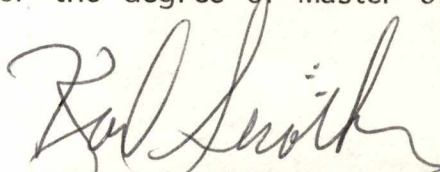


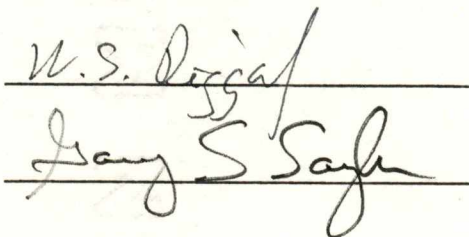
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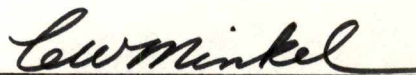


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COMPARATIVE SPECIFICITY OF GENOMIC VERSUS SUBGENOMIC PROBES
OF A PUTATIVE ARTHROBACTER SPECIES IN ENVIRONMENTAL
APPLICATIONS OF COLONY HYBRIDIZATION

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Carole Lynne Harris

March 1987

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ABSTRACT

This study tests the efficacy of colony hybridization to detect a model groundwater microorganism designated as AHS24, a putative Arthrobacter species isolated from a pristine groundwater aquifer located in Lula, Oklahoma. This application of colony hybridization represents one of the newly evolving technologies which addresses the need to develop more accurate and rapid ways of evaluating the maintenance and proliferation of certain populations of microorganisms in the environment.

Radioactively labeled AHS24 whole genomic probes were compared to constructed AHS24 subgenomic radioactive DNA probes for specificity and sensitivity in detecting AHS24 target DNA amidst the presence of target DNAs from other isolates present on the hybridization membrane. Initially, eight groundwater isolates showing consistent positive hybridization signals with AHS24 whole genomic probe under varying hybridization stringency of 50 mM to 1000 mM NaCl were chosen for comparative specificity experiments with constructed AHS24 subgenomic probes. These eight isolates, along with AHS24 and other ATCC strains of interest were utilized as target DNAs for comparing the specificity and relative sensitivity of the constructed AHS24 probes and the AHS24 whole genomic probe in hybridizations under two different stringency washes. Another minor set of comparative experiments in which the ability to detect AHS24 DNA sequences amidst AHS24 inoculated and uninoculated freshwater communities was also performed with AHS24 whole genomic and subgenomic probes.

These results showed that colony hybridization can be efficiently utilized to detect the presence of a particular groundwater microorganism, AHS24, with high sensitivity. In all sets of experiments, under varying stringencies and in the presence of known isolate target DNAs or AHS24 inoculated freshwater communities, AHS24 colonies yielded the most intense signals with either AHS24 whole genomic probes or AHS24 subgenomic probes. The study has also shown that subgenomic probes, although highly sensitive, have to be carefully chosen in order to be more discriminatory than whole genomic probes. Only one of four subgenomic probes, p2MLO, appeared to demonstrate consistent narrowed specificity.

An unexpected cross-hybridization between AHS24 probe DNA and Pseudomonas strain target DNAs was observed. This was unanticipated since the genus Arthrobacter and the genus Pseudomonas are considered to be highly divergent according to traditional taxonomy. If cross-hybridization is indicative of ancestry, AHS24 may represent a microorganism whose genome arose not necessarily from a single line of descent, but rather from the blending of more than one line of ancestry.

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I. INTRODUCTION

The development of highly discriminatory probes for subsets of genes in environmental samples represents a relatively new application of DNA-DNA colony hybridization technologies. This is mainly due to the fact that colony hybridization was first described only ten years ago by Grunstein and Hogness (20). They developed a new technique which allowed direct detection of recombinant plasmids containing specific cloned genes by affixing the plasmid-containing colonies to nitrocellulose, hybridizing the membrane with labeled homologous RNA, and visualizing the clones containing the specific genes of interest by autoradiography. Using this procedure, Grunstein and Hogness could screen approximately one thousand colonies at a time. In 1980, Hanahan and Meselson (23) demonstrated that recombinant plasmids containing specific cloned genes could be detected at colony densities of 1×10^5 or greater. This high density screening procedure made screening for unique eukaryotic DNA sequences from representative libraries possible with only a few high density filters. Thus, the use of colony hybridization in recombinant DNA library screening has become a fundamental application of the technique due to its efficiency.

Other applications of colony hybridizations have been developed in addition to its utilization in recombinant DNA library screenings. For example, DNA-DNA colony hybridizations have been used to detect the presence of enterotoxigenic E. coli genes directly from stool samples of patients suffering from acute diarrhea (33, 38). Colony hybridization has been used to detect Leishmania species in cutaneous tissues (49)

and similar systems have been applied to screen serum, cell lines, or urine for hepatitis B virus, cytomegalovirus, and Epstein Barr Virus, respectively, (2, 8, 4). Contaminated food samples have also been screened for the presence of Salmonella, virulent Yersinia enterocolitica, and enterotoxigenic E. coli using colony hybridization techniques (16, 25, 24).

The screening of environmental communities for specific DNA sequences represents a relatively untouched application of DNA-DNA colony hybridization technologies whose development is gauged mainly by how quickly the appropriate probes for a DNA sequence or set of sequences can be derived in the laboratory. These specific probes can then be utilized to track the survival and maintenance of microorganisms carrying those sequences in the environment. The dynamic nature of specific bacterial populations in the environment makes colony hybridization technology a particularly attractive method of monitoring and assessing these populations due to the efficiency at which colony hybridizations can be performed. DNA-DNA colony hybridizations are simple technically in that they require only the presence of target DNA from the particular colonies of a population on a nitrocellulose or nylon hybridization support and the single stranded radiolabeled DNA probe, therefore many environmental samples can be screened at once. This simplicity also has some inherent advantages over more conventional methods which rely on gene expression, causing a particular phenotype such as the synthesis of a surface antigen or the synthesis of a group of enzymes which allow growth on a single carbon source,

as summarized in Table 1 (42). These types of conventional analyses do not allow the detection of cryptic or "silent" genes that may be present in a population of microorganisms due to the reliance of these methods on phenotypic expression. In contrast, by analyzing the potential to express these subsets of genes in the environment via colony hybridization, a new perspective for research in microbial ecology has been developed in that the selective conditions or other criteria imposed by classical studies are minimized in their influence upon the accurate assessment of the frequency of specific indigenous or genetically engineered microorganisms in environmental bacterial populations (42). It is important to note also that colony hybridization represents only one of many new ways to evaluate the presence or proliferation of environmental microorganisms. Some of these approaches have been summarized in Table 2. Due to its simplicity and high sensitivity, however, colony hybridization will most likely become one of the more popular ways for assessment of the presence of specific bacterial populations, and that other techniques such as plasmid profile analysis or protein analysis will serve as supplementary methods to support data acquired initially from colony hybridization experiments, especially in those cases where colony hybridization proves to be the superior method.

Colony hybridization offers an additional advantage over traditional methods of enumerating bacterial populations in environmental samples in that the discovery of new information regarding genes involved in the expression of a particular phenotype is more likely. For

Table 1. Summary of conventional approaches to evaluate microbial presence or proliferation.

Approach	Phenotypic requirement
Primary Cultivation	General organismal growth
Enrichment	Growth on specific nutrients or existence of a discriminatory logical activity
- Direct Plating - Selective Medium - Differential Medium	
Dilution to Extinction	Growth or specific physiological activity
Serology/Fluorescent Antibodies	Synthesis of specific antigens
Phage Sensitivity	Infectibility

Table 2. Approaches for evaluating the presence or proliferation of genetically engineered microorganisms.

Approach	Method
Plasmid Analysis	Plasmid Profiles Restriction Profiles
Phenotypic Analysis	Genetically Linked Traits--DNA Segments Coding for: Antibiotic Resistance Chromogenic Substrates Unique Cellular Constituents (e.g., specific antigens)
Chemical Analysis	RNA, DNA Gene Probes
Sequence Analysis	Ribosomal RNA

example, traditional enumeration methods for detection of catabolic phenotypes in environmental samples involves plating populations upon single carbon source plates such as toluene vapor plates (43). In a particular colony hybridization experiment designed to assess the utility of [^{32}P] DNA probing methods for detecting catabolic genes in environmental populations, Sayler and his co-workers reported:

For all sediments, an average of 19.2% of the total CFU on YEPG agar were $^{32}\text{P-TOL}^+$ CFU. Since the plasmid TOL is a general probe that also hybridizes to other catabolic plasmids, the same analysis was carried out for colonies developing on toluene vapor minimal agar plates. $^{32}\text{P-TOL}$ probes of these toluene-grown CFU should indicate the true portion 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 $^{32}\text{P-TOL}^+$ CFU were 1 to 2 order of magnitude less than reported for YEPG agar. These results may indicate that most YEPG-grown $^{32}\text{P-TOL}^+$ CFU are auxotrophs incapable of growth on toluene minimal medium. (43)

From these results, it is apparent that the isolation of potentially useful auxotrophic mutants for particular catabolic phenotypes could be greatly facilitated by colony hybridization used in conjunction with traditional methods for determination of auxotrophy. The ability to detect new genes involved in catabolic pathways is also magnified by colony hybridization techniques. Particular environmental isolates may demonstrate through chemical analyses such as gas chromatography that they are positive for toluene catabolism, but may not probe positive with known probes for toluene degradation genes. These hypothetical results would indicate that there may be significantly different (in reference to DNA sequence homology) plasmid or chromosomally-encoded genes involved in toluene degradation in these isolates. Using

traditional analysis alone, these new and interesting genes might not be detected.

Colony hybridization may be especially helpful in the future in providing some guidelines with regard to taxonomic considerations. New environmental isolates and older ones of uncertain affiliation may be better characterized more quickly using a series of highly specific probes for designated species. The basis for this conjecture stems from the fact that the phylogenetic relatedness of organisms is positively correlated with the amount of DNA sequence homology shared (6). Newer approaches to deriving more accurate taxonomic relationships such as DNA-DNA hybridization and 16s RNA analysis may in fact reveal that traditional taxonomic criteria such as cell morphology and other phenotypic traits may be limited in their ability to designate the appropriate category for a microorganism. In a recent review on the use of 16s RNA analysis to establish phylogenetic relationships in prokaryotes, Fox addresses this particular discrepancy in traditional taxonomy:

While in many instances the present genealogies are consistent with traditional classifications, it is clear that in others the classical taxa are not phylogenetically valid units. The heavy emphasis traditionally placed on morphological characteristics is seen not to be justified. Spherical shape is a principal offender. All spherical bacteria so far examined fall into phylogenetic categories defined in terms of non-spherical organisms. Examples are Paracoccus (a specific relative of Rhodopseudomonas capsulata), Sporosarcina (a specific relative of Bacillus pasteurii), and Micrococcus (the species of which are genealogically intermixed with those of Arthrobacter). (17)

It is apparent that there will be differences in taxonomic relationships based on phenotypic similarities and those based upon genotypic

similarities, and that genotypic relatedness may be a stronger tool in better defining taxonomic groups of environmental isolates. This more complicated, but perhaps more accurate, assessment of the phylogenetic relatedness of microorganisms will allow for the development of highly accurate methods for tracking the survival and maintenance of distinct groups of microorganisms, more specifically their genes, in environmental settings.

In this study, colony hybridization is presented in conjunction with the use of subgenomic probes as one of a new, highly quantitative molecular approach for assessing presence of artificially introduced genes (via recombinant DNA techniques) or specific indigenous genes in the environment. Of primary importance in this study is the determination of the sensitivity and specificity of subgenomic probes versus whole chromosomal probes in these environmental samplings. Of particular interest for future specific probe development are those sequences which are unique to a species of microorganism or a very small family of closely related microorganisms in the environment. A putative Arthrobacter, AHS24, was chosen for the model microorganism in this investigation primarily for the reason that the Arthrobacters represent a very large heterogeneous population of soil and ground water microorganisms that have a nutritional versatility comparable to the genus Pseudomonas (9). The Arthrobacters, like Pseudomonas, therefore represent potentially important microorganisms to industry and to independent research groups which are involved in utilizing the catabolic capacities of these microorganisms in deriving

new ways to degrade recalcitrant environmental pollutants such as the PCBs.

II. THE EXPERIMENTAL APPROACH

In order to compare the specificity of subgenomic probes versus whole genomic probes in colony hybridization experiments, the following cloning scheme was used:

1. Library Generation

1. Isolation of chromosomal DNA from ground water isolate AHS24, a putative Arthrobacter species.
2. Partial digestion of the chromosomal DNA with four-hitter Sau 3A such that the fragment size favors adequate representation of the whole genome in a recombinant DNA library (44).
3. Ligation of the AHS24 chromosomal DNA to Bam H1 linearized pUC13 vector (Figure 1) and subsequent recircularization of the recombinant molecules. The vector pUC13 was chosen for its ability to accept large inserts due to its small size (2300 bp). It also has a high copy number and cells bearing recombinant plasmids are not selected against since the target DNA is clones into the multiple cloning site of M13 carried on the vector.
4. Transformation of E. coli DH1 with the recombinant plasmids to generate a recombinant DNA library of "gene bank" of AHS24 subgenomic fragments.

	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	5	6	7	8	
M13 mp11/pUC13	THR	MET	ILE	THR	pro	ser	leu	gly	cys	arg	ser	thr	leu	glu	asp	pro	arg	ala	ser	ser	ASN	SER	LEU	ALA	
(7)	ATG	ACC	ATG	ATT	ACG	CCA	AGC	TTG	GGC	TGC	AGG	TCG	ACT	CTA	GAG	GAT	CCC	CGG	GCG	AGC	TCG	AAT	TCA	CTG	GCC
					<u>Hind III</u>				<u>Pst I</u>		<u>Sal I</u>	<u>Xba I</u>	<u>Bam HI</u>				<u>Sma I</u> <u>Xma I</u>				<u>Sst I</u>	<u>Eco RI</u>			<u>Hae III</u>

Map of the pUC Family of Plasmids

The sequence of these plasmids was assembled based on the construction described by Viera and Messing (*Gene* 19: 259, 1982). There is some uncertainty in the sequence as an *Acc* I site was removed at position 518 by *Bal* 31 digestion. It is not known exactly how many bases were removed. For the present map it was arbitrarily assumed that six bases were removed. The numbering of the sites is based on the pUC18/19 pair of plasmids. For the sequence of the multiple cloning site (MCS) region, see page 13.

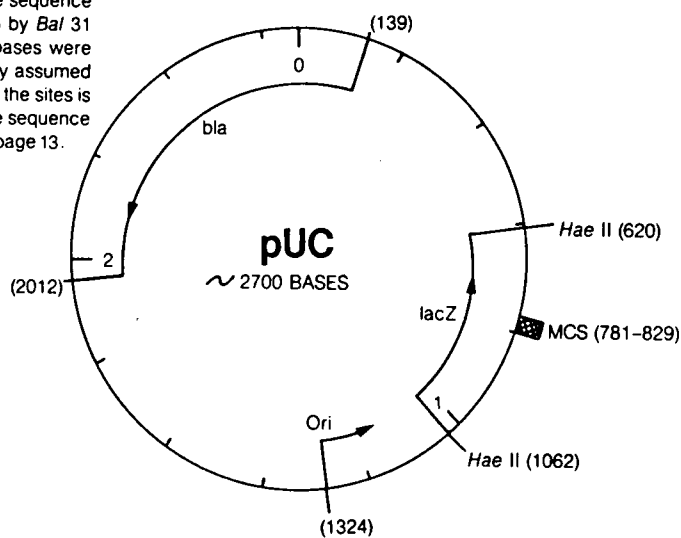


Figure 1. Map of the pUC13 plasmid and the multiple cloning site sequence (46).

