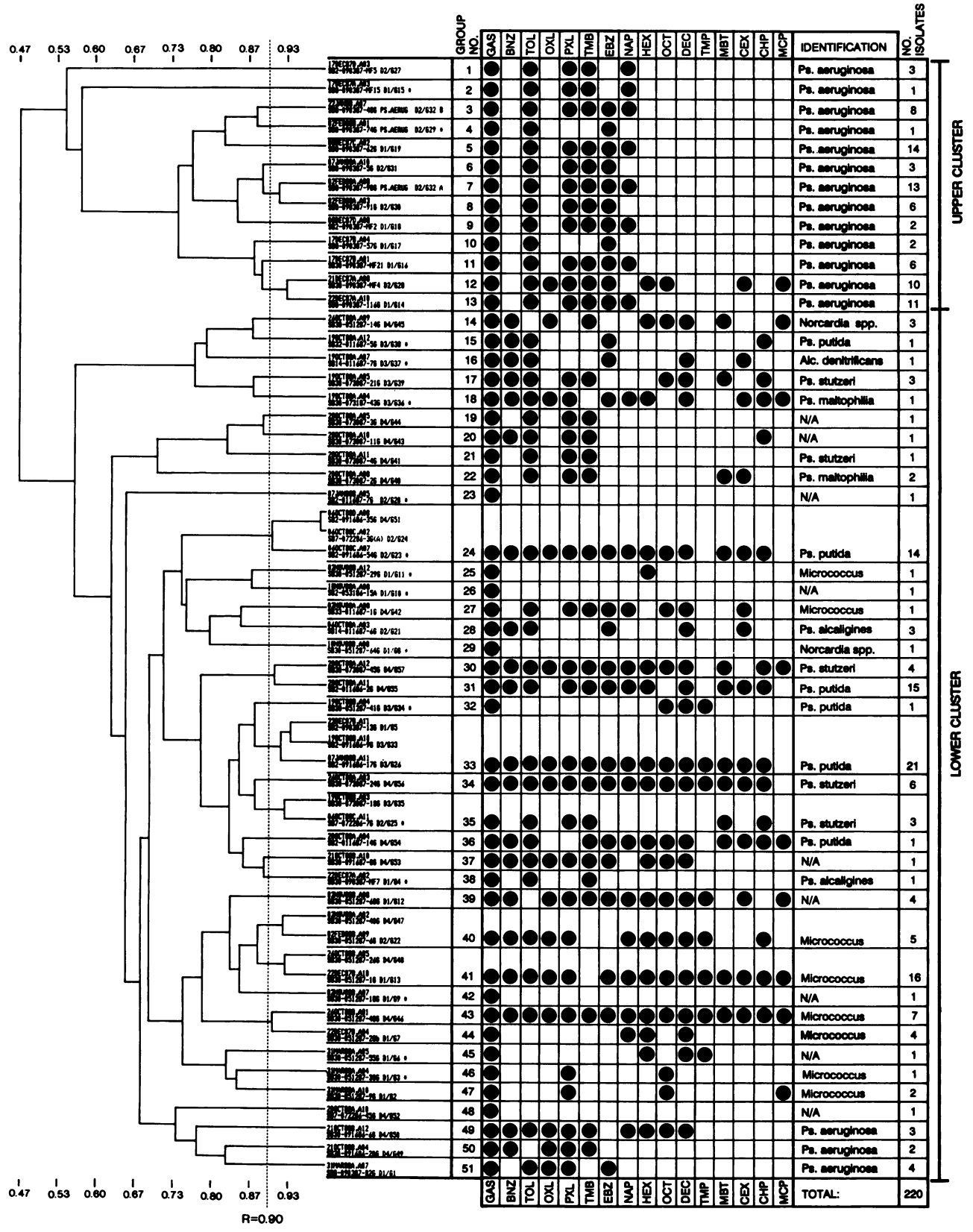
Growth screening also permitted determination of the frequencies at which specific test hydrocarbons were utilized by isolates. The frequencies of specific hydrocarbon degradation for 297 of the Seal Beach isolates are presented in Fig. 5. Alkylated aromatic hydrocarbons, such as toluene, *p*-xylene, ethylbenzene, and 1,2,4-trimethylbenzene were most frequently utilized. Approximately 75% of isolates were capable of growth on toluene vapors as a sole carbon and energy source. Benzene and naphthalene were less frequently degraded than most substituted aromatics with the exception of *o*-xylene, which was degraded less frequently than either compound or than its isomeric counterpart, *p*-xylene. It is interesting to note that more than 150 toluene-degrading isolates were unable to utilize toluene present as a contaminant in *o*-xylene (Table 2), suggesting that the concentrations of contaminating hydrocarbons were too low to support growth. The least frequently degraded compounds included the cyclic branched alkanes 2,2,4-trimethylpentane, 2-methylbutane, and methylcyclopentane. Unsubstituted linear and cyclic alkanes such as octane and cycloheptane were utilized at frequencies intermediate between those of the aromatics and branched alkanes.

