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Application of DNA-DNA Colony Hybridization to the Detection of Catabolic Genotypes in Environmental Samples

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The application of preexisting DNA hybridization techniques was investigated for potential in determining populations of specific gene sequences in environmental samples. Cross-hybridizations among two degradative plasmids, TOL and NAH, and two cloning vehicles, pLAFR1 and RSF1010, were determined. The detection limits for the TOL plasmid against a nonhomologous plasmid-bearing bacterial background was ascertained. The colony hybridization technique allowed detection of one colony containing TOL plasmid among 10^6 *Escherichia coli* colonies of nonhomologous DNA. Comparisons between population estimates derived from growth on selective substrates and from hybridizations were examined. Findings indicated that standard sole carbon source enumeration procedures for degradative populations lead to overestimations due to nonspecific growth of other bacteria on the microcontaminant carbon sources present in the media. Population estimates based on the selective growth of a microcosm population on two aromatic substrates (toluene and naphthalene) and estimates derived from DNA-DNA colony hybridizations, using the TOL or NAH plasmid as a probe, corresponded with estimates of substrate mineralization rates and past exposure to environmental contaminants. The applications of such techniques are hoped to eventually allow enumeration of any specific gene sequences in the environment, including both anabolic and catabolic genes. In addition, this procedure should prove useful in monitoring recombinant DNA clones released into environmental situations.

The enumeration of specific bacterial populations in environmental samples is hampered by the uncertainties of selective enrichment procedures. These uncertainties are further magnified in the analyses of those populations comprising the heterotrophic bacterial guild that is responsible for the catabolism of environmental contaminants (21). Although it is assumed that organisms capable of growth on agar medium containing an environmental pollutant as a sole carbon source must be capable of metabolism of that substrate, this assumption contains serious flaws that affect the reliability and utility of the approach. Potential factors affecting the results of this approach include the following: (i) poor or slow growth of the population due to the quality and concentration of the substrate; (ii) no growth due to co-oxidative or cometabolic utilization of the substrate, auxotrophic nutrient requirements, or toxicity of the substrate; (iii) interference growth (false-positives) due to oligotrophic growth on medium contaminants or micronutrients; (iv) cross-feeding; (v) lack of sensitivity for populations representing a small percentage of the total population; and (vi) colony/colony inhibition.

Additional problems exist in detecting poorly selectable phenotypes or specific genotypes that are not expressed on laboratory media. Such difficulties demonstrate the utility of being able to directly detect specific DNA of the organism in question without gene expression or host selection.

Nucleic acid hybridization techniques have been developed to detect and select specific DNA sequences in bacterial colonies (11, 12). These colony hybridization techniques have been applied to the detection of *Salmonella* spp. (7) and toxigenic strains of *Vibrio cholerae* (17), *Escherichia coli* (13, 22), and *Yersinia* spp. in foods (14). Theoretically, DNA-DNA colony hybridization should be a useful and sensitive tool for the nonselective detection of specific DNA sequences against the background of an environmental community. Such applications would make it possible to detect low frequencies of degradative bacteria or catabolic genes within natural or wastewater populations. It should also be possible to track the survival and maintenance of specific native or recombinant DNA within indigenous natural populations.

The objectives of this research were to evaluate the utility of DNA-DNA colony hybridization techniques for use in environmental populations and to develop an approach for determining the frequency, dispersal, and maintenance of catabolic plasmids or recombinant DNA in natural environments.

For these purposes, the toluene catabolic plasmid (TOL) harbored in *Pseudomonas putida* was used as the primary DNA probe for method development and application to environmental samples. A whole-plasmid probe of TOL was used as a general indicator of aromatic hydrocarbon catabolism in microcosm sediments exposed to synthetic oils (G. S. Sayler and T. W. Sherrill, *in Proceedings of an International Conference on the Environment and Lung Disease*, in press). This general probe was found to provide results that correlated well with levels of synthetic oil contamination, catabolism of aromatic hydrocarbons, conventional selective enumeration, and plasmid gene frequencies determined from a naphthalene plasmid DNA probe.

MATERIALS AND METHODS

Bacteria. Bacterial strains used for source plasmid DNA for the preparation of [^{32}P]DNA probes and as targets for colony hybridization are described in Table 1. *P. putida* ATCC 23973 carrying the TOL plasmid and *P. putida* NAH-7, originally obtained from D. Gibson (University of Texas),

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TABLE 1. Bacterial strains and associate plasmids used in this investigation

Strain	Plasmid (kilobase pairs)	Relevant plasmid characteristic[a]	Reference or source
P. putida ATCC 23973	TOL (117)	D	23
P. putida MR-2 2440	pKT530	CV, D	8
P. putida	NAH-7 (83.0)	D	D. Gibson, 27
E. coli C600	pLAFR1 (21.6)	CV	9
E. coli	RSF1010 (8.9)	CV	1
E. coli RC709 (153F)	RP4 (60.4)	R	4
P. aeruginosa PU21	None	R	G. Jacoby, 25
P. aeruginosa PU21	pMG1		G. Jacoby, 25
E. coli V517	pV517A-H (35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, 1.4)	C	20
Acinetobacter sp. strain ACS8	None		Shields and Sayler[b]
Alcaligenes sp. strain A2D	pSS20, pSS21 (52.2, 87)	D	Shields and Sayler[b]
Alcaligenes sp. strain BM2	None		O. Yagi
Acinetobacter sp. strain AX2	pSS2 (52.5)	D	Shields and Sayler[b]
Alcaligenes sp. strain A2	pSS2 (52.5)	D	Shields and Sayler[b]
Alcaligenes sp. strain A5	pSS50, pSS51 (52.5, 75)	D	Shields and Sayler[b]
Acinetobacter sp. strain P6	pKF1 (80.55)	D	10
Arthrobacter sp. strain M5	pKF1 (80.55)	D	10
P. putida 4Cba$^+$	pAC27 (110.0)	D	3
P. putida 3,5 Dcb$^+$	pAC31	D	3
E. coli J53	R388	R	G. Jacoby
E. coli J63	R46	R	G. Jacoby
E. coli Ec 290			G. Jacoby
E. coli Ec 295			G. Jacoby