2. Library Screening

5. Plating of library members to a colony density of 200–500 on selective ampicillin plates and transfer of these colonies to nylon membranes.
6. Alkaline lysis of the colonies to release target DNA, neutralization, and fixation by baking to the nylon support.
7. Nick translation of AHS24 chromosomal DNA with ^{32}P α -labeled dCTP.
8. Denaturation of ^{32}P -labeled probe by boiling to generate the single-stranded form for use in colony hybridizations.
9. Hybridization of the ^{32}P -labeled single stranded probe to the immobilized target DNA.
10. Washing of the nylon membranes at a known stringency to remove all nonhomologous or very weakly homologous probe.

3. Detection and Selection of Plasmids Containing Subcloned AHS24 Fragments

11. Exposure of X-ray film placed next to the hybridization support containing the radioactively labeled positive hybrids.
12. Detection of positive hybrids in the developed film by ^{32}P induced silver deposition.
13. Alignment of the X-ray film with the original colony containing plates and selection of single colonies which can be associated with a distinct autoradiographic signal.

14. Small or medium scale plasmid preparation from single colonies yielding positive autoradiographic signals and determination of AHS24 inert size by restriction endonuclease analysis.
4. Screening of Groundwater Isolates and AHS24-Dosed and Undosed Environmental Samplings
 15. Plating of individual groundwater isolates or plating of environmental samplings at colony densities of 10^2 - 10^4 colonies per plate and transfer of these colonies to nylon membranes.
 16. Alkaline lysis of the colonies to release target DNA, neutralization, and fixation by baking to the nylon support.
 17. ^{32}P -labeling by nick translation of chromosome AHS24, AHS23, P. putida DNA's and isolated plasmid DNA containing AHS24 inserts.
 18. Denaturation of ^{32}P -labeled probe by boiling to generate the single-stranded form for use in colony hybridizations.
 19. Hybridization of ^{32}P -labeled single-stranded probe to immobilized target DNA.
 20. Washing of the nylon membranes at a known stringency to remove nonhomologous or very weakly homologous probe.

5. Detection of Groundwater Isolates Showing Homology to Whole Chromosomal and Subcloned Probes

21. Exposure of X-ray film placed next to the hybridization support containing the radioactively labeled positive hybrids.
22. Detection of positive hybrids in the developed film by ^{32}P -induced silver deposition.
23. Alignment of autoradiographic signals from the X-ray film to those colonies on original culture plates showing positive hybridization signals.

These steps are summarized in pictorial fashion in Figures 2-5.

This experimental approach is by no means an entirely novel method for developing discriminatory probes for a particular microorganism, but a model representing a novel environmental application for the detection and monitoring of AHS24 in environmental samplings. The precedent for using randomly cloned genomic fragments as highly discriminatory probes was set by Salyers et al. (41), who demonstrated that closely related Bacteriodes species could be discriminated between by performing whole genomic digests of their DNAs and subsequently hybridizing these digests to a series of subgenomic plasmid probes. Two out of nine subgenomic probes were found to be primarily species specific, but not strain specific (41). Figure 6 illustrates the possible cross-homologies of such subclones probe inherent to the cloning procedure and demonstrates the necessity to screen several subgenomic probes for species specificity. The generation of a highly

GENERATION OF SUBCLONES

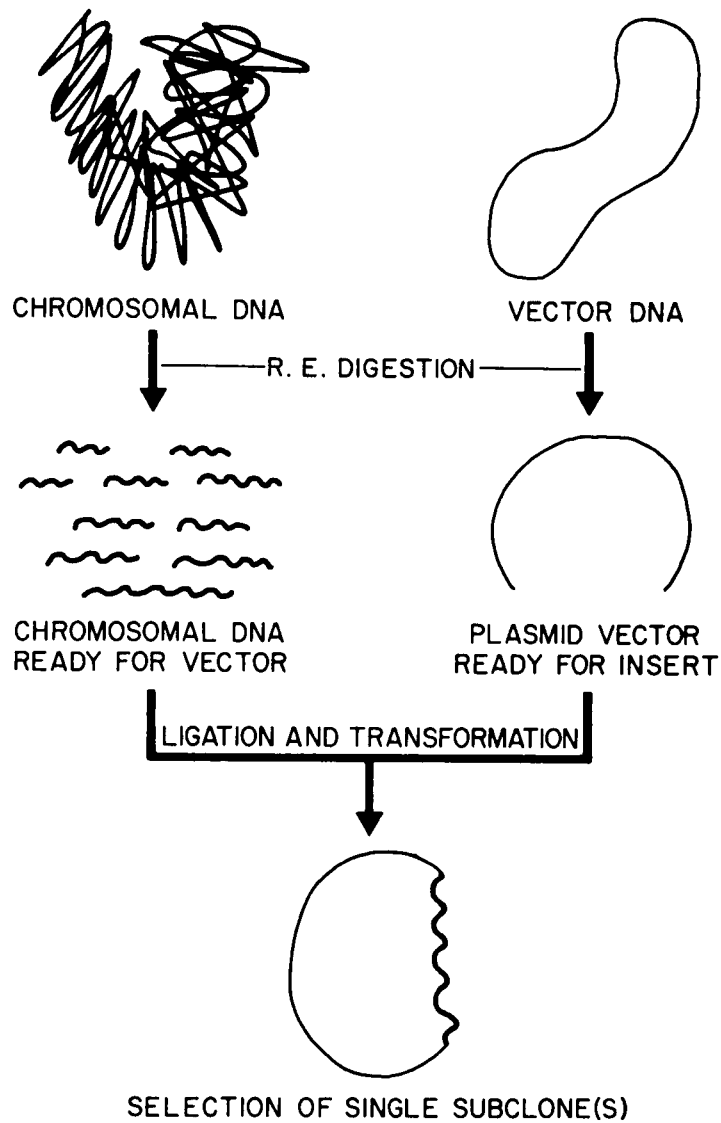


Figure 2. Generation of subclones.

PREPARATION OF DNA FROM TARGET BACTERIA

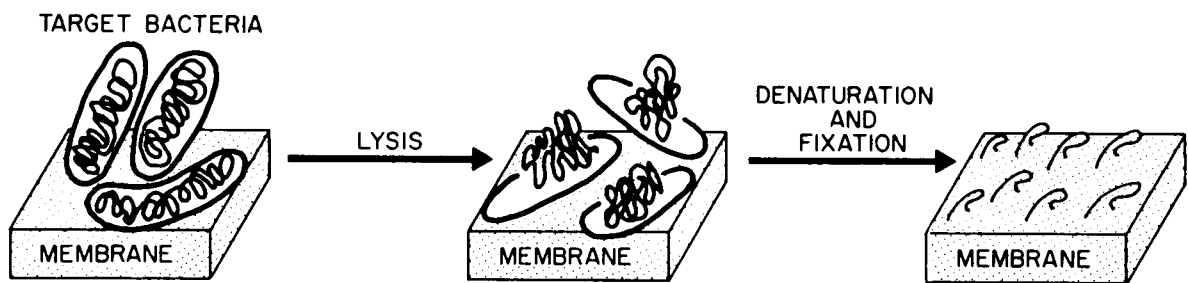


Figure 3. Preparation of DNA from target bacteria.

SCREENING

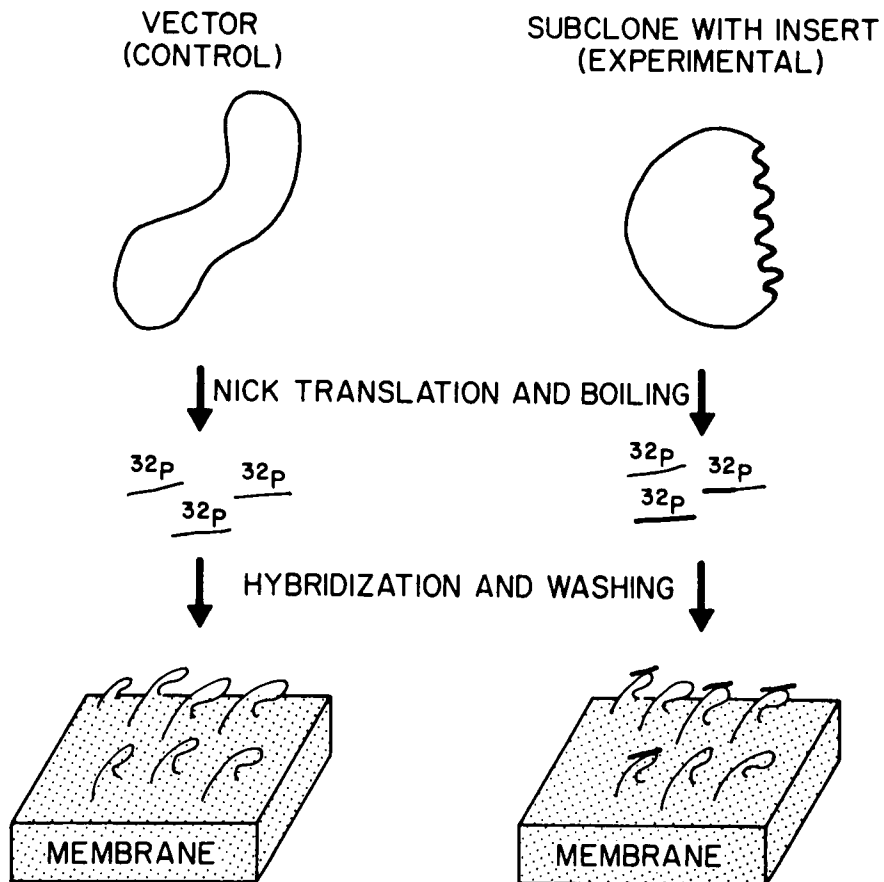


Figure 4. Screening procedure.

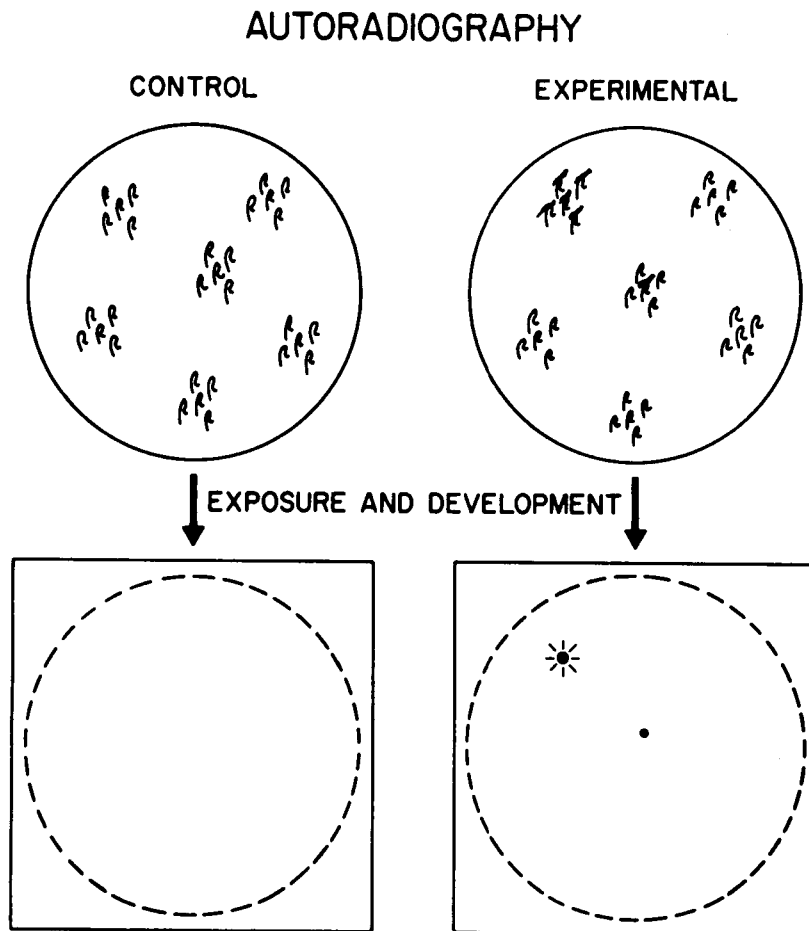


Figure 5. Autoradiography, exposure, and development.

PROBING GENOME

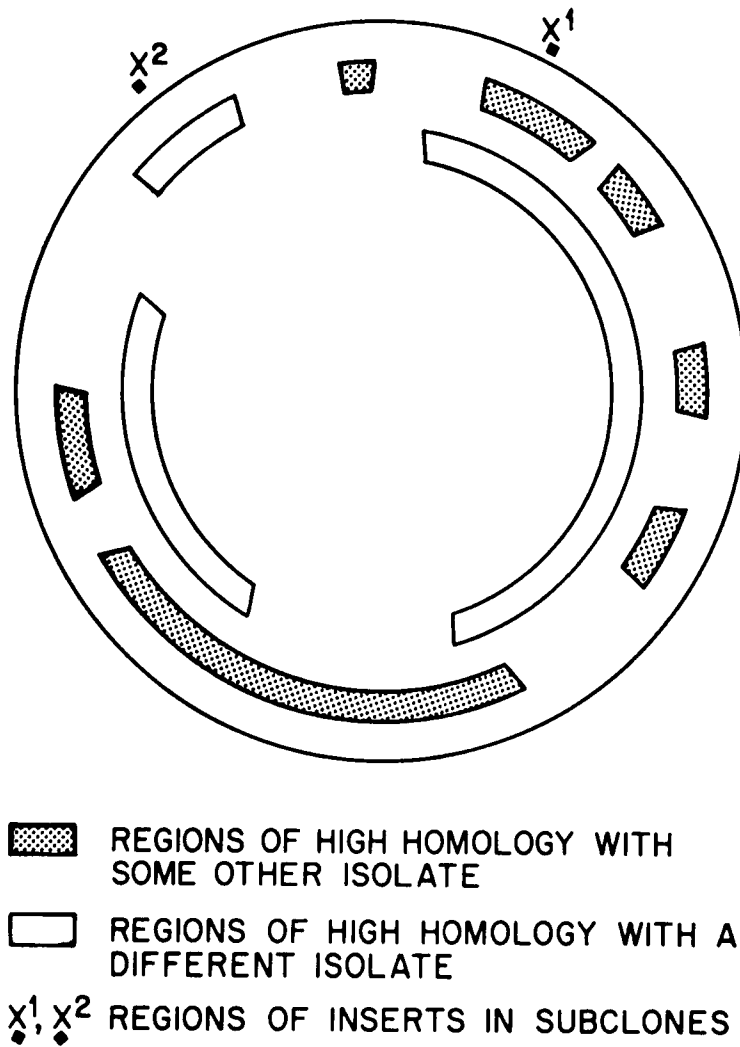


Figure 6. Homology relationships between groundwater isolates and the probing genome.

representative recombinant DNA library of AHS24 may not be essential to generate a specific subgenomic probe for comparison to a rather small number of groundwater isolates, but in the screening of total environmental samplings such as lake water, the number of different species of microorganisms is greatly increased at least upon the order of 100-fold. Thus, a highly representative library may prove to be quite essential as a back-up bank of subclones that could be screened for high specificity and would also be quite useful for future physiological studies. In environmental screenings, it may even be necessary to develop not only one, but a battery of highly specific probes for each species of interest.