Polypeptide-banding pattern analysis of isolates. A total of 220 gasoline-degrading isolates was characterized by computer-programmed SDS-PAGE analysis to further explore genetic and taxonomic heterogeneity among the sample population. The dendrogram-generating algorithms which were employed restricted analyses to 75 isolates, which necessitated construction of four separate dendrograms to accommodate the 220 isolates. Bacteria composing the four initial dendrograms were sorted into tentative groups (AMBIS groups), each exhibiting intracorrelations of 0.90 or greater. Representative strains (totaling less than 75) from each of the tentative AMBIS groups were subsequently used to construct a fifth, composite dendrogram to determine which tentative groups were unique. Using this iterative strategy, a total of 51 distinct AMBIS groups were resolved, and the relationship between these is indicated in Fig. 6. Confirmatory dendrograms (not shown) were performed on each of the 51 AMBIS groups to ensure that members of each were correlated above the 0.90 level.

Inspection of the dendrogram shown in Fig. 6 indicates that the Seal Beach isolates were organized into two broad clusters which were discriminated by a correlation value of about 0.47. The upper cluster consisted of the first 13 AMBIS groups, while the lower cluster comprised the remainder of the 51 groups. Each cluster was composed of members correlated with one another at a level of about 0.55 or higher. The largest AMBIS group, 33, contained 21 members, whereas 21 groups contained only one member isolate.

Test hydrocarbons collectively utilized by members composing each AMBIS group are also indicated in Fig. 6. The most catabolically diverse bacteria composed the lower cluster. These isolates were able to catabolize all 15 test hydrocarbons. AMBIS groups 24, 30, 31, 33, 34, 36, 39, 41, and 43 included members which were particularly versatile in terms of hydrocarbon usage. In contrast, the upper cluster, which comprised the first 13 AMBIS groups, included isolates which were more restricted catabolically (and taxonomically; see below) than those in the lower cluster. Alkanes served as growth substrates for only one of the upper-cluster groups (AMBIS group 12), the remainder degrading only substituted aromatics. However, benzene and *o*-xylene were biodegraded more frequently by members of the lower cluster. Because members of single AMBIS groups often displayed different growth responses on a given test hydrocarbon, it was not feasible to reliably predict catabolic activity from protein-banding information.

A total of 244 isolates (including the 220 analyzed by SDS-PAGE) were identified by conventional nutritional and biochemical tests to determine whether useful phylogenetic information could be extracted from protein-banding and catabolic-screening data. Nearly half (48.4%) of the 244