[a] D, Degradative; CV, cloning vehicle; R, resistance; C, cryptic;
[b] Submitted for publication.

were used as source strains for TOL and NAH plasmid DNA and their respective [^{32}P]DNA probes. An *E. coli* strain carrying plasmid RSF1010 and a second *E. coli* C-600 strain harboring the cosmid cloning vector pLAFR1 were the respective sources for DNA to prepare ^{32}P-labeled probes for each of the cloning vehicles. *E. coli* V517, containing eight stable cryptic plasmids, was used as a source for plasmid molecular weight markers in agarose gel electrophoresis. Additional strains (see Table 1) were obtained from other investigators or isolated by this laboratory.

Culture conditions. *P. putida* (TOL$^+$ or NAH$^+$) was maintained on 50 mM *m*-toluate or naphthalene, respectively, in minimal agar medium containing (in grams per liter): $NaNO_3$, 4.0; KH_2PO_4, 1.5; $FeSO_4 \cdot 7H_2O$, 0.0011; $MgSO_4 \cdot 7H_2O$, 0.2; Na_2HPO_4, 0.5; $CaCl_2 \cdot 2H_2O$, 0.01; pH to 7.0. When *m*-toluate (Eastman Kodak Co., Rochester, N.Y.) was to be used as the carbon source, it was autoclaved in the minimal medium; when naphthalene was used, it was added later in acetone after the medium had cooled to 45 to 50°C. Toluene vapor plates consisted of minimal medium agar plates incubated at room temperature in a toluene-saturated atmosphere. All other strains were maintained on yeast extract-peptone-glucose (YEPG) agar (24).

Replication. Bacteria were grown to an optical density (OD) (λ = 550 nm) of 0.5 to 1.0 in 6 ml of YEPG broth. Samples (0.2 ml) of these cultures were transferred to individual wells of presterilized, 96-well tissue culture plates (Microtest II; Falcon Plastics, Oxnard, Calif.). This was done to facilitate a reproducible transfer matrix when using a replicator block consisting of a plastic block with 48 imbedded stainless-steel pins that matched the pattern of one-half of the microliter plate and fit within an 82-mm-diameter circle. The pegs were sterilized by immersion in 5.25% sodium hypochlorite (full-strength Clorox) for 2 min. The residual hypochlorite was removed by three sequential immersions (with agitation) of the replicator pins in sterile distilled water. The replicator block was then used to transfer 1 droplet from each microtiter well in a single step. The inoculated surface consisted of a sterile 82-mm-diameter nitrocellulose disk (type HATF; Millipore Corp., Bedford, Mass.) which overlaid three sterile 90-mm filter paper disks (Whatman no. 40) saturated with 10.0 ml (total) of YEPG broth. Cells transferred to this support were allowed to mature to colonies at room temperature.

Microcosm samples. Sediment samples were obtained from 1-liter pond sediment microcosms previously described (Sayler and Sherrill, in press). These microcosms had been exposed to synthetic oils (SO) at concentrations (vol/vol) of 0, 0.001, 0.01, 0.1, and 1% for >2 years at 25°C in the dark. The purpose of these samples stems from the fact that they demonstrate enhanced rates of oxidation of aromatic hydrocarbons due to the SO exposure (Sayler and Sherrill, in press) and may demonstrate bacterial populations containing catabolic genes homologous to the TOL and NAH-7 plasmids.

Sediment samples were diluted and spread-inoculated directly onto YEPG or minimal agar plate surfaces or onto hybridization supports overlaid on the respective medium. The inoculated plates were incubated at room temperature until colony development occurred: ca. 2 weeks for minimal media with toluene vapor, naphthalene, or *m*-toluate added. For hybridization, the respective supports (i.e., nitrocellulose or GeneScreenPlus; New England Nuclear Corp., Boston, Mass.) were removed and processed as described below. In some cases, colonies growing on the agar surface were replicated to the appropriate filter by placing the filter

support on the colonies and pulling it off after 10 to 15 min. These "pulls" were subjected directly to hybridization with no further incubation of the cell mass transferred during the replicating process.

Buffers. The following buffers were used: 20× SET—3 M NaCl, 0.6 M Tris-hydrochloride (pH 7.9), 0.02 M disodium EDTA; 1× Denhardt—0.02% Ficoll (molecular weight, 400,000), 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin; hybridization buffer—5× SET, 1× Denhardt, 0.1% sodium dodecyl sulfate (SDS); single-stranded salmon sperm carrier (10 mg/ml)—boil 50 mg of DNA from salmon testes (Sigma Chemical Co., St. Louis, Mo.) in 4 ml of NaOH for 5 min and then add 750 μl of 2 M Tris-hydrochloride, 250 μl of concentrated HCl, and 20 μl of 0.5 M disodium EDTA; G-50 column buffer (pH 7.9 ± 0.3)—0.25 M NaCl, 0.05 Tris, 0.002 M disodium EDTA, 0.5% SDS; high-stringency wash buffer—10 mM NaCl, 20 mM Tris, 1 mM disodium EDTA, 0.5% SDS.