III. EXPERIMENTAL METHOD

1. Bacterial Strains and Plasmids

Table 3 lists the bacterial strains and plasmids used in these investigations. Arthrobacter sp. strains AHS24, ABS10, and AHS23 as well as groundwater isolates of undetermined genera were isolated in Lula, Oklahoma, from uncontaminated aquifer core material. E. coli DH1 was used in transformation with pUC13 containing AHS24 chromosomal inserts. All Arthrobacter strain isolates were maintained on one tenth strength yeast extract peptone glucose agar (42). P. putida was maintained on 50 mM m-toluate in minimal agar medium containing (in grams per liter): NaNO_3 , 4.0; KH_2PO_4 , 1.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0011; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; NaHPO_4 , 0.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01; pH to 7.0. The carbon source m-toluate (Eastman Kodak Co., Rochester, NY) was autoclaved in the minimal agar before use. Citrobacter freundii, Enterobacter aerogenes, Klebsiella pneumoniae and P. aeruginosa were maintained on nutrient agar slants (in grams per liter): beef extract, 3.0; tryptone, 5.0; pH adjusted to 7.0. E. coli JM107 was maintained on M9 minimal salt agar supplemented with 1 mM thymine (per liter: 6 g Na_2HPO_4 , 3 g $\text{K}_2\text{H}_2\text{PO}_4$, 0.5 g NaCl , 1 g NH_4Cl ; adjust pH to 7.4, autoclave and add 2 ml 1 M MgSO_4 , 10 ml 20% glucose, .1 ml .1 M CaCl_2). Transformed DH1 was maintained on LB-amp agar medium (45 μg ampicillin/ml).

Table 3. Bacterial strains and plasmids used in this study.

Bacteria	Plasmid	Plasmid Remarks	Source or Reference
<u>Arthrobacter</u> sp. AHS24	-	-	This study
<u>Arthrobacter</u> sp. AHS23	-	-	This study
<u>Arthrobacter</u> sp. ABS10	-	-	This study
<u>Arthrobacter</u> <u>crystalpoietes</u> ATCC 15481	-	-	ATCC
<u>Arthrobacter</u> <u>globiformis</u> ATCC 8010	-	-	ATCC
<u>Citrobacter</u> <u>freundii</u> ATCC 8090	-	-	"
<u>Enterobacter</u> <u>aerogenes</u> ATCC 13048	-	-	"
<u>Klebsiella</u> <u>pneumoniae</u> ATCC 13883	-	-	"
<u>P. putida</u> ATCC 23973	pWVO	TOL plasmid	ATCC
<u>P. aeruginosa</u> ATCC 27853	-	-	Viera and Messing, 1982
<u>E. coli</u> JM107	pUC13	cloning vector	Hanahan, this study (22)
<u>E. coli</u> DH1	p1CA3	-	"
	p2MLO	-	"
	p2ML2	-	"
	p498	-	"
		2.3 kb	
		4.5 kb	
		5.7 kb	
		10.5 kb	
		5.7 kb	

2. Environmental Samples

Freshwater lake samples were collected locally from the surface of Cherokee Reservoir and Douglas Reservoir of the Tennessee Valley Authority. One freshwater sample was collected from the Holston River at Knoxville, TN. Microcosms were derived from groundwater aquifer core material from a depth of approximately 5 m in the saturated zone from groundwater wells at Lula, OK (courtesy of Dr. John Wilson, U.S. EPA, Ada, OK). The aquifer material is typical of water table aquifers in the south central United States and does not contain any chemical contaminants (41). The individual groundwater isolates used in the initial screening of the AHS24, AHS23, and P. putida whole genomic probes were also from a collection of isolates from this same zone. All freshwater samples from the Knoxville area were collected aseptically in sterile 50 ml polypropylene tubes kept at 4°C and diluted in 1/10 YEPC and plated immediately.

3. Genomic DNA Isolation

One liter cultures of AHS24, AHS23, and Pseudomonas putida ATCC 23973 were grown to an OD ($\lambda 600$) of approximately 1.0 at room temperature. Cells were harvested by centrifugation at 5,000 rpm for 10 minutes. Lysis and DNA purification were similar to the procedure of Marmur (37). The pellets were dried on ice and then resuspended in 6.25 ml of lysozyme buffer (0.1 mM NaCl, .1 M Tris, 1. M EDTA, 5 mg/ml lysozyme). Resuspended pellets were combined respectively for each strain in large 150 ml Corex tubes and the volume was

brought to 50 ml with lysozyme buffer. The tubes were then incubated at 37°C for approximately 10 minutes until increasing viscosity of the cellular mixture was observed. One hundred and twenty-five microliters of 20 mg/ml stock of proteinase K and 1 ml of 25% SDS were then added to each Corex tube. Cesium chloride was added to achieve a density of 1.7 and genomic DNA's were then purified by isopycnic ultracentrifugation using a Ti60 rotor (Beckman, Palo Alto, CA) at 35,000 rpm for 26.75 hours at 22.9°C. Purified DNAs were transferred dialysis tubing and dialyzed for 18 hours in 0.5 M NaCl and TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA pH 8.0) with three buffer changes. The dialysis buffer was then switched to TE and dialyzed over a period of two days with five changes of buffer. A stock solution of RNase A at 1 mg/ml was added to each DNA sample to achieve a concentration of 100 µg/ml and these were allowed to incubate at 37°C for 1 hour. DNA samples were then extracted with an equal volume of Tris-equilibrated phenol and chloroform 1:1 v/v) in 25 ml Corex tubes. Samples were spun down at 5,000 rpm for 10 minutes and supernatant aqueous phases were reextracted with equal volumes of chloroform (24:1 v/v chloroform:isoamyl alcohol). DNA samples were placed in dialysis tubing and dialyzed for 18 hours in 0.5 M NaCl and TE buffer with one buffer change. Concentration and purity of the DNAs was determined by absorbance spectroscopy.

4. Restriction Endonuclease Digestion of Genomic DNA and Vector DNA

Although three genomic DNAs were isolated, only one strain, AHS24, was utilized for library construction. Five micrograms of

AHS24 DNA was partially digested with Sau 3AI for one hour. The reaction volume consisted of 416.7 μ l of 0.05 μ g/ μ l AHS24 genomic DNA, 60 μ l of 10X TA (10X TA is 0.3 M Tris base, 0.6 M potassium acetate, 0.1 M magnesium acetate, 5 mM dithiothreitol, pH to 8.6; upon dilution to 1X, it will give a pH of 7.8, 60 μ l of 10X BSA (10X BSA is 1 mg/ml bovine serum albumin), 60.3 μ l of sterile distilled H₂O, and 3.0 μ l of Sau 3AI (2 units/ μ l) (New England Biolabs, Beverly, MD). Twenty micrograms of pUC13 vector DNA was linearized with Bam H1 for 30 minutes in a reaction volume of 10 μ l of pUC13 (2 μ g/ μ l), 10 μ l 10X TA, 10 μ l 10X BSA, 62 μ l of sterile distilled H₂O, and 8 μ l of Bam H1 (5 units/ μ l) (New England Biolabs, Beverly, MD). Both reactions were carried out 15 37°C in an incubated water bath. Reactions were stopped with one fifth reaction volume of 200 mM EDTA. Both DNAs were phenol-chloroform extracted, ethanol precipitated, and resuspended in TE buffer.

5. Ligation Conditions

The ligation reaction was carried out in a 20 μ l volume consisting of 7.9 μ l AHS24 Sau 3AI fragments (0.63 μ g/ μ l), 0.5 μ l Bam H1 linearized pUC13, 1.0 μ l of 1 mg/ml bovine serum albumin, 1.0 μ l of 0.2 M dithiothreitol, 1.0 μ l of 20X ligation buffer (1 M Tris pH 7.8, 200 mM MgCl₂), 2.0 μ l 10 mM ATP, 2.0 μ l of T₄ DNA ligase (0.01 weiss units/ μ l, New England Nuclear, Boston, MA), and 4.6 μ l sterile distilled H₂O. The ratio of AHS24 insert DNA to pUC13 vector DNA was determined to be approximately thirteen to one (5 μ g AHS24 fragments to 0.38 μ g pUC13 DNA). The reaction was allowed to incubate at 14°C

for one half hour and then was diluted with 80 μ l of ligation dilution buffer (1 ml stock is 50 μ l 20X ligation buffer, 50 μ l 20X BSA, 50 μ l 20X DTT, 100 μ l 10 MM ATP, and 750 μ l sterile distilled H_2O). The 100 μ l reaction was then left at 14°C overnight to promote recircularization. A control ligation reaction of only the pUC13 vector without AHS24 DNA was also performed under the same conditions previously described, but 12.5 μ l of sterile distilled H_2O was used to bring the reaction volume to 20 μ l.

6. Transformation

A procedure similar to that of Dagbert and Ehrlich (11) was used to transform E. coli DH1 with the products of the ligation reaction mixture. An overnight culture of E. coli DH1 was grown up in 50 ml of LB broth (10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl) at 37°C. Four 150 ml Ehrlenmyer flasks of LB were each inoculated with 0.5 ml of the overnight culture. The cells were incubated at 37°C, 225 rpm for 2.5 hours until they reached a concentration of approximately 20 Klett units ($\lambda 600 = 0.2$). The cells were then collected in four 150 ml Sorvall tubes and pelleted by centrifugation at 5,000 rpm for 5 minutes. The supernatants were poured off and the pellets were resuspended in cold 0.1 M $CaCl_2$. Fifteen mls of cold 0.1 M $CaCl_2$ was added to each tube and the cell suspensions were allowed to sit on ice for 20 minutes. The cells were centrifuged again at 5,000 rpm for 5 minutes, supernatants drained and pellets resuspended in 0.5 ml of cold 0.1 M $CaCl_2$ each. The volume per tube was brought to 1.5 ml with cold 0.1 M $CaCl_2$ which resulted in a one

hundred-fold concentration of the cells from the original volume of 150 ml. The tubes were then placed for 1 hour on ice.

For each transformation reaction, 0.1 ml of the one hundred-fold concentration of cells was considered to be one discreet transformation. To test the competency of the cells, 0.0 ng, 0.1 ng, 1.0 ng, 10 ng and 100 ng of pUC13 in 10 μ l volumes of TE buffer were added to five discreet transformations respectively. The remaining stock of cells was stored at 4°C for 20 hours. The trial transformations were placed on ice for 30 minutes and then at 37°C for 10 minutes to allow the cells to take up the pUC13 plasmid DNA. Then 0.9 ml of LB broth was added to each reaction and the cells were kept at 37°C for approximately 45 minutes before plating. Ten microliters of the competent cells which had taken up the DNA were then plated on LB-amp agar (50 μ g amp/ml) and the number of the transformants were scored within 16 hours. Transformation efficiencies were determined by the number of transformants per microgram of supercoiled pUC13 DNA. When the transformation efficiency was found to be within the range of 10^6 to 10^7 transformants per microgram of DNA, the remaining competent cells which had been stored at 4°C were used in the transformations with the recombinant pUC13 plasmids containing AHS24 inserts.

Since the products of the ligation reactions were phenol-chloroform extracted, ethanol precipitated and resuspended in TE buffer, there was uncertainty as to whether or not the T_4 DNA ligase and other components of the ligation reaction mixture would affect the efficiency of transformation. Therefore, varying concentrations of the

ligation reaction mixture of pUC13 with AHS24 inserts were used to transform the competent cells. The 100 μ l ligation reaction mixture was empirically divided and added to varying volumes of competent cells. Table 4 summarizes the division of the 100 μ l ligation reaction mixture and the volumes of competent cells added to each transformation reaction. Each of these reactions was respectively designated as the 2 ML, 1H2, 49H2, 1CA, 49CA and "vector alone" reactions. These reactions were all placed on ice for 30 minutes and then heat pulsed for 10 minutes at 37°C in a water bath as previously described. Twenty milliliters of sterile LB broth was added to the 2ML reaction and 0.9 ml was added to the other four reactions. The cells were allowed to recover for 45 minutes at 37°C before plating 10 μ l of each reaction on LB-amp agar. The control transformations of 0.0 ng, 0.1 ng, 1.0 ng, 10.0 ng, and 100 ng of pUC13 DNA were repeated as previously described to assure that the competent cells were transformed at high efficiencies of 10^6 - 10^7 transformants per microgram of DNA. Transformants from the library-generating reactions 2ML, 1H2, 49H2, 1CA, and 49CA as well as the "vector alone" were scored within 16 hours after incubation.

7. Library Amplification and Preservation

After plating 10 μ l of transformants from the 2ML, 1H2, 49H2, 1CA, 49CA and vector alone reactions, the remaining cells from each reaction were added to 50 ml of LB respectively and grown for 1 hour at 37°C. Ampicillin was then added to achieve a final concentration of 45 μ g/ml to ensure that the recombinant plasmids were retained in

Table 4. Summary of transformation reaction components.

Transformation Reaction	Volume and Type of Ligation Reaction Mixture	Volume and Type of Diluent	Volume of Diluted Ligation Mixture Used	Volume of Competent Cells	Total Volume per Discreet Transformation
2ML	80 μ l pUC13 + AHS24 inserts	-	-	2 ml	2.1 ml
1H2	{ 10 μ l pUC13 + AHS24 inserts	40 μ l H ₂ O	1 μ l	100 μ l	101 μ l
49H2			49 μ l	500 μ l	549 μ l
1CA	{ 10 μ l pUC13 + AHS24 inserts	40 μ l 0.1M CaCl ₂	1 μ l	100 μ l	101 μ l
49CA			49 μ l	500 μ l	549 μ l
vector alone	10 μ l pUC13	-	-	150 μ l	160 μ l

the population of growing cells. After 1 hour, DNase I was added to achieve a concentration of 10 $\mu\text{g/ml}$ in order to degrade DNA from lysed cells so that clumping of viable cells would not occur. The cells were then allowed to grow overnight at 37°C and then pelleted and resuspended in 3.0 ml of 85% LB-amp broth-15% glycerol mixture. Aliquots of the concentrated cells were then placed into 1.5 ml Eppendorf tubes and stored at -70°C. Although the four transformation reactions with recombinant pUC13 plasmids containing AHS24 inserts were derived from the same ligation mixture, they are designated as separate libraries for the purpose of clarity.

8. Determination of Library Representativeness

Cultures of the transformed DH1 strain containing the four libraries stored at -70°C were made and each inoculated into 50 ml of LB-amp media and grown overnight on a rotary shaker at 37°C. The cultures were then diluted serially in LB broth and plated immediately on LB-amp media to achieve a colony density of approximately 200-300 colonies per plate. The colonies were allowed to mature overnight. In determining library representativeness, 12 to 20 colonies were selected randomly from each library and plasmids were isolated and analyzed for inserts as described below. The fraction of plasmids containing inserts was determined for each library and used to calculate the representativeness.