0.47 0.53 0.60 0.67 0.73 0.80 0.87 0.93
R=0.90
GROUP NO.
GAS BNZ TOL OXL PXL TMB EBZ NAP HEX OCT DEC TMP MBT CEX CHP MCP
IDENTIFICATION
NO. ISOLATES
Ps. aeruginosa 3
Ps. aeruginosa 1
Ps. aeruginosa 8
Ps. aeruginosa 1
Ps. aeruginosa 14
Ps. aeruginosa 3
Ps. aeruginosa 13
Ps. aeruginosa 6
Ps. aeruginosa 2
Ps. aeruginosa 2
Ps. aeruginosa 6
Ps. aeruginosa 10
Ps. aeruginosa 11
Norcardia spp. 3
Ps. putida 1
Alc. denitrificans 1
Ps. stutzeri 3
Ps. maltophilia 1
N/A 1
N/A 1
Ps. stutzeri 1
Ps. maltophilia 2
N/A 1
Ps. putida 14
Micrococcus 1
N/A 1
Micrococcus 1
Ps. alcaligines 3
Norcardia spp. 1
Ps. stutzeri 4
Ps. putida 15
Ps. putida 1
Ps. putida 21
Ps. stutzeri 6
Ps. stutzeri 3
Ps. putida 1
N/A 1
Ps. alcaligines 1
N/A 4
Micrococcus 5
Micrococcus 16
N/A 1
Micrococcus 7
Micrococcus 4
N/A 1
Micrococcus 1
Micrococcus 2
N/A 1
Ps. aeruginosa 3
Ps. aeruginosa 2
Ps. aeruginosa 4
TOTAL: 220
UPPER CLUSTER
LOWER CLUSTER

isolates were recovered from groundwater samples from well 8; the remainder were obtained from five other wells (wells 2, 7, 14, 30, and 32). Of 121 gasoline-degrading isolates recovered from well 8 groundwater, 118 were identified as *Pseudomonas aeruginosa*. In all, nine species of pseudomonads were identified, including *P. aeruginosa* (55.7%), *P. putida* (18.4%), *P. stutzeri* (7.4%), *P. fluorescens* (0.82%), *P. acidovorans* (0.82%), *P. pickettii* (0.41%), *P. maltophilia* (1.2%), *P. alcaligenes* (1.6%), and *P. cepacia* (0.41%). Other identifications included *Alcaligenes denitrificans* (0.82%), *Micrococcus* spp. (10.7%), and *Nocardia* spp. (1.2%).

The majority of *P. aeruginosa* strains exhibited similar protein-banding patterns and hydrocarbon catabolic activities (see above), cosegregating into the upper cluster of the dendrogram (Fig. 6), where they formed the first 13 AMBIS groups. A few *P. aeruginosa* isolates, however, displayed protein-banding patterns which differed significantly from the main body of isolates included in the upper cluster, and these cosegregated near the bottom of the dendrogram (AMBIS groups 49 to 51). *P. aeruginosa* isolates composing AMBIS groups 12 and 49 exhibited unusual catabolic activities compared with other *P. aeruginosa* groups, since they grew on selected alkanes, including hexane, octane, decane, cyclohexane, and methylcyclopentane in addition to the more frequently catabolized aromatics. Inclusion of these catabolically anomalous strains in *P. aeruginosa* is, therefore, suspect. *P. putida*, *P. stutzeri*, and the *Micrococcus* spp. were capable of catabolizing most alkanes and were distributed throughout the mid to lower regions of the dendrogram (Fig. 6).

DISCUSSION

The principal advantage of vapor-phase growth tests such as those employed in this investigation is that they can be performed much more rapidly than more complex chemical analytical methods, thereby permitting the examination of large numbers of isolates with multiple hydrocarbon substrates in a reasonable time frame. Unlike batch culture techniques in which a single hydrocarbon concentration is typically employed, tests using hydrocarbon vapors offer organisms a range of substrate concentrations, if vapors are introduced gradually as was done in this study. Such a technique increases the likelihood of a positive growth response within some favorable concentration range of hydrocarbon. Assuming purity of the carbon source and inclusion of appropriate negative controls (e.g., incubation in the absence of substrate), a positive result is unambiguous and may be interpreted as catabolic (i.e., biodegradative) activity coupled to cellular growth.