Plasmid isolation and probe preparation. Large-scale isolation of pLAFR1 and RSF1010 was performed by the method of Kado and Liu (15) with a minor modification of the lysis buffer. The buffer was prepared by adjusting 50 mM Tris (Sigma)–3% sodium lauryl sulfate (Sigma) to 0.062 N sodium hydroxide. This buffer was prepared fresh and used within 3 h. TOL plasmid was prepared by the method of Wheatcroft and Williams (26). NAH-7 plasmid isolation was by the Birnboim and Doly procedure (2). Routine detection of plasmid DNA was by the method of Kado and Liu (15).

Purified DNA for probe preparation was obtained by dye-buoyant density ultracentrifugation in CsCl gradients (at 1.05 g ml^{-1}, containing ethidium bromide at 0.94 mg ml^{-1}) with a Ti50 rotor at 40,000 rpm for 48 h and 20°C. Plasmid bands obtained from this procedure were extracted with 5 M NaCl-saturated isopropanol to remove contaminating ethidium bromide and precipitated with 95% ethanol according to established procedures (5).

[^{32}P]DNA probe was prepared by nick translation of the appropriate purified plasmid DNA, using the protocol supplied with the commercial nick translation reagent kit (Bethesda Research Laboratories, Gaithersburg, Md.). [^{32}P]dCTP at 3,000 Ci $mmol^{-1}$ (New England Nuclear) was used as the sole labeling nucleotide. [^{32}P]dCTP was separated from the ^{32}P-labeled probe by differential rates of elution through a 3-cm^3 Sephadex G-50 column (previously equilibrated) with column buffer (see buffers given above). Fractions (0.5 ml) were monitored with a Geiger counter (model 3708; Dosimeter Corp., Cincinnati, Ohio). The fractions making up the first radioactive peak were pooled and used as probe DNA. Quantification of activity was by liquid scintillation spectrometry.

During the course of these experiments, three hybridization supports (filters) were examined for their general utility. These included nitrocellulose HATF, GeneScreenPlus, and cellulose filters (white ribbon; Schleicher & Schuell, Keene, N.H.). In preliminary studies the cellulose support was found unsuitable due to high autoradiographic background; consequently, all further hybridizations were conducted with the nitrocellulose or GeneScreenPlus hybridization supports.

Colonies on nitrocellulose disks were lysed by placing the disk (colony side up) on a 1.5-ml pool of 0.5 M NaOH on plastic wrap for 1 min. The filter was then blotted briefly on filter paper (Whatman 3MM) and the entire procedure was repeated once more with 0.5 M NaOH, twice with 1 M Tris (pH 8.0), and once with 1.5 M NaCl in 1 M Tris, pH 8.0 (with blotting before each new rinse). The nitrocellulose filters were then air dried and baked in vacuo at 70°C for 1.5 h. Colonies on GeneScreenPlus filters were lysed according to the manufacturer's protocol, blotted dry, and used directly.

Up to five filters at a time were prehybridized by placing them in a heat-sealable plastic bag containing the appropriate prehybridization solution. For nitrocellulose this solution contains 5× SET, 1× Denhardt, 0.1% SDS, and 0.1 mg of salmon sperm carrier DNA ml^{-1}. For GeneScreenPlus, prehybridization was conducted according to the manufacturer's instructions. The sealed bags were then incubated for 12 to 16 h at 65°C.

[^{32}P]DNA probe was heated in a boiling-water bath for 5 min, cooled, and injected into the sealed plastic bags with a 21-gauge needle and tuberculin syringe to give a final activity of 1.5×10^5 dpm ml^{-1} (ca. 10 μg of DNA ml^{-1}) of the hybridization buffer for nitrocellulose filters. For GeneScreenPlus filters, the denatured [^{32}P]DNA probe and salmon sperm carrier DNA were injected into the sealed bags to attain a final concentration of 1.5×10^5 dpm ml^{-1} and 1.0 mg ml^{-1}, respectively. These bags were incubated (with agitation) for 16 h at 65°C. Filters were then removed from the bags and washed twice in the high-stringency wash buffer (75 ml of buffer $disk^{-1}$) at 68°C for 2 h. This was followed by two 3 mM Tris washes (2 h each) with 75 ml $disk^{-1}$ at room temperature. The filters were then blotted to remove excess fluid and mounted between two sheets of plastic wrap before they could dry.

Autoradiography. Filter disks with ^{32}P-labeled plasmid probe bound were autoradiographed with Kodak X-Omat X-ray film and a single intensifier screen (Cronex, Lighting Plus; Dupont). The cassettes were exposed at −70°C for 8 to 24 h.

RESULTS

Probe cross-hybridization. Cross-hybridization analysis was performed to assess the cross-reactivity of a given probe towards several target bacterial lysates of known and unknown DNA homologies. The purpose, therefore, was to test the likelihood of cross-hybridization interferences due to common DNA sequences. Bacterial strains used as targets and sources of probe templates are listed in Table 1. Table 2 gives the results of hybridizations with ^{32}P-labeled TOL (^{32}P-TOL), ^{32}P-NAH-7, ^{32}P-RSF1010, and ^{32}P-pLAFR1 plasmid probes.

(i) ^{32}P-TOL. Hybridization signals were detected for the following: TOL plasmid; the NAH-7 plasmid, which is known to share at least four regions of strong homology with TOL (18); pKT530, which is a clone of the TOL *meta*-cleavage operon (8); and the pAC27 and pAC31 plasmid-bearing strain which harbors the TOL transposon (3). The only unexpected hybridization occurred with *Alcaligenes* sp. strain A2D. A2D is capable of 4-chlorobiphenyl mineralization which is plasmid mediated (M. S. Shields and G. S. Sayler, submitted for publication). Apart from this, the TOL plasmid probe shows no cross-reactivity with any cloning vector, antibiotic resistance, or cryptic plasmid-bearing strain surveyed.