9. Plasmid Isolation

Randomly selected plasmids used in the determination of library representativeness and those used specifically in the generation of

subcloned AHS24 probes; pICA3, p2MLO, p498, and p2ML2 were isolated from the DH1 libraries according to a modified procedure of Birnboim and Doly (3) described by Ish-Horowitz (26). Individual colonies were inoculated into 3 ml of sterile LB-amp broth and grown overnight on a rotary shaker at 37°C. The cultures were spun down in 5 ml polypropylene tubes at 9,000 rpm for 10 minutes. The supernatant was removed and the pellets were each suspended in 100 μ l of Solution I (50 mM glucose, 25 mM TRIS, pH 8, 10 mM EDTA, pH 7.5, 4-5 mg/ml lysozyme added fresh). The tubes were vortexed lightly and incubated at room temperature for 5 minutes. Two hundred microliters of Solution II (0.2 N NaOH, 1% SDS) was added to each tube and these were mixed by rocking the tubes sharply. The tubes were then incubated on ice for 5 minutes. One hundred and fifty microliters of precooled Solution III (3 M KOAc, pH 6.0 with glacial acetic acid) was added. Caps were sealed well, tubes were inverted and mixed gently on the lid, vortexed, reinverted and vortexed again briefly. The tubes were incubated on ice for 5 minutes and spun at 9,000 rpm for 5 minutes. The supernatants were transferred to clean microfuge tubes, an equal volume (about 400 μ l) of phenol:chloroform (1:1 phenol:chloroform) was added to each tube, and mixed thoroughly. The tubes were then spun down in a microfuge for 2 minutes. The aqueous phases were transferred to clean microfuge tubes and equal volumes of chloroform (24:1 chloroform:isoamyl alcohol) were added and mixed. The tubes were spun for 2 minutes and the supernatant was transferred to clean microfuge tubes. One milliliter of ethanol and 1/10 vol. of 3 M Na acetate was added and mixed thoroughly. The

supernatants were carefully removed and the pellets were rinsed with 70% ethanol. The pellets were then dried in a vacuum for 2-5 minutes and were dissolved in 50 μ l of 1/10 TE buffer containing 1/10 volume of 100 ng/ μ l of RNase A. For those recombinant plasmids used as subcloned probes, p1CA3, p2ML2, p2MLO, p498, 25 mls of cells rather than 3 mls of cells were prepared for plasmid isolation such that approximately eight times as much plasmid DNA would be recovered.

10. Determination of Plasmid Insert Sizes

The AHS24 inert containing plasmids were digested with Eco RI and Hin dIII. These enzymes each have unique restriction sites flanking the Bam H1 site of the pUC13 multiple cloning site into which the AHS24 inserts were originally cloned (Figure 1, p. 10). One μ l of plasmid midipreps (approximately 1 μ g) of pUC13, p1CA3, p2M10, p498, and p2ML2 were digested with 10 units of Hin dIII (0.5 μ l) and 10 units of Eco RI (1.0 μ l) for 1 hour at 37°C in 2.5 μ l of 10X TA, 2.5 μ l of 10X BSA and 17.5 μ l of sterile distilled H₂O. The remaining plasmids not used for development of subcloned probes were digested from aliquots of plasmid minipreparations. Twenty microliters of each plasmid miniprep (approximately 2 μ g) was digested with 10 units of Hin dIII (p.5 μ l) and 10 units of Eco RI (1.0 μ l) for 1.5 hours at 37°C in 5.0 μ l 10X TA (10X TA buffer in 330 mM Tris base, 660 mM K acetate, 100 mM Mg acetate, 5 mM dithiothreitol, pH 7.8), 5.0 μ l 10X BSA (10X BSA is 1 mg/ml), and 18.5 μ l of sterile distilled H₂O. Insert sizes were determined by electrophoresis in 1% agarose gels in 1X TE buffer (10X stock is 0.4 M Tris Base, 0.05 M Na acetate, 0.01 M

EDTA, pH to 7.8 with acetic acid). Remaining plasmids that were used to determine library representativeness but were not digested in order to determine insert size were run on 1% agarose gels from minipreps. Five microliters of each plasmid miniprep was run in parallel with 5 μ l of an undigested noninsert containing pUC13 miniprep. Electrophoretic mobility of the undigested plasmids were compared to determine the fraction of total plasmids with AHS24 inserts.

11. Preparation of Genomic and Subcloned Probes

Purified AHS24, AHS23, ABS10, and P. putida whole genomic DNAs were labeled by nick translation according to the protocol of Bethesda Research Laboratory that is supplied with the commercial nick translation kit (Bethesda Research Laboratories, Gaithersburg, MD). Plasmid DNAs p1CA3, p2ML0, p498, p2ML2 and pUC13 were also prepared in this manner. It was found that the plasmid DNA need not be purified past the Birnboim and Doly procedure for use in nick translation reactions in order to obtain sufficiently labeled plasmid DNAs of approximately 10^7 - 10^8 cpm/ μ g. Alpha 32 P dCTP at 3,000 Ci mMole $^{-1}$ (ICN Radiochemicals, Irvine, CA) was the sole labeled nucleotide used in the nick translation reactions. 32 P-labeled probe was separated from the unincorporated dCTP using Elutip-d disposable purification columns according to the manufacturer's protocol (Schleicher and Schuell, Keene, NH). The specific activity of the labeled probes were determined by liquid scintillation spectrometry. The specific activity of each labeled DNA in each set of colony hybridization experiments is listed in Appendix B.

12. Transfer of Colonies to Nylon Membranes and DNA Fixation

Colonies growing on the agar surface to Pall Biotrans A nylon membranes (Pall Ultrafine Filtration Corporation, Glen Cove, NY) by placing the membranes directly on the plates for 15 minutes. In one particular experiment, colonies were grown directly on presterilized filters. Membranes were oriented for colony hybridization and autoradiography by spotting colonies of AHS24, P. putida, or E. coli JM107 at designated points on the edges of the membrane after transfer. Filters were processed according to the Pall Biotrans Protocol for RNA and DNA transfer to Biotrans A. This procedure involved placing the membranes colony side up for 5 minutes on 3 MM paper that was saturated with denaturation solution (1.5 M NaCl, 0.5 M NaOH) and then placing the membranes on 3 MM paper saturated with neutralizing solution (3 M Na Acetate, pH 5.5). The membranes were then blotted on 3 MM paper and air dried for 30 minutes and baked for 1 hour at 70°C.

13. Prehybridization and Hybridization

Each filter was placed separately in a heat-sealable plastic bag along with 5 mls of prehybridization solution (80% v/v, 1.5X Buffer, 20% v/v 50X Denhardt's Solution, and 0.5% single stranded carrier DNA; 1.5X Buffer is 1.3 M NaCl, 0.07 M Tris base, and 0.001 M EDTA, pH 7.9 $\pm$ 1.5; Denhardt's Solution is 1% Pentax BSA, 1% Polyvinylpyrrolidone, and 1% Ficoll; carrier DNA is 10 mg/ml Salmon Sperm DNA prepared as stock by boiling 50 mg of DNA from salmon sperm

testes in 4 ml of NaOH for 5 minutes and adding 750 μ l of 2M Tris-HCl, 250 of reagent grade HCl and 20 μ l of 0.5 m EDTA). The bags were sealed and prehybridized in a self-rotating oven for 3 hours. 32 P-labeled probe DNA was heated in a boiling water bath for 5 minutes and then added to the sealed bags by cutting a small corner from the bag and micropipetting the appropriate amount of labeled probe. In most experiments the final probe activity concentration was approximately 10^5 - 10^6 CPM/ml of the hybridization buffer. These bags were then incubated and rotated intermittently in a 65°C oven for 16 hours. The filters were removed and rinsed twice for 5 minutes each in 1X Low wash buffer (0.05 M NaCl, 0.05 M Tris, pH 7.9, 1% Sarkosyl [Sigma Chemical Co.] and 10 mM EDTA). In an experiment to determine which groundwater isolates showed homology with the AHS24, AHS23, and P. putida probes, the wash buffer varied in concentration to create different levels of stringency. These concentrations were .1X Low, 1X Low, and 4X Low, respectively. One set of hybridizations from this experiment were washed in hybridization buffer previously described. The filters were then rinsed in wash buffer six times with each rinse lasting 20 minutes. Filters were then dried colony side up for 1 hour on 3 MM paper at room temperature.

14. Authoradiography

Filters were autoradiographed with Kodak X-Omat X-ray film with two intensifier screens (Crone, Lighting Plus: Dupont). The cassettes were exposed at -70°C for 24-96 hours.

IV. EXPERIMENTAL RESULTS

1. AHS24 Library Representativeness

In order to determine the utility of the AHS24 libraries for use in the development of an adequate bank of subgenomic probes, transformants from the libraries were screened randomly for the presence of inserts in the pUC13 plasmid. Approximately 10-20 plasmid minipreps from each of the four libraries 2ML2, 1CA, 49CA, and 1H2 were screened by comparing the electrophoretic mobility of the plasmid isolate with that of a miniprep of the monomer, dimer and trimer of the pUC13 vector without an insert. These forms of the pUC13 plasmid were obtained by transforming DH1 with a control ligation mixture that contained only pUC13 linearized plasmids. Visible differences in migratory distances of the library plasmid with that of the control in 1% agarose minigels indicated the potential presence of an AHS24 subgenomic insert (data not shown). These estimates of the total number of plasmids containing inserts are summarized in Table 5. Some of these plasmids which were chosen for possible use as subgenomic probes were later characterized for insert size by removing the insert with an Eco RI and Hind III cut. These sizes of AHS24 inserts are summarized in Table 5 as well. The estimates of the fraction of total plasmids containing inserts were then used to approximate the representativeness or "genome equivalent" for each library; the genome equivalent referring to the designated fraction of the genome has been cloned into the recipient colony bank of E. coli (10). These results are summarized in Table 6. The determination of the genome

Table 5. Colonies analyzed from 1H2, 49CA, ICA, and 2ML2 libraries for plasmids by miniprep method.

Colony/Plasmid Name		Insert	Approximate size (kb)
<u>1H2</u>			
	p1H ₂ A	-	-
	p1H ₂ B	-	-
	p1H ₂ C	+	1.5
	p1H ₂ D	lost	-
	p1H ₂ E	+	4.7
	p1H ₂ F	lost	-
	p1H ₂ G	-	-
	p1H ₂ H	-	-
	p1H ₂ I	lost	-
	p1H ₂ J	+	4.5
	p1H ₂ 1	+	1.8
	p1H ₂ 2	+	5.8
	p1H ₂ 3	lost	-
	p1H ₂ 4	lost	-
TOTAL	14	5	
<u>49CA</u>			
	p49CA A	-	-
	p49CA B	+	1.8
	p49CA C	-	-
	p49CA D	-	-
	p49CA E	+	1.5
	p49CA F	+	2.0
	p49CA G	+	3.6
	p49CA a	lost	-
	p49CA b	+	a
	p49CA c	+	a
	p49CA 8	+	5.7

Table 5 (continued)

	Colony/Plasmid Name	Insert	Approximate size (kb)
	p49CA 1	lost	-
	p49CA 2	-	-
	p49CA 3	lost	-
	p49CA 4	+	a
TOTAL	15	8	
<u>ICA</u>			
	p1CA A	+	8.5
	p1CA B	+	5.4
	p1CA C	+	1.1
	p1CA D	-	-
	p1CA E	-	-
	p1CA P	+	a
	p1CA S	+	a
	p1CA R	+	a
	p1CA M	+	a
	p1CA 1	lost	-
	p1CA 2	+	9.8
	p1CA 3	+	4.5
TOTAL	12	9	
<u>2ML2</u>			
	p2ML A	-	-
	p2ML B	+	a
	p2ML C	-	-
	p2ML D	lost	-
	p2ML E	lost	-
	p2ML F	-	-
	p2ML G	-	-
	p2ML H	-	-

Table 5 (continued)

Colony/Plasmid Name	Insert	Approximate size (kb)
p2ML I	-	-
p2ML J	-	-
p2ML K	-	-
p2ML L	-	-
p2ML X	+	a
p2ML O	+	5.7 kb
p2ML Z	-	-
p2ML 1	+	12.4 kb
p2ML 2	+	10.5 kb
p2ML 3	+	1.2 kb
p2ML 4	lost	-
TOTAL	19	6

^aNot determined.

Table 6. Determination of AHS24 library representativeness.

Library name	Transformation efficiency (transformants/ μ g of DNA)	Number of independent transformants*	Total individuals tested for inserts using miniprep	Total plasmids with inserts	Fraction containing inserts	Estimated genome equivalent*
2ML	1.2×10^5	3.7×10^4	19	6	$5/16^a$	2.3
1CA	6.6×10^5	5.0×10^2	12	9	$9/11^b$	0.08
49CA	2.7×10^4	1.0×10^3	15	8	$8/12^c$	0.13
1H2	1.1×10^7	8.0×10^3	14	5	$5/9^d$	0.89

*See Appendix A for sample calculation.

^aThree plasmids were lost in the miniprep procedure.

^bOne plasmid was lost in the miniprep procedure.

^cThree plasmids were lost in the miniprep procedure.

^dFive plasmids were lost in the miniprep procedure.

equivalent is explained in Appendix A. The approximation of the genome equivalent is only a rough value based on a conservative estimate of the AHS24 genome size which is not known, but taken to be around the average size of a bacterial genome, or 5,000 kb. The genome size of free living bacteria varies from about 1×10^9 to 8×10^9 molecular weight which corresponds to a range of 1,000 to 12,000 kilobases (5). The approximation of the representativeness of each library is also most likely to be a conservative estimate if the average insert size is assumed to be 1 kb. The average insert size estimated from the data in Table 5 indicates that it is approximately 5 kb. More accurate estimates of the genome equivalent of these libraries would be necessary in the future if the libraries were utilized for example to isolate single genes from AHS24.

2. Selection of AHS24 Subgenomic Probes

Colony hybridizations were performed using plates of the members of each library. Each plate contained approximately 200-500 colonies. These colonies were transferred to nylon membranes and hybridized to ^{32}P -labeled whole genomic AHS24 DNA for 16 hours. Figure 7 shows the 24 hour autoradiographs of these colony hybridizations. The labels in each of the autoradiographs designate the library probed or the appropriate controls of AHS24 colonies or DH1 containing pUC13 plasmid (without an AHS24 insert). Membranes, plates, and autoradiographs were aligned with respect to signal orientation by spotting individual colonies of AHS24 on the membrane prior to lysis at 0° (1 spot), 90° (2 spots), and 180° (3 spots) from the top of the

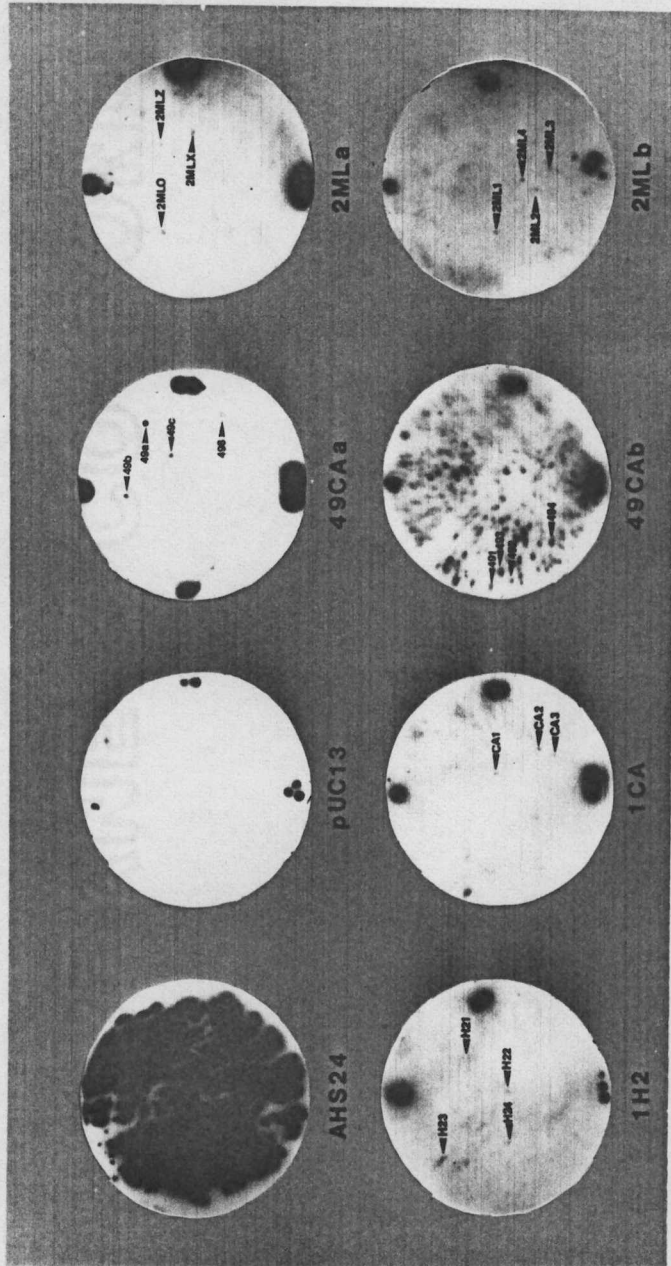


Figure 7. AHS24 library screening. 49CA, 2ML, 1H2 and 1CA libraries were screened for plasmids containing AHS24 inserts. Clones analyzed for plasmids are indicated with an arrow.