If test hydrocarbons are not absolutely pure, then a positive growth response could result from utilization of contaminant hydrocarbons. However, this possibility was unlikely, since more than 90 isolates which grew on toluene as a sole carbon source were unable to utilize this same substrate when it was present as a contaminant in ethylbenzene at a level of approximately 0.8% (wt/wt) (Fig. 5; Table 2). Similarly, nearly 150 toluene-degrading isolates failed to utilize toluene present as a contaminant in *o*-xylene (the least-pure test substrate [Table 2]) at a concentration of 2.7% (wt/wt). These observations suggest that the concentrations of contaminant hydrocarbons were insufficient to support the growth of gasoline-degrading isolates examined in this study.

As in all studies that employ growth responses on single-carbon substrates, a negative result may be interpreted as (i) lack of a functional catabolic pathway or transport system, (ii) insufficient concentration of the test substrate (i.e., inadequate bioavailability), or (iii) biotoxicity. In addition, a negative growth response does not imply that a compound cannot be cometabolized or syntrophically utilized. It may be argued that inadequate bioavailability was not responsible for negative results in the present study, since all isolates grew on gasoline vapors in which the concentrations of individual hydrocarbon components were lower than those of the single test hydrocarbons subsequently employed. In addition, test hydrocarbons could have been expected to approach equilibrium concentrations at the surfaces of the agar plates given the lengthy incubation periods employed.

Inadequate bioavailability may also be discounted on grounds that different isolates in the same subspecies group (e.g., *P. putida* in AMBIS group 33) often displayed different catabolic patterns, such that one or more hydrocarbons were utilized by one strain but not by another. To account for such discrepancies in substrate utilization, it must be postulated (i) that significant differences existed among otherwise closely related strains with regard to hydrocarbon affinity, transport kinetics, or other factors that could restrict bioavailability or (ii) that strains exhibited markedly different susceptibilities to the biotoxic effects of the same hydrocarbon. Whereas strain differences in specific-hydrocarbon affinity or toxicity are admittedly feasible, they have not been documented nor would they necessarily be expected in such closely related isolates. Moreover, assuming that such differences did exist, there is no a priori reason to suspect that they would have been of sufficient magnitude to account for the marked differences in growth responses of different strains on the same hydrocarbon. Furthermore, it may be inferred that none of the 15 test hydrocarbons was generally cytotoxic at levels employed in this study; otherwise, no isolate would have exhibited growth on that compound. In contrast, every test hydrocarbon was found to support the growth of at least one gasoline-degrading isolate.

However, since biotoxicity or bioavailability was not rigorously proven or disproven, the growth response data tend to provide a conservative estimate of catabolic potential. Thus, catabolic groups defined by using vapor-phase growth tests might have hydrocarbon catabolic activities other than those actually observed. Despite this inherent conservatism, all gasoline hydrocarbon categories tested were found to have been directly catabolized under aerobic conditions. For purposes of predicting bioremediative feasibility at a hydrocarbon-contaminated site, a conservative estimate of catabolic potential provides a safer margin for error than a more liberal estimate of degradative ability.

Given the qualifications discussed above, results of growth-screening tests and other methods employed in this investigation indicate that there was considerable physiologic and genetic heterogeneity among gasoline-degrading bacteria associated with the Seal Beach site. It is interesting to note that the growth-screening technique employed provided greater phenotypic resolution than one-dimensional protein-banding information, since a total of 111 catabolic

FIG. 6. Composite dendrogram showing 51 AMBIS groupings made on the basis of computerized analysis of protein-banding patterns. Upper- and lower-cluster regions are indicated. Test hydrocarbons degraded within each AMBIS group are indicated by filled circles. Numbers and identifications of isolates are also provided. Abbreviations for hydrocarbons are given in the legend to Fig. 2.

groups were defined compared with 51 AMBIS groups. This difference was not entirely unexpected, since some catabolic enzymes having different substrate specificities might exhibit similar or identical mobilities in polyacrylamide gels. Furthermore, many catabolic enzymes may be present in cells at levels too low to be readily detectable in whole-cell protein preparations, thereby precluding their use as discriminatory signals in polyacrylamide gels.