(ii) ^{32}p-NAH-7. The pattern of hybridization for ^{32}p-NAH-7 was identical to that for the ^{32}P-TOL plasmid probe. This is not surprising since NAH and TOL plasmids are known to share a high degree of homology.

(iii)^{32}P-RSF1010. RSF1010 is a broad-host-range plasmid cloning vector used in the construction of pKT530. The only expected hybridizations were with RSF1010 and pKT530. Three unexpected hybridizations also occurred: two with EC290 and EC295 and one weak reproducible hybridization

TABLE 2. Cross-hybridization of [^{32}P]DNA probes in colony hybridization

Strain	Target plasmid	Probe plasmid							
		^{32}P-TOL		^{32}P-NAH		^{32}P-RSF1010		^{32}P-pLAFR1	
		f[a]	r[b]	f	r	f	r	f	r
ATCC 23973	TOL	1.00	6/6	0.68	6.6		0/6	0.64	6/6
	NAH-7	0.30	6/6	1.00	6/6	0.47	6/6	0.82	6/6
	pLAFR1	NDG[c]		NDG		NDG		1.00	5/0[d]
C600	RSF1010		0/4		0/6	1.00	4/6	2.87	6/6
Mt-2 2440	pKT530	0.81	6/6	0.27	6/6	1.22	6/6	0.19	5/6
PU21	None		0/6		0/6		0/4		0/6
PU21	pMG1		0/6		0/6		0/2		0/6
V517	pV517A-H		0/6		0/6		0/5	2.97	6/6
ACS8	None		0/6		0/6		0/6		0/5
BM2	None		0/4		0/4		0/3		0/6
AX2	pSK2		0/6		0/6		0/6		0/6
A2	pSK2		0/6		0/6		0/6		0/6
A2D	pSK20 & 21	1.05	6/6	0.21	6/6		0/6	0.13	6/6
A5	pSK50 & 51		0/6		0/6		0/3		0/6
P6	pKF1		0/5		0/6		0/6		0/6
M5	pKF1		0/6		0/6		0/6	0	0/6
	pAC27	0.42	6/6	0.12	6/6		0/6	0	0/6
	pAC31	0.23	6/6	0.13	6/6		0/4	0	0/6
J53	R388		0/5		0/5		0/6	2.91	6/6
J53	R46		0/3		0/3		0/6	4.85	6/6
Ec 290			0/4		0/4	0.58	6/6	4.83	6/6
Ec 295			0/6		0/6	0.77	6/6	4.46	6/5[d]
RC709	RP4		0/6		0/6		0/6	3/59	6/6

[a] f = Autoradiogram spot intensity relative to that of the self-hybridized probe (i.e., density of the autoradiogram spot divided by the density of that probe's autoradiogram spot resulting from hybridization to itself). Data based on scanning densitometry tracings.
[b] r = Ratio of number of colonies positive for hybridization/number of colonies observed at ×35 magnification.
[c] NDG, No detectable growth.
[d] Detectable hybridization despite lack of detectable growth on the nitrocellulose filters.

with the strain containing NAH-7 plasmid (unexplainable in view of the fact that the reverse hybridization [i.e., RSF1010 with the ^{32}P-NAH-7 plasmid probe] was not detected, despite good growth of RSF1010 target colonies).

(iv) ^{32}P-pLAFR1. pLAFR1 is a broad-host-range cosmid cloning vector originally constructed for the subcloning of *Rhizobium meliloti* chromosomal DNA and constructed from lambdoid coliphage DNA and pRK290, a broad-host-range plasmid (9). The ^{32}P-pLAFR1 probe demonstrates a wide range of hybridization, i.e., 52% of all strains examined and 60% of all plasmid-bearing strains (Table 2). The ^{32}P-pLAFR1 probe hybridized with strains harboring the TOL, NAH-7, and RSF1010 plasmids, whereas none of these plasmids (when used as probes) hybridized with pLAFR1. This result is explainable. pLAFR1-containing cells failed to yield detectable growth on any of the filters surveyed. Consequently, the quantity of pLAFR1 target DNA was insufficient to yield a detectable signal when hybridized to marginally homologous probe. ^{32}P-pLAFR1 self-hybridization to the location on the filter where pLAFR1 colonies should have appeared indicates a high sensitivity of the technique for detecting gene sequences from a cell mass below the limits of observable growth (at ×35 magnification).

Probe sensitivity. This stage of the investigation was undertaken to assess the minimal detectable frequency of specific plasmid-bearing cells against a bacterial background of nonhomologous DNA. The ^{32}P-TOL plasmid was used to probe a mixed population of *P. putida* ATCC 23973 (bearing the TOL plasmid) and the eight-plasmid *E. coli* V517. *E. coli* V517 was chosen because it failed to hybridize with the ^{32}P-TOL plasmid probe (Table 2). The *E. coli* and *P. putida* cultures were separately prepared in YEPG broths and bacterial concentrations were estimated by OD_{550}. They were then diluted and mixed to give approximate ratios and spread onto sterile nitrocellulose filters atop the growth media. Two general approaches were undertaken: (i) a low density of *E. coli* V517 cells as background, with a high frequency of *P. putida* ATCC 23973 cells; and (ii) a high density of *E. coli* V517 cells as background, with a low frequency of *P. putida* ATCC 23973 cells.

The first case (Table 3) was designed to test the fidelity of the ^{32}P-TOL probe for morphologically recognizable *P. putida* colonies known to harbor the target TOL plasmid DNA. Figure 1 is a representative autoradiogram after hybridization of ^{32}P-TOL plasmid to *P. putida* within low and high *E. coli* (V517) backgrounds. In Fig. 1A, *E. coli* was present at approximately 5×10^5 CFU per plate; in Fig. 1B, *E. coli* was present at 77 CFU per plate. The results presented in Table 3 indicate a correlation approaching 100% between ratios based on colony morphology and those based on autoradiographic data at target frequencies of less than 50 colonies per plate. Since colony morphology was tedious and difficult to score, a certain error in the morphological determinations was to be expected. These results indicate that the ^{32}P-TOL plasmid probe hybridizes exclusively to TOL plasmid-bearing cells in this experiment.