autoradiogram. This procedure also provided an internal positive control for each hybridization. Those colonies which could be associated with distinct signals on the autoradiograph were analyzed for the presence of plasmids containing AHS24 inserts. The signals of those colonies are marked individually for library in the respective autoradiographs in Figure 7. The plasmids isolated from these colonies giving signals are included in Table 5 in determining library representativeness. Two of the colonies giving autoradiograph signals p2ML2 and p49CA2 yielded plasmids without inserts and plasmids p49CAa, p49CA1, p49CA3, p2ML4, p1H23, p1H24, and 1CA1 were lost in the miniprep procedure perhaps due to the effect of the AHS24 insert functioning in deleterious ways upon the copy number of the plasmid. This might result in poor precipitation of the DNA in ethanol during the miniprep procedure. No obvious correlation of signal intensity with AHS24 insert size could be determined due to variability in the amount of cells present per colony as well as variability in the transfer and subsequent lysis of each colony on the membrane. Only 15 of the total 22 colonies analyzed were found to have plasmids, therefore any correlation between signal strength and AHS24 insert size would be difficult to make due to the small sample size of total plasmids checked for inserts. Four of the eleven plasmids shown to have inserts, plasmids p1CA3, p2ML0, p2ML2, and p498, were chosen randomly to be used in subsequent environmental colony hybridization experiments. The sizes of the inserts were determined by an Eco R1-Hin dIII double restriction which would remove the AHS24 insert cloned

into the Bam HI site and would also restrict the insert at internal Eco R1 or Hin dIII sites. An ethidium bromide-stained gel of the restriction pattern of the plasmids plus inserts is shown in Figure 8. The prominent 2.6 kb single band in lane 2 of this gel corresponds to the linearized pUC13 vector without an insert. The restricted plasmid p1CA3 is shown in lane 3. There are three prominent bands at 4.5 kb, 3.5 kb, and 1.0 kb. If the 3.5 kb band and 1.0 kb band represent a partial digestion of the 4.5 kb band, then the total insert size is 4.5 kb. The lower intensities of the 3.5 and 1.0 kb tend to support that these bands are indeed a partial digest of the 4.5 kb band. Five insert bands are evident in lane four corresponding to fragments of 5.7, 3.0, 1.5, 0.8, and 0.4 kb. It appears that the 3.0, 1.5, 0.8, and 0.4 kb bands represent partial digestion of the 5.7 kb band, thus the insert size of p2ML0 is assumed to be 5.7 kb. Four AHS24 insert fragments from p2ML2 can be seen in lane 5. These bands at 4.3 kb, 4.0 kb, 1.4 kb, and 0.8 kb add up to an AHS24 insert size of 10.5 kb, making p2ML2 the largest plasmid used as a subgenomic probe. Three major bands of 3.6, 1.7, and 0.4 kb are evident in lane 6 which contains plasmid p498. These insert fragments add up to an insert size of 5.7 kb.

3. Selection of Groundwater Isolates Which Can
Cross-hybridize to AHS24, AHS23, and P. putida
Whole Genomic Probes

To better test the specificity of the subgenomic versus the AHS24 whole genomic probes, 48 groundwater isolates, including AHS24 from the groundwater aquifer in Lula, Oklahoma, were probed with whole

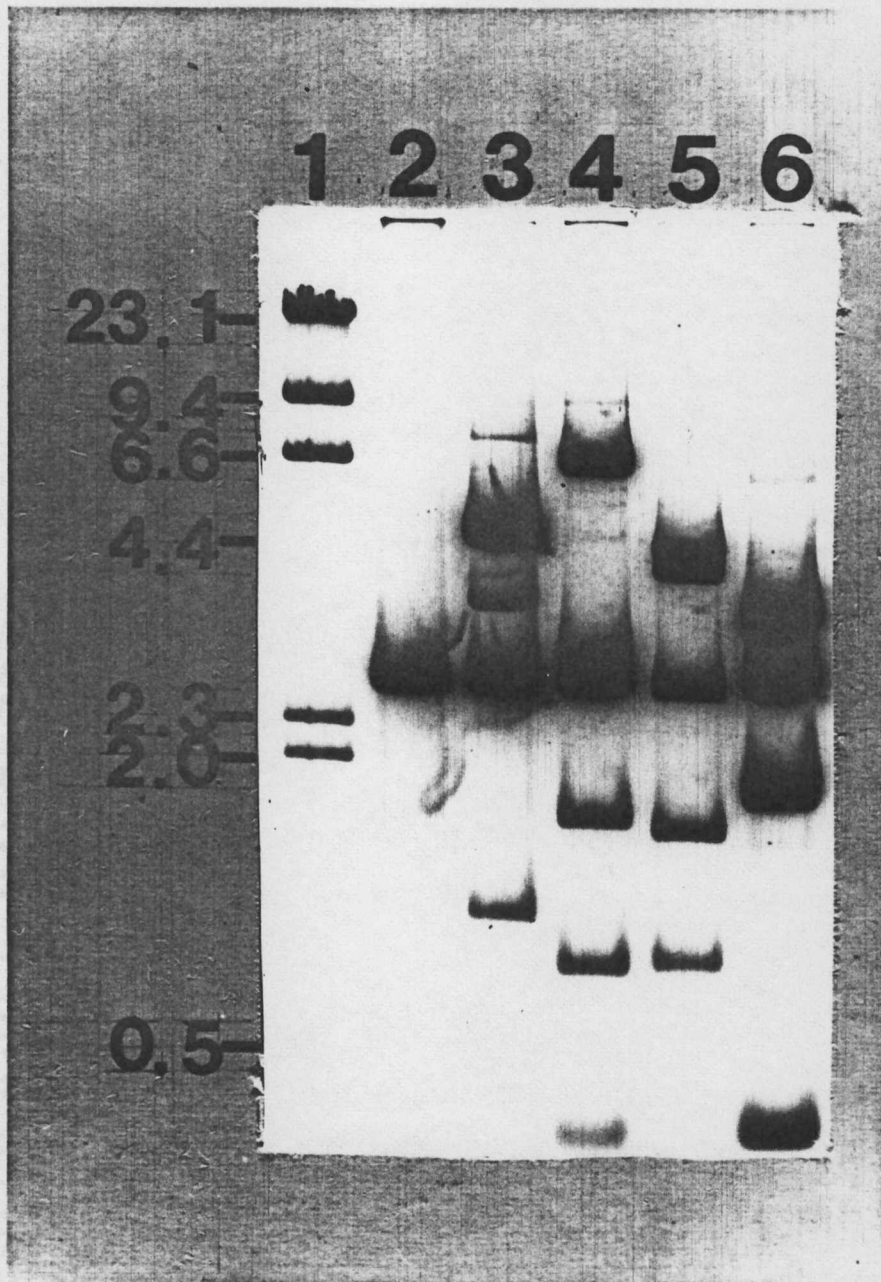


Figure 8. Eco R1/Hin dIII restriction pattern of plasmids containing AHS24 inserts. Lanes (1) λ Hin dIII markers, (2) pUC13 with no insert, (3) p1CA3-4.5 kb insert, (4) p2MLO-5.7 kb insert, (5) p2ML2-10.5 kb insert, (6) p498-5.7 kb insert.

genomic DNAs from AHS24, AHS23, and P. putida under varying conditions of stringency. The stringency in these experiments was varied by adjusting the salt concentrations of the wash buffer. Wash buffer in concentrations of .1X Low, 1X Low, 4X Low, (defined on page 34), along with one wash in fresh hybridization solution corresponded to sodium concentrations of 5 mM, 50 mM, 200 mM, and 1,000 mM, respectively. The groundwater isolates were grown on the same YEPG agar plate by transferring with a sterile 50-pronged block. Maturation of all colonies was observed within 3-5 days. Some slight variability was observed in transfer of colonies to the Pall-Biodyne Membrane due to varying colonial morphologies. However, membranes were checked before lysis and it appeared that at least colonial surface cells were transferred. Upon baking, transfer was obvious due to a glistening appearance of the remaining cellular debris affixed to the membrane. Hybridizations were performed for 16 hours and washed at 65°C as previously described. A summary of the groundwater isolates which hybridize to three different whole genomic probes under varying stringencies is shown in Table 7. Figure 9 shows the 24-hour autoradiograms of these hybridizations. The labels AHS24, P. putida and AHS23 in Figure 9 indicate the whole genomic probe used in the hybridizations. The numbers which follow the labels, numbers 1-4, indicate NaCl stringencies of 5 mM, 50 mM, 200 mM, and 1000 mM, respectively. AHS24 colonies were spotted at the margins of the agar plates and transferred to the membranes to serve as internal hybridization controls for AHS24 probe and as tests for cross-hybridization of

Table 7. Groundwater isolates hybridized with AHS24, AHS23, and *P. putida* whole genomic DNA under varying stringencies.

Groundwater isolate number	5mm NaCl	50mm NaCl	200mM NaCl	1000mM NaCl
<u>AHS24 probe</u>				
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	(+)	(+)	(+)	+
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	(+)	(+)	(+)	-
14	-	-	-	(+)
15	-	-	-	-
16	-	-	-	-
17	-	(+)	(+)	+
18	-	-	(+)	-
19	-	-	-	-
20	-	-	-	-
21	-	-	-	-
22	-	-	-	(+)
23	-	(+)	(+)	+
24	-	-	-	-
25	(+)	(+)	(+)	+
26	(+)	(+)	(+)	(+)
28	-	-	-	-
29	-	-	-	-
32	+	+	+	+
33	-	-	-	-
34	-	-	-	-
35	-	-	-	-
36	-	-	-	-
37	-	-	-	(+)
38	-	-	-	-
39	-	-	-	-
40	-	-	-	-
41	-	-	-	-
42	-	-	-	-
51	-	(+)	(+)	+
53	-	-	-	-
58	-	(+)	-	(+)
59	-	-	-	-
TM1	-	-	-	-
7S41	(+)	(+)	(+)	(+)
7S46	-	-	-	-
7S15	-	-	-	-
7S25	-	-	-	-
AHS24	+	+	+	+
TOTAL	7	11	11	13
<u>AHS23 probe</u>				
1	(+)	(+)	(+)	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-

Table 7 (continued)

Groundwater isolate number	5mm NaCl	50mm NaCl	200mM NaCl	1000mM NaCl
5	(+)	(+)	(+)	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	(+)	(+)	(+)	(+)
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	-	-	-	-
14	(+)	(+)	(+)	-
15	(+)	(+)	(+)	-
16	-	-	(+)	-
17	(+)	(+)	(+)	(+)
18	-	-	-	-
19	-	-	-	-
20	-	-	-	-
21	-	-	-	-
22	-	-	-	-
23	(+)	+	-	(+)
24	-	-	-	-
25	(+)	(+)	(+)	+
26	-	(+)	-	(+)
28	-	-	-	-
29	-	-	-	-
32	+	(+)	(+)	+
33	-	-	-	-
34	-	-	-	-
35	-	-	-	-
36	-	-	-	-
37	-	-	(+)	(+)
38	(+)	(+)	(+)	(+)
39	-	-	-	-
40	-	-	-	-
41	-	-	-	-
42	(+)	(+)	-	(+)
51	-	-	(+)	-
53	-	-	-	-
58	-	-	(+)	(+)
59	-	-	-	-
TM1	-	-	-	-
7S41	-	-	-	(+)
7S46	-	-	-	-
7S15	-	-	-	-
7S25	-	-	-	-
AHS24	(+)	(+)	(+)	+
TOTAL	12	13	14	12
<u>P. putida probe</u>				
1	-	-	-	-
2	-	-	-	(+)
3	(+)	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	(+)	(+)	+	+
10	-	-	-	-
11	-	-	-	-

Table 7 (continued)

Groundwater isolate number	5mm NaCl	50mm NaCl	200mM NaCl	1000mM NaCl
12	-	-	-	-
13	-	-	-	-
14	-	-	-	-
15	-	-	-	-
16	-	-	-	-
17	-	-	(+)	(+)
18	-	-	-	-
19	-	-	-	-
20	-	-	-	-
21	-	-	-	-
22	-	-	-	-
23	-	-	(+)	+
24	-	-	-	-
25	-	(+)	(+)	+
26	(+)	(+)	(+)	(+)
28	-	-	-	-
29	-	-	-	-
32	+	+	+	+
33	-	-	-	-
34	-	-	-	-
35	-	-	-	-
36	-	-	-	-
37	-	-	-	(+)
38	-	-	-	-
39	-	-	-	-
40	-	-	-	-
41	-	-	-	-
42	-	-	-	(+)
51	-	-	-	+
53	-	-	-	-
58	-	-	(+)	(+)
59	-	-	-	-
TM1	-	-	-	-
7S41	-	-	-	+
7S46	-	-	-	-
7S15	-	-	-	-
7S25	-	-	-	-
AHS24	(+)	(+)	(+)	+
TOTAL	<u>5</u>	<u>5</u>	<u>8</u>	<u>13</u>

Figure 9. Autoradiographs of groundwater isolates screened with AHS24, Pseudomonas putida and AHS23 whole genomic probes under varying stringencies: (1) 5 mM NaCl, (2) 50 mM NaCl, (3) 200 mM NaCl, and (4) 1000 mM NaCl. Groundwater isolate showing a positive signal are labeled by their identification numbers.