It is noteworthy that different members of the same species (e.g., *P. aeruginosa* or *P. putida*) often differed significantly with respect to their protein-banding patterns. This result is primarily attributable to the somewhat arbitrary use of a conservative correlation limit of 0.90 in constructing dendrograms and defining isolate groups. Delineation of AMBIS groups on the basis of a lower correlation value (e.g., 0.75) would have resulted in broader taxonomic groupings; however, significant discrepancies between banding pattern groups and conventional identifications would still have occurred. Thus, the use of one-dimensional protein-banding pattern analysis to reliably infer phylogenetic relationships was limited in this study. Evidently, protein-banding information is not always adequately conserved for this purpose, even at the species level. Nevertheless, the protein-banding patterns were reproducible, and isolates composing each of the 51 AMBIS groups were consistently identified to the same species or subspecies. Thus, protein-banding information must be viewed in this study primarily as a means of resolving strain or subspecies differences, not as a technique for inferring phylogenetic relationships.

Results of growth tests indicated that isolates displayed a predilection for aromatic hydrocarbons. Aromatic hydrocarbons constitute a major fraction of gasoline (24), and these compounds are more water soluble and less volatile than many aliphatic constituents. These characteristics favor the prevalence of aromatic hydrocarbons in groundwater contaminated by gasoline (5, 18, 29), and previous GC analyses of groundwater samples from the Seal Beach site are consistent with these concepts (unpublished data). Given the age of the plume (>6 years), it seems plausible that bacteria at the site might have had adequate time for significant physiologic and genetic adaptation to the aromatic fraction, which is consistent with the observed preference of the isolates for this hydrocarbon category.

The majority of isolates were able to use only 2 or 3 different test hydrocarbons as growth substrates (Fig. 3). This observation is intriguing, since it may reflect an optimum level of catabolic potential that is inherently maintained by cells from this environment. Aside from arguments concerning unfavorable energetics, there is no a priori reason to expect that more catabolic activities could not have been maintained. Indeed, a number of catabolic groups included individual isolates that degraded nearly all of the 15 test hydrocarbons, but these bacteria represented a small minority. It is interesting to speculate on the purpose of maintaining multiple degradative pathways for compounds that have little resemblance to one another (e.g., an alkane and an aromatic). It is not known whether these catabolic pathways are simultaneously expressed and active in the environment or whether certain pathways are repressed while others remain functional. Additional biochemical and physiological studies will be needed to resolve these questions.

Frequencies of test hydrocarbon utilization by the Seal Beach isolates differed somewhat from those of 32 gasoline-degrading isolates analyzed by Jamison and co-workers (16, 17). For example, 2,2,4-trimethylpentane was the most frequently biodegraded test hydrocarbon in the Jamison study (frequency of degradation, 60%), whereas this same substrate was utilized by about 7% of the 297 Seal Beach isolates examined. In addition, while *p*-xylene was infrequently utilized by the Jamison isolates (<15%), it was the second-most-utilized compound among the Seal Beach bacteria. Toluene and ethylbenzene were among the most frequently utilized hydrocarbons in both investigations. Bacteria able to catabolize *o*-xylene, methylcyclopentane, and cyclohexane were not isolated by Jamison, though each of these hydrocarbons was biodegraded by a consortium growing on gasoline, suggesting cooxidative or syntrophic processes. It is possible that bacteria existed at the Jamison site that could directly degrade these hydrocarbons, but they were not detected because of the low number of isolates screened.

In the Jamison investigation, isolates were obtained from groundwater following in situ injection of nutrients and oxygen. Thus, growth of aerobic gasoline-degrading bacteria may have been enhanced relative to the Seal Beach aquifer, which is anoxic and largely depleted of inorganic nutrients (e.g., nitrogen and phosphorus [unpublished data]). In situ bioremediation via nutrient injection and recycling establishes subsurface conditions which favor development of certain hydrocarbon-degrading phenotypes while suppressing the growth of others. Quantitative data are needed to better understand and delineate the relationship between microbial community structure and perturbation of in situ conditions by invasive bioremediative techniques.

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