In the second case only a very few *P. putida* (TOL) cells were plated amid a large population of *E. coli* V517 cells (Fig. 1A). This was designed to ascertain the limits of detectability of the target DNA in the presence of two potential limitations. One possible limitation at high cell densities (i.e., 5×10^2 to 5×10^6 CFU per plate) is the size of a *P. putida* colony. It will necessarily be much smaller than colonies grown in a low-cell-density plate (i.e., <300 CFU per plate) due to competition. Second, a high popula-

TABLE 3. Accuracy of ^{32}P-TOL DNA colony hybridization in a low-density reconstructed population

Approximate *E. coli*/*P. putida* ratio[a]	CFU/plate[b]	Colony morphology		Autoradiography[c]	
		E. coli/*P. putida* ratio	*P. putida* frequency	*E. coli*/*P. putida* ratio	*P. putida* frequency
1,000:1	122	122:0	0	122:0	0
100:1	125	123:2	1.60×10^{-2}	123:2	1.60×10^{-2}
10:1	210	185:25	1.19×10^{-1}	186:24	1.14×10^{-1}
3:1	88	56:32	3.64×10^{-1}	62:26	2.95×10^{-1}
2:1	77	42:35	4.55×10^{-1}	43:24	4.42×10^{-1}
1:1	191	73:118	6.18×10^{-1}	99:92	4.82×10^{-1}

[a] Based on dilutions of broth whose CFU density was estimated by OD_{550}.
[b] YEPG agar.
[c] Probe was ^{32}P-TOL from *P. putida* 23973 used in these reconstructed populations.

tion of nonhomologous cells might reduce the ability of a probe to find its target DNA due to a masking phenomenon caused by the abundance of nonhomologous DNA. The results of this experiment are presented in Table 4. The frequencies of cells bearing target DNA (as measured by hybridization to the ^{32}P-TOL probe) was an average of 73.2% of the frequencies estimated from OD_{550} measurements and subsequent dilutions. The detection limits appear to be as low as 1 target colony per 10^6 nonhomologous background colonies per plate.

Microcosm sediments. Experimental sediment microcosms were used to assess the utility of [^{32}P]DNA probing methods for detecting catabolic genes in environmental populations. Sediment microcosms previously exposed to a range of SO contamination (0% to 1.0%) demonstrated a direct correlation between SO contamination level and rates of polynuclear aromatic hydrocarbon mineralization (Sayler and Sherrill, in press). Sediment samples were diluted and plated directly on nitrocellulose filters overlaid on YEPG or minimal agar with toluene vapor as the carbon source. After a 2-week incubation the filters were hybridized with ^{32}P-TOL probe (Fig. 2A). Total CFU on YEPG agar and colonies that hybridized to the ^{32}P-TOL plasmid probe (^{32}P-TOL$^+$ CFU) increased in direct relationship to increased SO content (Table 5). Approximately 3% of the total CFU (YEPG agar) demonstrated positive hybridization with the ^{32}P-TOL DNA probe in untreated sediments compared with 13.8 to 31.8% in SO-treated sediments. A 20-fold increase in total CFU on YEPG was recorded for sediments treated with 1% SO versus those treated at the 0.1% level. Comparatively, 50-fold increase in ^{32}P-TOL$^+$ CFU was observed for the same microcosm sediments.

For all sediments, an average of 19.2% of the total CFU on YEPG agar were ^{32}P-TOL$^+$ CFU. Since the plasmid TOL is a general probe that also hybridizes to other catabolic plasmids (Table 2), the same analysis was carried out for