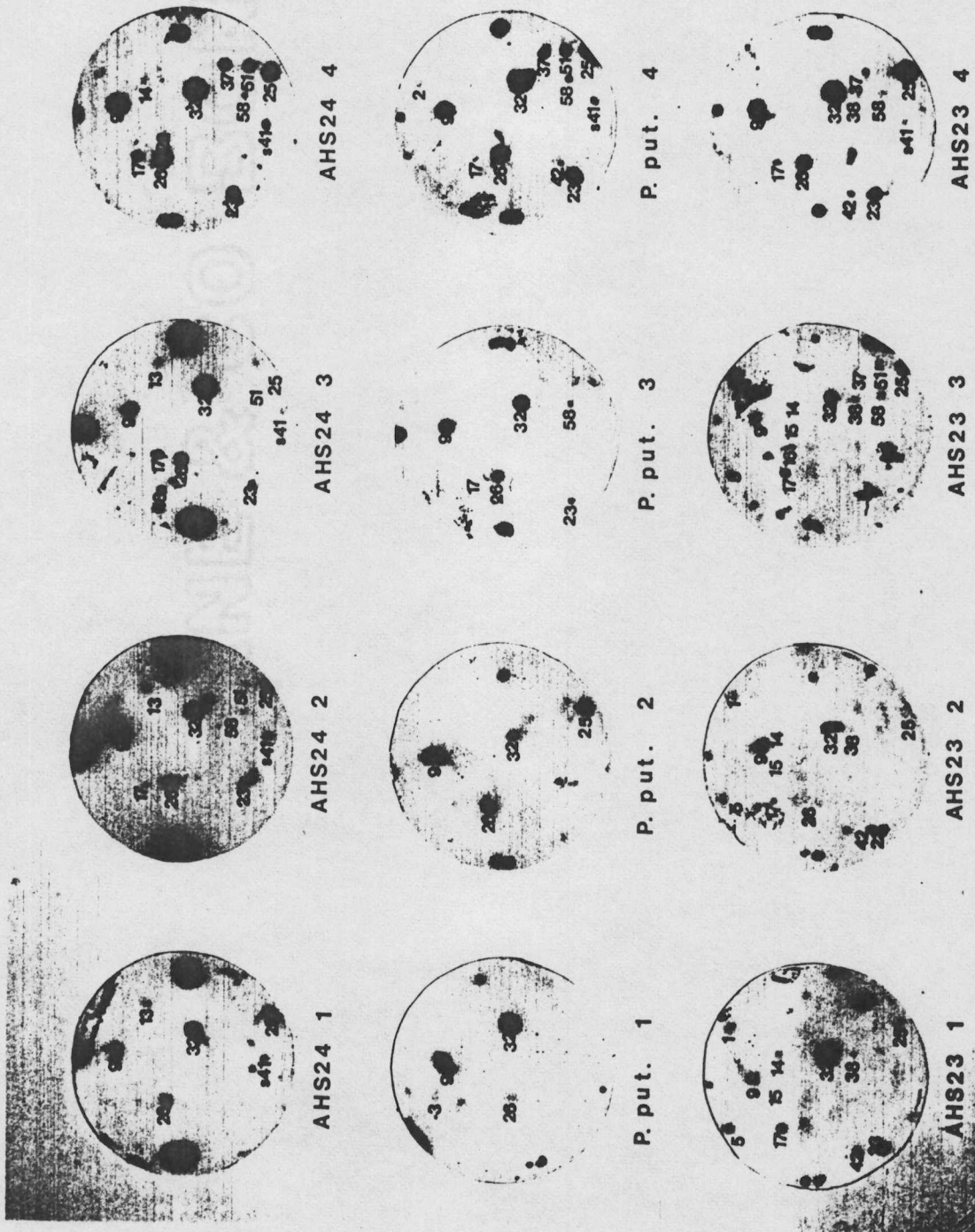


Figure 9

the P. putida probes and AHS23 probes which is only consistently seen at the lowest stringency of 1000 mM sodium (Figure 9, P. putida 4 and AHS23 4). A pattern of increasing number of groundwater isolates which cross-hybridize to the whole genomic probes at increasingly lower stringencies is most evident in AHS24-probed and P. putida probed membranes, whereas AHS23 probed membranes do not appear to demonstrate this trend.

Groundwater isolates were then grown directly on the Pall-Biodyne membranes to determine whether this would improve clarity and strength of hybridization signals due to the presence of more homologous DNA being present on the filters. From the previous set of groundwater isolate hybridization experiments, it was apparent that AHS24 showed no visible cross-hybridization with Arthrobacter TM1, but did cross-hybridize with several of the same isolates cross-hybridizing with P. putida and AHS23. The tentative genera identifications of some of these isolates were Citrobacter, Enterobacter, and Klebsiella for isolate numbers 9, 26, and 37, respectively. However, the true taxonomic classification of these organisms is still not certain. Therefore, ATCC strains of Citrobacter freundii, Enterobacter aerogenes, Klebsiella pneumoniae, P. putida, P. aeruginosa, and Arthrobacter TM1 were spotted directly from individual colonies onto the membranes and were allowed to mature for 3 days along with the groundwater isolates. Prior to lysis, AHS24 colonies were spotted directly on each filter at 0° (1 spot), 90° (2 spots), and 270° (3 spots) with respect to the top of the filter. Pseudomonas putida colonies were also spotted on each filter at 225° (2 spots) with respect to

the top of the filter. These colonies only give signals with their homologous DNA which is evident in the longer 44 hour exposures P. putida f and P. putida h of Figure 10. The results of this experiment are summarized in Table 8 and shown in Figure 10. From these data, it is evident that several groundwater isolates previously shown to cross-hybridize with AHS24, P. putida, and AHS23 genomic DNA are reproducibly cross-hybridizing to these DNAs (isolate numbers 17, 26, 32, 38, and 7S41). More interestingly, these isolates give fair or strong signals with each of the whole genomic probes AHS24, P. putida, ABS10, and AHS23, which indicates that these genomic DNAs may share a high degree of homologous sequences. It is interesting to also note that AHS24, ABS10, and AHS23 do not cross-hybridize with the known reference Arthrobacter TMI at concentrations of 0.1 μ g of whole genomic probe DNA. It is likely that the Arthrobacters AHS24, ABS10, and AHS23 could share many common sequences since they were isolated from the same environment and share common selection pressures; however, it is totally unanticipated that these putative Arthrobacter strains share DNA homologies with P. putida and P. aeruginosa. These data are supported by an independent discovery in Dr. Sayler's lab by Alex Breen that the ABS10 strain has been shown to cross-hybridize with P. putida. AHS24, ABS10, and AHS23 were evaluated for the presence of plasmids; however, none were ever isolated from these strains, therefore the source of these homologies is most likely resident in the main chromosomal DNA. AHS24 does seem to weakly cross-hybridize with Enterobacter aerogenes and Klebsiella

Figure 10. Autoradiograph of groundwater isolates screened with AHS24, ABS10, P. putida and AHS23 whole genomic DNAs in which the isolates were grown directly on the membrane. Membrane sets e and g represent 24 hour exposures, membrane sets f and h represent 44 hour exposures. Positive groundwater isolates in autoradiographs of the first two rows are indicated with their identification number. Groundwater isolates in the autoradiographs of the last two rows are identified by row number: (1) 7s15, (2) 7s25, (3) 7s46, (4) 7s41. Reference strains are marked by small letters: (a) P. aeruginosa, (b) Citrobacter freundii, (c) Enterobacter aerogenes, (d) Klebsiella pneumoniae, (e) ABS10, (f) Arthrobacter TM1, (g) AHS24.

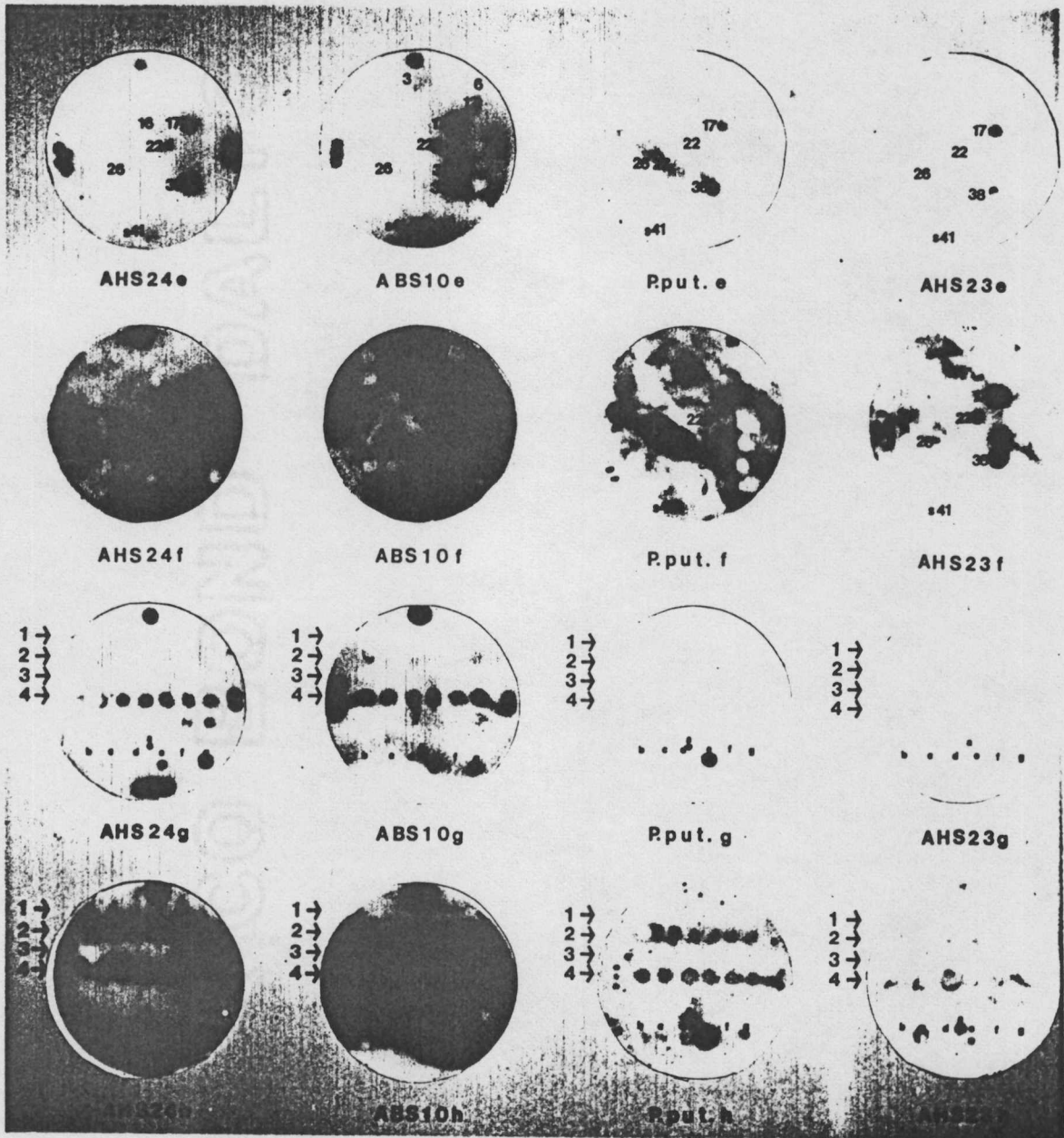


Figure 10

Table 8. Groundwater isolates probed with whole genomic DNAs after growth on membranes.

Groundwater isolate number	Probe DNA			
	AHS24	ABS10	<u>P. putida</u>	AHS23
1	-	-	-	-
2	-	-	-	-
3	-	(+)	-	-
4	-	-	-	-
5	-	-	-	-
6	-	(+)	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	(+)	-	-
13	-	-	-	-
14	-	-	-	-
15	-	-	-	-
16	(+)	-	-	-
17	(+)	+	(+)	+
18	-	-	-	-
19	-	-	-	-
20	-	-	-	-
21	-	-	-	-
22	(+)	+	(+)	+
23	-	-	-	-
24	-	-	-	-
25	-	-	-	-
26	(+)	(+)	(+)	(+)
28	-	-	(+)	-
29	-	-	-	-
32	(+)	(+)	(+)	-
33	-	-	-	-
34	-	-	-	-
35	-	-	-	-
36	-	-	-	-
37	-	-	-	-
38	+	+	+	+
39	-	-	-	-
40	-	-	-	-
41	-	-	-	-
42	-	-	-	-
51	-	-	-	-
53	-	-	-	-
58	-	-	-	-
59	-	-	-	-
TM1	-	-	-	-
7S41a	(+)	+	(+)	(+)
7S15	-	-	-	-
7S25	(+)	(+)	(+)	-
7S46	-	-	-	-
7S41b	+	+	(+)	(+)
AHS24	+	+	(+)	-
ABS10	(+)	(+)	+	(+)
ATCC strains spotted				
<u>P. putida</u>	-	-	+	-
<u>P. aeruginosa</u>	(+)	(+)	(+)	+
<u>Citrobacter</u>				
<u>freundii</u>	-	-	-	-
<u>Enterobacter</u>				
<u>aerogenes</u>	(+)	-	-	(+)
<u>Klebsiella</u>				
<u>pneumoniae</u>	(+)	-	-	-
TM1	-	-	-	-
TOTAL	14	14	13	9

pneumoniae, but this was only evident at very long exposure times of 44 hours (Figure 10, AHS24 h, points c and d). AHS23 only weakly cross-hybridized to Enterobacter aerogenes and this was visible only upon the longer exposure as well, therefore the tentative identifications of the groundwater isolates 9, 26, and 37 as being Citrobacter, Enterobacter, and Klebsiella may not be accurate. The cross-hybridization of AHS24 and AHS23 to these reference strains does not appear to be significant compared to the cross-hybridization to P. putida and P. aeruginosa, but more accurate analysis of this observation could be followed by determination of DNA-DNA homologies using the membrane filter technique or the method of De Ley (12) which employs spectrophotometric determination of DNA hybridizations from renaturation rates.

In comparing the autoradiographs of this experiment (Figure 10) in which the groundwater isolates were grown directly on the filter and the autoradiographs of the previous experiment (Figure 9, p. 49) in which the colonies were grown on the agar plates and transferred to the membrane just prior to lysis, it is obvious that higher background and less clear signals result as of growing the colonies directly on the membrane and then processing for hybridization. A possible explanation of this could be the accumulation of various organic compounds on the membrane which were secreted by the groundwater isolates or the adherence of compounds incorporated into the media which all could result in the nonspecific binding of labeled DNA to the membranes upon hybridization. The spreading of the cells themselves could also

result in nonspecific binding of labeled DNA. In some cases, growth of the colonies on the membrane was inhibitory (isolates 9, 13, 23, 25, 51 and 53) to the point that growth was not visible. Table 9 compares the results of the experiment in which the groundwater isolates were grown directly on the filters to the experiment in which colonies were transferred from agar prior to lysis. The data used to generate this table are from Tables 6 and 7 (pp. 39 and 46) from columns where the probes AHS24, P. putida, and AHS23 were used to probe groundwater isolates and hybridizations were washed at standard stringencies as previously described. From the table, consistencies in the groundwater isolates hybridizing the AHS24 in both of the experiments are obvious for isolates 17, 26, 32, and 7S41. The fact that isolates 9, 13, 23, 25, 51, and 58 did not visibly grow on the membrane may explain why no signals were observed when hybridized to AHS24 but were observed in the earlier experiment. For the P. putida probes, isolates 26, 32, and AHS24 show consistent signals in both experiments. Again, the missing signals for isolate numbers 9 and 25 could be explained by the fact that no visible growth for these isolates was observed when grown directly on the nylon membrane. When AHS23 was used as a probe in both experiments, isolates 17, 26, and 38 showed consistent signals. Lack of growth for isolates 9 and 25 may again account for missing signals in the autoradiographs of the latter experiment; however, the weak signal strength, thus weak hybridization of 9 and 25 at 50 mM NaCl stringency (Figure 9, p. 49, AHS23 2) may explain why these signals were not observed in the autoradiograph

Table 9. Comparison of groundwater isolate hybridization when colonies were (a) transferred to membranes prior to lysis or (b) when colonies were grown directly on the membrane.