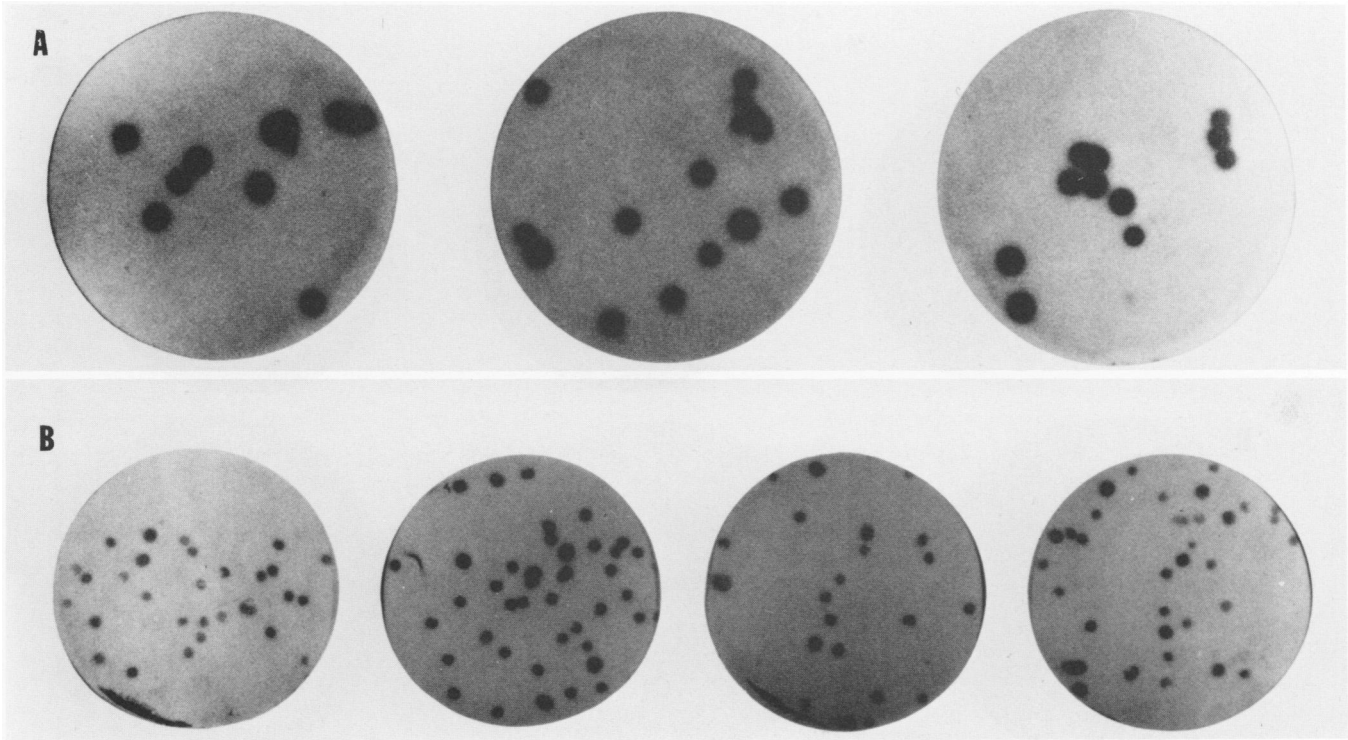

FIG. 1. Sensitivity and fidelity of ^{32}P-TOL DNA probes in *E. coli*/*P. putida* (TOL) reconstructed populations. (A) Low-frequency *P. putida*, high *E. coli* background (see 2.33×10^{-5} frequency of *P. putida* at 5×10^5 cells per plate in Table 4); (B) high-frequency *P. putida*, low-background *E. coli* (see 2:1 ratios in Table 3).

TABLE 4. Sensitivity of ^{32}P-TOL DNA colony hybridization at low-frequency high-density populations

Approximate CFU/plate[a]	Frequency of *P. putida*		
	Predicted by dilution	Observed by autoradiography[b]	Observed predicted (%)
5×10^6	5.3×10^{-7}	1.04×10^{-6}	196
5×10^6	5.3×10^{-6}	3.45×10^{-6}	65
5×10^6	5.3×10^{-5}	2.11×10^{-5}	40
5×10^5	5.3×10^{-6}	5.45×10^{-7}	10
5×10^5	5.3×10^{-5}	2.53×10^{-5}	48
5×10^4	5.3×10^{-5}	5.46×10^{-5}	103
5×10^4	5.3×10^{-4}	4.36×10^{-4}	82
5×10^3	5.3×10^{-3}	3.33×10^{-3}	63
5×10^2	5.3×10^{-2}	2.78×10^{-2}	52
5×10^6	0	0	

[a] Based on OD_{550} estimations from standard curves of *E. coli* and *P. putida*; *E. coli* representing the nonhomologous background.
[b] Same probe as in Table 3.

colonies developing on toluene vapor minimal agar plates. ^{32}P-TOL probes of these toluene-grown CFU should indicate the true proportion of this population that actually contains toluene-degradative genes and is capable of growth on toluene. Total CFU on toluene vapor plates were 2 to 3 orders of magnitude less than for YEPG agar, whereas the toluene vapor-grown ^{32}P-TOL$^+$ CFU were 1 to 2 orders of magnitude less than that reported for YEPG agar (Table 5). These results may indicate that most YEPG-grown ^{32}P-TOL$^+$ CFU are auxotrophs incapable of growth on the toluene minimal medium. For the toluene-grown cells, approximately 20- and 100-fold increases in both total CFU and ^{32}P-TOL$^+$ CFU, respectively, were observed in sediments exposed to 1% SO compared with 0.1% SO. This was similar to the increased population response observed for ^{32}P-TOL$^+$ CFU recovered from YEPG. For the 1.0% SO-treated microcosms, 35% more ^{32}P-TOL$^+$ CFU than the total CFU on toluene vapor plates were detected. The reason for this apparent discrepancy was the greater ease and accuracy of counting ^{32}P-TOL$^+$ CFU by hybridization than counting the small transparent colonies developing on the nitrocellulose support.

From the data presented in Table 2, the knowledge of TOL, NAH, and SAL genetic relatedness (18, 27), and the previous microcosm analyses, it is apparent that ^{32}P-TOL$^+$ CFU do not necessarily indicate the presence of TOL plasmid DNA in microcosm sediments. For these reasons, a population of cells with NAH-7 plasmid homologous DNA and growth capability on naphthalene minimal agar plates was investigated.

Comparative population and hybridization analyses (Fig. 2B) of the control microcosm sediments (no SO) and the 1.0% SO-treated sediment indicated that both the CFU developing on naphthalene minimal medium and ^{32}P-NAH-7$^+$ were 1 to 3 orders of magnitude higher than comparable toluene-grown CFU or ^{32}P-TOL$^+$ CFU (Table 6).Toluene vapor-grown CFU represented 1% or less of the total YEPG-grown CFU in microcosms. The naphthalene-grown CFU accounted for 37% of the total YEPG-grown CFU in the untreated sediment and 92% in the 1% SO-treated sediment. For untreated sediment, <0.05% of the naphthalene-grown CFU scored positively as ^{32}P-NAH-7$^+$ CFU, whereas in the 1% treated sediment 74% of the naphthalene-grown CFU were found to be ^{32}P-NAH-7$^+$ CFU.