Groundwater isolate number	Probe used					
	AHS24		<u>P. putida</u>		AHS23	
	a	b	a	b	a	b
1	-	-	-	-	(+)	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	-	-	-	-	-	(+)
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	-	-	-	-	-	-
9	(+)	-	(+)	-	(+)	-
10	-	-	-	-	-	-
11	-	-	-	-	-	-
12	-	-	-	-	-	-
13	(+)	-	-	-	-	-
14	-	-	-	-	(+)	-
15	-	-	-	-	(+)	-
16	-	(+)	-	-	-	-
17	(+)	(+)	-	(+)	(+)	+
18	-	-	-	-	-	-
19	-	-	-	-	-	-
20	-	-	-	-	-	-
21	-	-	-	-	-	-
22	-	(+)	-	(+)	-	+
23	(+)	-	-	-	-	-
24	-	-	-	-	-	-
25	(+)	-	(+)	-	(+)	-
26	(+)	(+)	(+)	(+)	(+)	(+)
28	-	-	-	-	-	-
29	-	-	-	-	-	-
32	+	(+)	+	(+)	(+)	-
33	-	-	-	-	-	-
34	-	-	-	-	-	-
35	-	-	-	-	-	-
36	-	-	-	-	-	-
37	-	-	-	-	-	-
38	-	+	-	+	(+)	+
39	-	-	-	-	-	-
40	-	-	-	-	-	-
41	-	-	-	-	-	-
42	-	-	-	-	(+)	-
51	(+)	-	-	-	-	-
53	-	-	-	-	-	-
58	(+)	-	-	-	-	-
59	-	-	-	-	-	-
TM1	-	-	-	-	-	-
7S41	(+)	+	-	(+)	-	(+)
7S46	-	-	-	-	-	-
7S15	-	-	-	-	-	-
7S25	-	(+)	-	(+)	-	-
AHS24	+	+	(+)	(+)	-	-
TOTAL	11	9	5	8	11	5

(+) = weak signal observed in hybridization to colonies transferred to nylon membrane.

+ = strong signal observed in hybridization to colonies transferred to nylon membrane.

of the later experiment if there was a nonvisible amount of cells growing on the filter. The fact that no signals were observed for membrane isolates number 13, 14, and 15 may be explained by the observation that these signals in the previous experiment (Figure 9, p. 49, AHS23 2) were extremely faint (possibly artifactual) and do not show increased signal strength even at low stringencies (Figure 9, AHS23 2-4). This may also be the case for isolate number 13 when probed with AHS24 since it too gives a very weak signal even at lower stringencies (Figure 9, AHS24 2-4). One case in which a membrane grown isolate, such as isolate 22, did give a signal when probed with AHS24, P. putida, and AHS23 but did not give a signal when probed previously could be accounted for by the fact that it did not transfer as readily to membranes when grown on agar, but did grow well on the membrane. Membrane-grown isolates 38 and 7S25 gave weak signals when probed both with AHS24 and P. putida DNA, but did not give signals in the earlier experiment even though they did transfer well to the membrane; therefore these results cannot be explained except in the case of 7S25, where a very faint hybridization signal was observed in 24 hour exposures but became much stronger at later 44 hour exposures. Since the autoradiographs of the earlier experiment in which the colonies were transferred to membranes were 24 hour exposures, signals to 7S25 would not be observed. The fact that membrane-grown 7S41 gave a signal when probed with P. putida and AHS23 at the 50 mM NaCl standard stringency, but did not give a signal in the preliminary experiment at the same stringency, yet did

give weak signals at lower stringencies (Figure 9, p. 49, P. putida 2-4, and AHS23 2-4) may be explained by possible variability in the amount of available DNA present on the membrane.

From this series of experiments, it was determined that groundwater isolates 9, 17, 26, 25, 7S41, 38, and 23, which all gave hybridization signals to AHS24 probe, would be utilized in further experiments to better characterize the specificity of AHS24 subcloned probes versus whole genomic AHS24 probes.

4. Comparison of the Specificity of AHS24 Subgenomic Probes and AHS24 Whole Genomic Probes in Individual Isolate Hybridizations

In the previous set of hybridization experiments, it was discovered that the whole genomic probes AHS24, P. putida and AHS23 demonstrated a wide specificity with regard to which groundwater isolates they cross-hybridized to. These three whole genomic probes also seemed to cross-hybridize to a common subset of groundwater isolates. This suggested that the whole genomic probes themselves share regions of homology which was not expected. Therefore, a series of subgenomic probes were utilized in efforts to develop a set of probes which would be highly discriminatory for AHS24.

The previously mentioned groundwater isolates, along with P. putida, P. aeruginosa, Arthrobacter TM1 and E. coli, were hybridized with subcloned probes p1CA3, p2MLO, p498 as well as whole genomic probes AHS24 and P. putida. The plasmid pUC13 which does not carry an AHS24 insert was utilized as a negative control to

characterize those groundwater isolates showing cross-hybridizations to the subgenomic probes as potentially sharing regions of homology with the vector itself and not the AHS24 inserts. AHS23 whole genomic DNA was not utilized further in these studies. Groundwater isolates along with other reference strains were transferred in triplicate to the membranes as previously described. In order to better compare signal intensities between membranes, Arthrobacter cells at an approximate OD of 1.0 ($\lambda = 600$ nm) were concentrated 10-fold and 5 microliters were spotted at 0° (a spot), 90° (2 spots) and 150° (3 spots) in reference to the top of the membrane. E. coli JM107 was grown to the same density, concentrated 10-fold and 5 μ l were spotted at 270° (1 spot) on the membranes. Two different stringencies were employed in this set of experiments, and two separate probe concentrations were utilized to determine the effect of these parameters upon signal intensity of cross-hybridizing groundwater isolates.

The results of the first comparative experiment are shown in Figure 11 and summarized in Table 10. These filters were rinsed at 70°C in 1X Low buffer with a probe concentration of $0.10\ \mu\text{g}$ of DNA at a specific activity of around 10^7 CPM/ μg of DNA (Appendix B). From these data it is apparent that all of the subcloned probes except p498 show a narrower range of specificity as compared to the AHS24 whole genomic probe. The whole genomic AHS24 probe's lack of specificity is demonstrated here in that it hybridized with both P. putida and P. aeruginosa. It also showed cross-homology with ABS10, but not Arthrobacter TM1. The fact that isolates 17, 9, and 26 do not

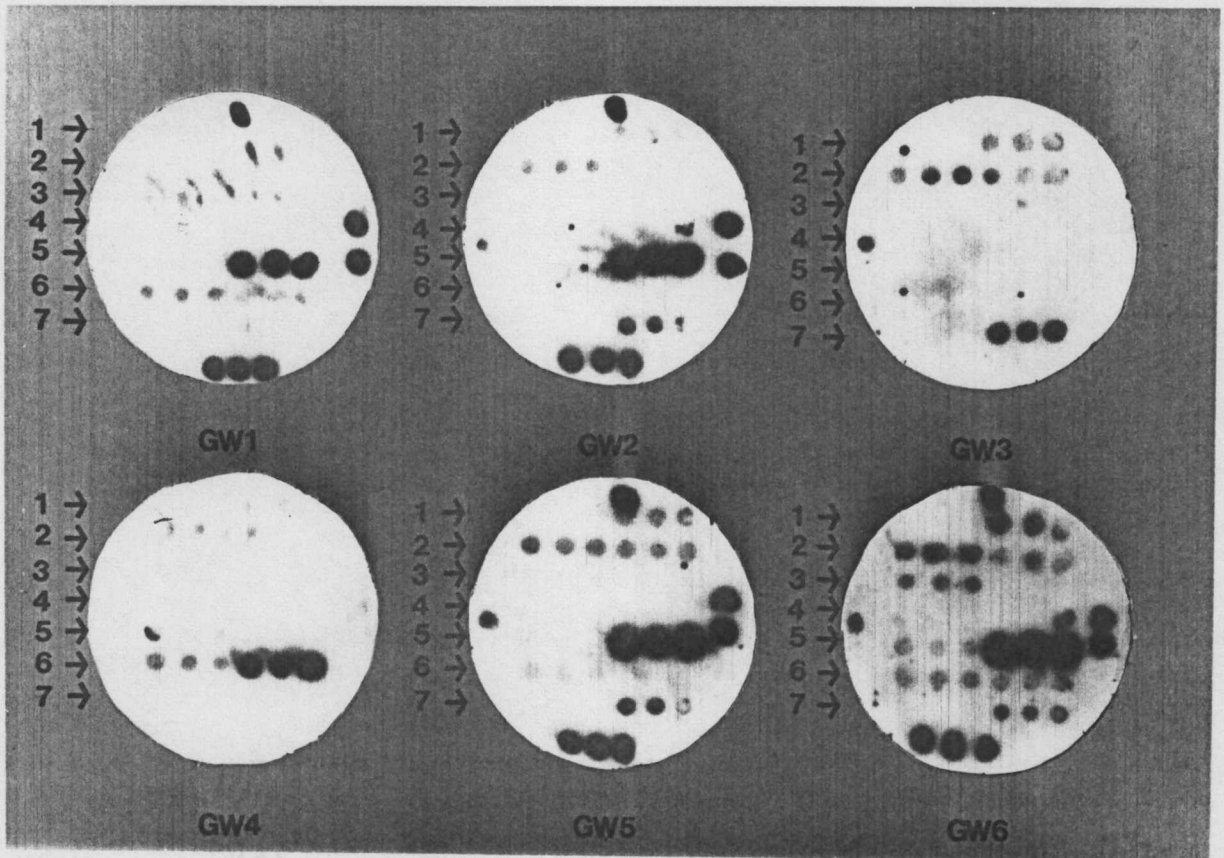


Figure 11. Comparative specificity of *Arthrobacter* AHS24 chromosomal and subclones gene probes in colony hybridization at 70°C stringency. Rows 1-7 bacterial isolates in triplicate colonies (two isolates per row). Row (1) GW17 and 9; (2) GW26 and 25, (3) GW 7s41 and 38, (4) GW23 and *Arthrobacter* TM1, (5) ABS10 and AHS24, (6) *P. aeruginosa* and *P. putida*, (7) *E. coli* JM107. Filters GW1-6 DNA probes GW1 chromosomal AHS24; GW2 p1CA3; GW3, pUC1B; GW4, *P. putida* chromosomal DNA; GW5 p2MLO, and GW6, p498.

Table 10. Comparative specificity of AHS24 whole genomic and sub-genomic DNA probes at higher stringency wash of 70°C.

Target DNA	Probe DNA					
	AHS24	p1CA3	p2MLO	p498	pUC13	<u>P. putida</u>
AHS24	+	+	+	+	-	-
ABS10	(+) ^b	-	-	(+)	-	(+)
GW ^a 17	-	-	-	-	-	-
GW9	-	-	(+)	(+)	(+)	(+)
GW26	-	(+)	(+)	(+)	(+)	-
GW25	(+)	(+)	(+)	(+)	(+)	-
GW7s41	(+)	(+)	-	(+)	-	-
GW38	(+)	-	-	-	-	-
GW23	(+)	-	-	-	-	-
<u>Arthrobacter</u> TM1	-	-	-	-	-	-
<u>P. aeruginosa</u>	(+)	-	-	(+)	-	(+)
<u>P. putida</u>	(+)	-	-	(+)	-	+
<u>E. coli</u> JM107	-	+	+	(+)	+	-
TOTAL	8	5	5	9	4	4

^aGW indicates uncharacterized groundwater isolate from Lula, Oklahoma aquifer.

^b() indicates weak positive hybridization.

^cTotal strong positive and positive hybridizations.

hybridize with whole chromosomal AHS24 DNA as seen previously may be due to the increased stringency as well as the increased amount of homologous DNA present on the filter competing with the DNA from isolates 17, 9, and 26 for probe. Using this information in conjunction with the fact that isolates 9 and 26 cross-hybridize to the pUC vector control demonstrates that the signals from isolates 9 and 26 and perhaps isolate 25, when using the subclones probes p1CA3, p2MLO and p498, are actually due to homologous sequences of enteric origin from which the vector was derived, or possible E. coli chromosomal DNA contamination of the plasmid preparation. Also, the probe p498 is potentially useful in that it hybridizes with the known Pseudomonas strains and ABS10 which could provide information regarding any isolate which may possess Pseudomonas character. Probe p1CA3 shows a much narrower specificity, but its use to exclusively detect AHS24 is compromised in that it cross-hybridizes to isolate 7S41. Probe p2MLO only shows cross-hybridization due to enteric sequences. Therefore the insert fragment of p2MLO is predicted to provide the most specific probe for AHS24.

Table 11 and Figure 12 summarize the next comparative experiment in which the filters were rinsed at 65°C in 1X Low buffer with a probe concentration of 0.10 µg DNA at a specific activity of 10^7 CPM/µg of DNA. At the lower stringency, isolates 9 and 26 now demonstrate weak signals to the AHS24 whole genomic probe. It is unknown why isolates 38 and 23 do not hybridize to the whole genomic AHS24 probe as they did at the higher stringency unless the amount

Table 11. Comparative specificity of AHS24 whole genomic versus subgenomic probes at lower stringency wash of 65°C.

Target DNA	Probe DNA						
	AHS24	p1CA3	p2ML0	p498	pUC13	<u>P. putida</u>	p2ML2
AHS24	+	+	+	+	-	(+)	+
ABS10	(+) ^b	(+)	-	(+)	-	(+)	(+)
GW ^a 17	-	-	-	-	-	-	-
GW9	(+)	(+)	(+)	(+)	(+)	(+)	(+)
GW26	(+)	(+)	(+)	(+)	-	(+)	(+)
GW25	(+)	(+)	(+)	(+)	-	(+)	(+)
GW7s41	(+)	(+)	-	(+)	-	(+)	(+)
GW38	-	-	-	-	-	-	-
GW23	-	-	-	-	-	(+)	(+)
<u>Arthrobacter</u> sp. TM1	-	(+)	(+)	(+)	(+)	(+)	(+)
<u>Arthrobacter</u> <u>globiformis</u> 8010	-	-	-	-	-	-	-
<u>Arthrobacter</u> <u>crystalpoietes</u> T5481	-	-	-	-	-	-	-
<u>P. aeruginosa</u>	(+)	(+)	-	(+)	(+)	(+)	(+)
<u>P. putida</u>	(+)	(+)	+	(+)	(+)	+	+
<u>E. coli</u> JM107	-	(+)	+	+	(+)	(+)	+
TOTAL	8	10	7	10	5	11	11

^aGW indicates uncharacterized groundwater isolate from Lula, Oklahoma aquifer material.

^b() indicates weak positive hybridization.

^cTotal strong positive and weak positive hybridizations.

Figure 12. Comparative specificity of Arthrobacter AHS24 chromosomal subgenomic gene probes in colony hybridization at 65°C stringency. Rows 1-7 bacterial isolates in triplicate colonies (two isolates per row). Row (1) GW 17 and 9, (2) GW26 and 25, (3) GW 7s41 and 38, (4) GW23 and Arthrobacter TM1, (5) ABS10 and AHS24, (6) P. aeruginosa and P. putida (7) Arthrobacter globiformis and E. coli JM107, (8) Arthrobacter crystalpoietes. Membranes are labeled according to the respective probes utilized, AHS24 or P. putida chromosomal probes or p1CA3, p2MLO, p498, and p2ML2 subgenomic probes. A pUC13 (no AHS24 insert) set of membranes is also shown. Membrane set (a) represents a 36-hour exposure, set (b) a 96-hour exposure.