DISCUSSION

A primary consideration in the use of DNA probes to detect specific organisms or DNA in primary cultivation of

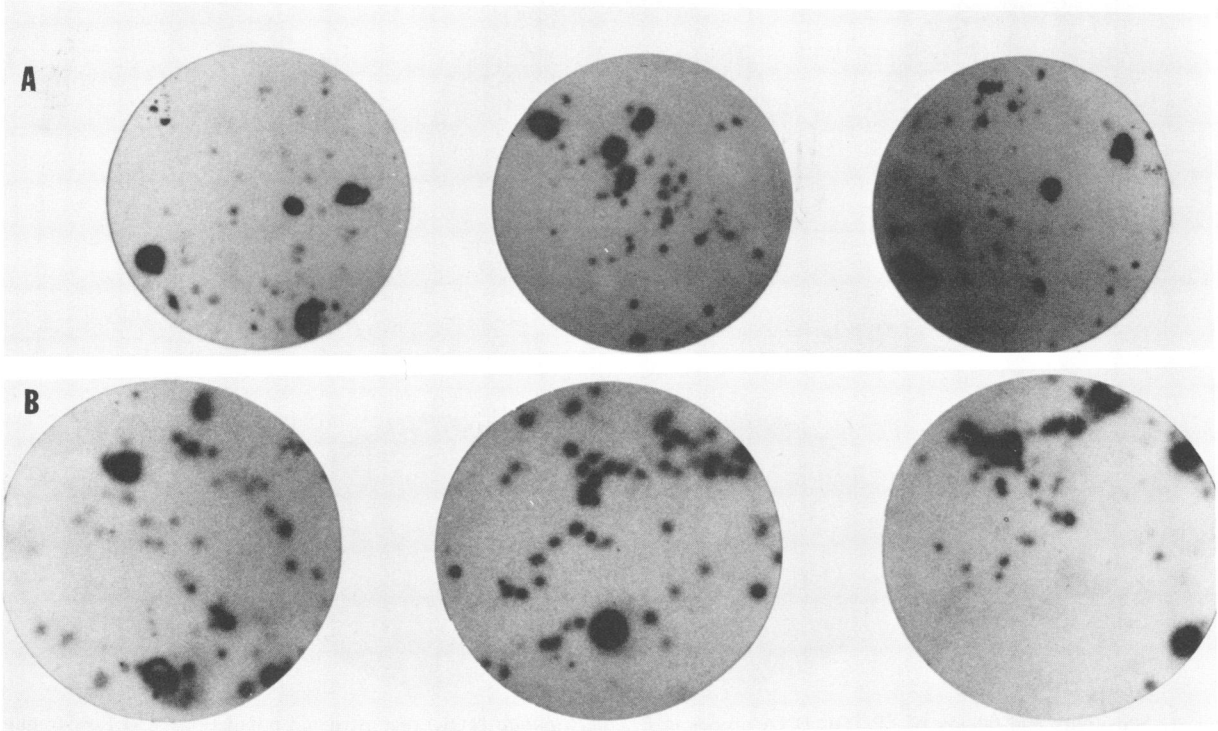

FIG. 2. Comparison of ^{32}P-TOL and ^{32}P-NAH DNA probe colony hybridization for populations from 1% SO-treated microcosm sediments. (A) TOL probe; (B) NAH probe.

TABLE 5. Comparison of ^{32}P-TOL colony hybridization among SO-treated microcosm sediments[a]

Medium	SO treatment (%)	Total CFU ml of sediment^{-1}	CFU filter^{-1} examined[b]	^{32}P-TOL filter^{-1}[b]	^{32}P-TOL positive (%)[b]	32P-TOL$^+$ CFU ml of sediment^{-1}
YEPGA	0	5.3×10^5	68	1.8	2.6	1.4×10^4
	0.001	3.0×10^5	40	5.5	13.8	4.1×10^4
	0.01	3.5×10^5	58	9.5	16.4	5.7×10^4
	0.1	9.7×10^5	214	32	15.0	1.5×10^5
	1.0	1.9×10^7	192	61	31.8	6.0×10^6
TOL-VAPOR[c]	0	6.1×10^3	87	16	18.4	1.1×10^3
	0.001	4.9×10^3	64	10	15.6	7.6×10^2
	0.01	3.5×10^3	73	13	17.8	6.2×10^2
	0.1	3.9×10^3	70	18	25.7	1.0×10^3
	1.0	6.4×10^4	60	81	135.0	8.6×10^4

[a] Microcosms exposed to SO for approximately 600 days originally described by Sayler and Sherrill (in press).
[b] Mean values of three or four replications.
[c] Toluene vapor plates.

environmental samples is the probe specificity required to test the experimental hypothesis. In initiating this research, whole-plasmid DNA was deliberately chosen for preparation of DNA probes. This decision was based on the intended use of the probes to detect homologous and related catabolic plasmids or genes in a microcosm community that previously demonstrated a broad-spectrum catabolism of aromatic hydrocarbon substrates (Sayler and Sherrill, in press). As indicated in the preliminary calibration of the colony hybridization assay used in these studies, the catabolic DNA probes prepared from NAH and TOL plasmid DNA are not highly specific, demonstrating cross-hybridization among themselves as well as selected bacteria harboring other catabolic genes. This lack of absolute specificity does not necessarily reduce the utility of whole-plasmid probes, such as the TOL plasmid, if they can serve a general purpose of estimating catabolic potential of a microbial community for aromatic substrates. Finer detailed analysis of specific catabolic genes in environmental samples can be achieved by preparing probe DNA of specific DNA restriction fragments containing genes or DNA sequences of interest.

Potentially greater problems of probe specificity can arise from the use of whole probes derived from broad-host-range plasmids or cosmid cloning vehicles such as pLAFR1. The low specificity of this probe, as observed in this investigation, may be related to undetected chromosomal DNA contamination of the nick translation mixture. A more likely explanation is the broad-host-range nature of the pRK2 plasmid, from which pLAFR1 is derived, and genetic construction of the vector (6, 9). In the case of pLAFR1, pRK290, a deletion derivative of pRK2, was developed containing DNA sequences from pMK20 of enteric origin (16). pRK290 and Cos site were then used to construct pLAFR1 (6, 9), the result being that the mixed ancestory of the cloning vector can result in whole probes of low specificity. This necessitates subcloning to develop smaller probes of higher specificity.

It is apparent from these studies that probe sensitivity by the colony hybridization procedure is high, i.e., 1 colony in 10^6 colonies of a nonhomologous background. However, potentially 1 in 10^8 colonies may be detectable if modifications of DNA fixing to nitrocellulose hybridization supports are used (19). Since the primary target DNA in these studies were low-copy-number plasmids, smaller amplifiable plasmids or genes may potentially be detected at even greater sensitivity, using appropriate amplifying media. Such improvements in sensitivity and specificity in conjunction with a new generation of nonisotopic DNA probes can make this approach a useful and sensitive tool in a variety of ecological, environmental, and taxonomic investigations.

Sediments exposed to long-term SO contamination had previously demonstrated enhanced rates of naphthalene, phenanthrene, and benzo[*a*]pyrene mineralization, correlating with the level of synthetic oil contamination (Sayler and Sherrill, in press). Initially, this enhanced biodegradative activity corresponded to a net increase in heterotrophic bacteria. The results of the experiments reported here indicate that this response correlated well with a significant increase in colonies containing DNA of sufficient homology to hybridize with TOL and NAH plasmid DNA probes. This provides evidence for an overall enrichment of catabolic

TABLE 6. Degradative population estimates and naphthalene mineralization activity in SO-treated microcosms

SO treatment	Naphthalene mineralization k (h^{-1})	Cultivation medium	CFU (ml^{-1})	^{32}P-TOL$^+$ CFU (%)[a]	^{32}P-NAH$^+$ CFU (%)
None		YEPG	5.3×10^5	2.6	50.9
		Toluene-vapor	6.1×10^3	18.4	ND[b]
	4.5×10^{-4}	Naphthalene	2.0×10^5	ND	<0.05[c]
1%		YEPG	1.9×10^7	31.8	70.8
		Toluene-vapor	6.4×10^4	135.0	ND
	7.3×10^{-3}	Naphthalene	1.1×10^7	ND	73.6

[a] % of CFU.
[b] ND, Not done.
[c] No hybridizations were detected at the dilution tested.

genotypes greater than the rate of general population increase.

TOL probe analysis of microcosms indicates that all colonies developing on toluene vapor plates from samples of the 1.0% SO-treated microcosms were toluene-catabolic organisms. This compares to approximately 20% for the lower-dosage SO microcosms (Table 5). Furthermore, approximately 32% of the organisms cultivated on YEPG agar from the 1.0% SO-treated sediments demonstrated homology with the TOL plasmid. The combined results from YEPG and toluene cultivation of the 1% SO-treated sediments indicate that 98.6% [$(8.6 \times 10^4/6.0 \times 10^6) \times 100\%$] of the ^{32}P-TOL$^+$ CFU from YEPG-grown cells were incapable of utilization of toluene as a sole carbon source. Furthermore, only 0.3% [$(6.4 \times 10^4/1.9 \times 10^7) \times 100\%$] of the total YEPG-grown population is capable of toluene catabolism and growth. This is a considerably smaller proportion than was found to be positive for ^{32}P-TOL probe hybridization: 31.6% [$(6.0 \times 10^6/1.9 \times 10^7) \times 100\%$] (Table 5).

The combined results for TOL and NAH probe analysis indicate that the majority of organisms in untreated sediment capable of growth on toluene vapor or naphthalene minimal agar plates either do not utilize the aromatic substrates and exist on trace levels of organics or contain catabolic genes significantly different from the catabolic plasmid DNA used as the probe for colony hybridization (Tables 5 and 6). Conversely, organisms capable of aromatic metabolism appear to have been selected in oil-treated sediments and the majority demonstrate DNA sequence homology to the ^{32}P-labeled catabolic DNA probes used.

Through the use of nonselective and selective cultivation media, and ^{32}P-labeled NAH and TOL plasmid DNA probes, it appears that a response of the heterotrophic guild to SO contamination is a significant genetic selection or community adaptation for bacteria capable of sole carbon source utilization of naphthalene as a growth substrate and containing DNA homologous to NAH plasmid DNA. Evidence for this interpretation is the 15-fold increase in the naphthalene mineralization rates and the 30-fold increase in ^{32}P-NAH-7 CFU of the microbial community in the 1.0% SO-treated microcosms (Table 6). Although similar populations are present in the untreated microcosm sediments, their frequency and capacity for growth on naphthalene are significantly lower. These organisms, although containing DNA homologous to NAH (on nonselective cultivation), may not demonstrate equivalent growth on naphthalene as a result of co-oxidative metabolism or auxotrophic growth requirements. In addition, growth on naphthalene minimal medium may also be indicative of oligotrophic growth by organisms not actually utilizing naphthalene as a carbon source (Table 6). In the case of oil-treated sediments, 74% of the colonies capable of growth on naphthalene minimal medium are also positive targets for ^{32}P-NAH plasmid DNA.

It is not envisioned that DNA probe techniques would replace classical enrichment, selection, and differential cultivation procedures; it is, however, expected that they would augment these procedures. There are instances where colony hybridization may be superior to classical methods. Examples of such cases could include detection of debilitated organisms on primary nonselective cultivation or detection of organisms carrying poorly expressed or nonselectable genes of interest. In the latter case, organisms harboring recombinant DNA (in the original host or transferred to indigenous environmental organisms) would represent a likely case for the use of colony hybridization on primary nonselective cultivation of an environmental sample.

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