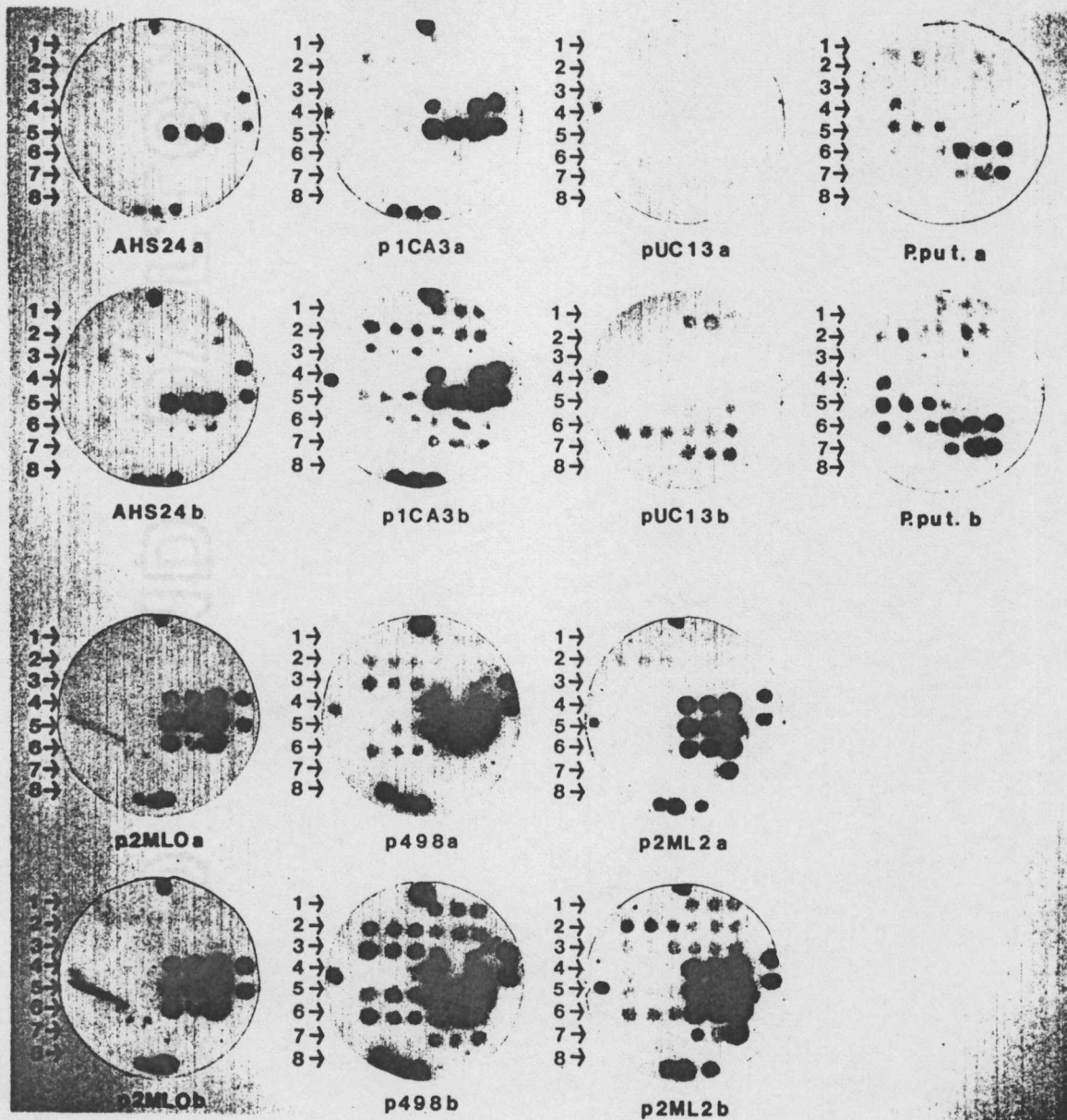


Figure 12

of probe DNA is limiting or perhaps isolates 38 and 23 did not lyse as efficiently on the filter; however, this was not observed during the procedure. By comparing the data in Table 11 to Table 10, it is apparent that 5° can significantly decrease the specificity of the subgenomic probes overall. Isolates 26 and 25 do not demonstrate hybridization to the pUC13 plasmid control, but did in the higher stringency experiment which can best be explained by comparing the signal intensity of the E. coli control spot at 270° on the pUC 13 membrane in Figure 12. Even at the higher exposure, the signal is not as intense as the same control signals in the subgenomic-probed membranes, thus the ability and reliability of the control for this experiment has been somewhat compromised. Also, the quality and reliability of the whole genomic AHS24 probed-membrane is slightly compromised in that the control AHS24 spots at 0° , 90° , and 180° were of equal intensity in comparison to the subgenomic probed filters only at the longer time exposures. Signals of groundwater isolates cross-hybridizing to AHS24 were only visible when the original autoradiographs were highly illuminated. At the lower stringency, it is also apparent that all of the subgenomic probes give intense hybridization signals to Arthrobacter TM1 and all subgenomic probes except p1CA3 give intense signals to P. putida. Cross-contamination of wells is not suspect in this case since the P. putida-probed membranes and AHS24 probed membranes do not demonstrate that this has occurred (Figure 12). (One possible well of cross-contamination is observed in the P. putida-probed membrane, but this is observed in

one E. coli lane and is not significant to change the interpretation of the experimental results.) Variability in signal strengths again may be due to limiting probe DNA or variability in the amount of availability of target DNA. Two ATCC reference Arthrobacter strains, Arthrobacter globiformis and Arthrobacter crystalpoietes, did not demonstrate cross-hybridization to any subgenomic probes or AHS24 and P. putida whole genomic probes at this stringency and DNA concentration.

In order to determine if the concentration of probe DNA was significantly limiting in these comparative studies, membranes were hybridized to 10X the amount of probe DNA previously used, or 1 μg in 5 ml prehybridization buffer at a specific activity of 5×10^7 cpm/ μg DNA. Only one subgenomic probe, p1CA3, was used to compare specificity with the AHS24 probe. The results of this experiment are shown in Table 12 and Figure 13. At this DNA concentration and stringency wash of 65°, all groundwater isolates, Arthrobacter TM1, A. globiformis, P. putida, and P. aeruginosa all gave visible hybridization signals with the AHS24 whole genomic and p1CA3 subgenomic probes. Interestingly, A. crystalpoietes did not demonstrate cross-hybridization to AHS24 whole genomic DNA. This experiment also confirms the earlier result of Arthrobacter TM1 and P. putida giving strong hybridization signals at 65°C wash stringency when probed with both AHS24 DNA and p1CA3 DNA. The significant observation about this experiment is that P. aeruginosa and P. putida give equally intense hybridization signals when probed with AHS24 DNA, but differ in intensity when probed with the subgenomic probe p1CA3

Table 12. Comparative specificity of AHS24 whole genomic and p1CA3 subgenomic probes with 10X (1 μ g) probe concentration hybridization and low stringency wash at 65°C.

Target DNA	Probe DNA	
	AHS24	p1CA3
AHS24	+	+
ABS10	(+)	(+)
GW17	(+)	-
GW9	(+)	(+)
GW26	(+)	(+)
GW25	(+)	(+)
GW7s41	(+)	(+)
GW38	(+)	-
GW23	(+)	-
<u>Arthrobacter</u> TM1	+	(+)
<u>Arthrobacter</u> globiformis 8010	+	-
<u>Arthrobacter</u> crystalpoietes 15481	-	-
<u>P. aeruginosa</u>	+	(+)
<u>P. putida</u>	+	+
<u>E. coli</u> JM107	(+)	(+)
TOTAL	14	10

Figure 13. Comparative specificity of Arthrobacter AHS24 chromosomal and p1CA3 subgenomic probe in colony hybridization 10X DNA probe concentration (1 μ g) at 65°C stringency. Rows 1-7 bacterial isolates in triplicate colonies (two isolates per row. Row (1) GW 17 and 9, (2) GW 26 and 25, (3) GW 7s41 and 38, (4) GW 3 and Arthrobacter TM1, (5) ABS10 and AHS24, (6) P. aeruginosa and P. putida, (7) Arthrobacter globiformis and E. coli JM107, (8) Arthrobacter crystalpoietes. Membranes are labeled according to the respective probes utilized, AHS24 or p1CA3. Membrane set (a) represents a 6-hour exposure, set (b) an 18-hour exposure, and set (c) a 96-hour exposure.

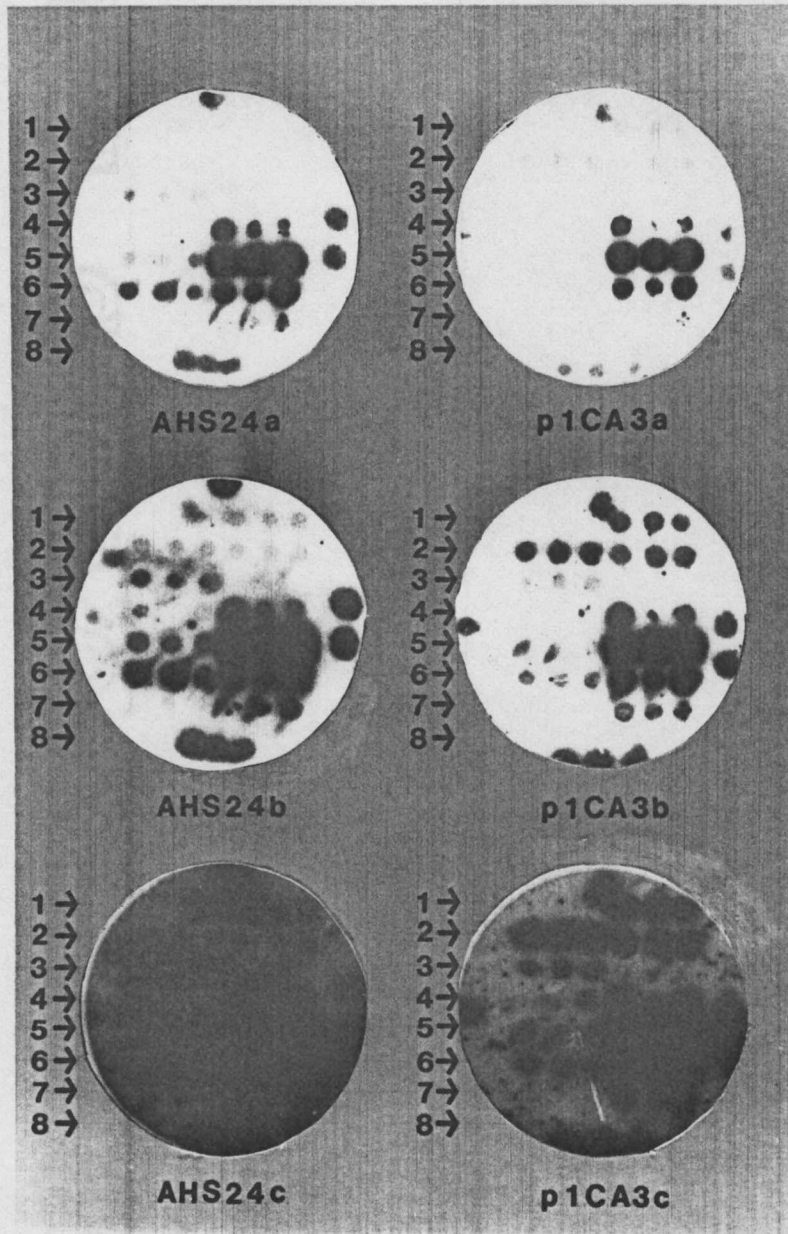


Figure 13

which is most evident in autoradiographs AHS24b and P1CA3b of Figure 13. This demonstrates the ability of subgenomic probe p1CA3 to distinguish between two very closely related species of Pseudomonas that are not distinguished by the whole genomic AHS24 probe.

The results of these three comparative specificity experiments are summarized in Table 13. From the combined data, it is evident that all of the subgenomic probes are highly sensitive for AHS24 in that they give strong hybridization signals, but that sensitivity of the probe, especially in the case of p1CA3 and p2MLO, depends upon the stringency of the wash conditions. The probe p498 did not demonstrate a significant narrowing of specificity at either stringency of 70°C or 65°C washes.

5. Probing of Local Freshwater and Microcosm Communities at High Colony Densities

To determine the sensitivity of the AHS24 whole chromosomal probe versus the subgenomic AHS24 probes in actual environmental applications, water samples from three TVA recreational lakes in addition to one microcosm soil sample from a pristine groundwater aquifer in Lula, Oklahoma, were screened at high colony densities with AHS24 whole genomic probe, the p1CA3 4.5 kb probe and the pUC13 (no AHS24 insert) probe control. Since these samples were not dosed with AHS24, signals arising from AHS24 would not be anticipated; however, spurious signals due to highly homologous AHS24-like bacteria would be expected. Positive AHS24 controls were performed internally for each membrane as previously described for the comparative specificity

Table 13. Summary of comparative specificity of whole genomic versus subgenomic AHS24 probes under varying stringencies.

Target DNA	Probe DNA																	
	AHS24			p1CA3			p2ML0			p498			pUC13			P. putida		
	12 ^a	11 ^b	10 ^c	12	11	10	12	11	10	12	11	10	12	11	10	12	11	10
AHS24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ABS10	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
GW17	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
GW9	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
GW26	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
GW25	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
GW7s41	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
GW38	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
GW23	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Arthrobacter TM1	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
A. globiformis 8010	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
A. crystallopoietes 15481	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
P. aeruginosa	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
P. putida	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
E. coli JM107	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
TOTAL	14	8	8	10	10	5	-	7	5	-	10	9	-	5	4	-	11	4

^a10X DNA probe conc. (1µg), 1X LOW 65°C wash from Table 12.^b1X DNA probe conc. (0.1 µg), 1X LOW 65°C wash from Table 11.^c1X DNA probe conc. (0.1 µg), 1X LOW 70°C wash from Table 10.^dNot determined.

experiments. The spotting of 5 μ l of 10-fold concentrated E. coli JM107 cells at 270° (1 spot) again serve as a positive control for the p1CA3 and pUC13 plasmids which contain part of the E. coli lacZ gene in the region of the multiple cloning site on the pUC13 parent vector. The membranes were rinsed in 1X Low buffer at the higher stringency of 70°C. Figure 14 shows the representative autoradiographs of the environmental sample filters with the designated probes. The results of this experiment are summarized in Table 14. The internal controls for each set of probed environmental samplings demonstrate the high sensitivity of the individual probes for their homologous sequences. On filters A1, B1, C1, and D1, the only intense signals observed (despite the average environmental colony density of 10^3) are those due to hybridization of the whole genomic AHS24 probe with AHS24 positive control spots in the periphery of the membrane. On filters A2, B2, C2, and D2, an intense signal corresponding to where JM107 was spotted is observed along with the signals corresponding to where AHS24 cells were placed. This demonstrates the sensitivity of the probe p1CA3 for AHS24 but also the cross-reactivity of the probe with E. coli due to the β -galactosidase enteric sequences present on the plasmid or contamination of the probe preparation with E. coli chromosomal DNA. The only intense control signals on filters A3, B3, C3, and D3 are those corresponding to the spots where E. coli JM107 was placed. These signals serve as negative controls to demonstrate that the pUC13 vector DNA used in the construction of the subgenomic probes shares little if no homology with the insert DNA. The tabulation with regard to the number of signals observed per membrane in

Figure 14. Probing of local freshwater and microcosm communities at high colony densities with AHS24 whole genomic probe and p1CA3 subgenomic probe at 70°C stringency wash. Membranes set (A) represent samples from the Holston River, (B) from Cherokee Lake, (C) from Douglas Lake, and (d) from an undosed microcosm. Membranes are labeled 1-3 with respect to the whole genomic or subgenomic probe used. Probe (1) AHS24 chromosomal DNA, (2) p1CA3 chromosomal DNA, (3) pU13 (no AHS24 insert) probe control. All autoradiographs were exposed 24 hours.

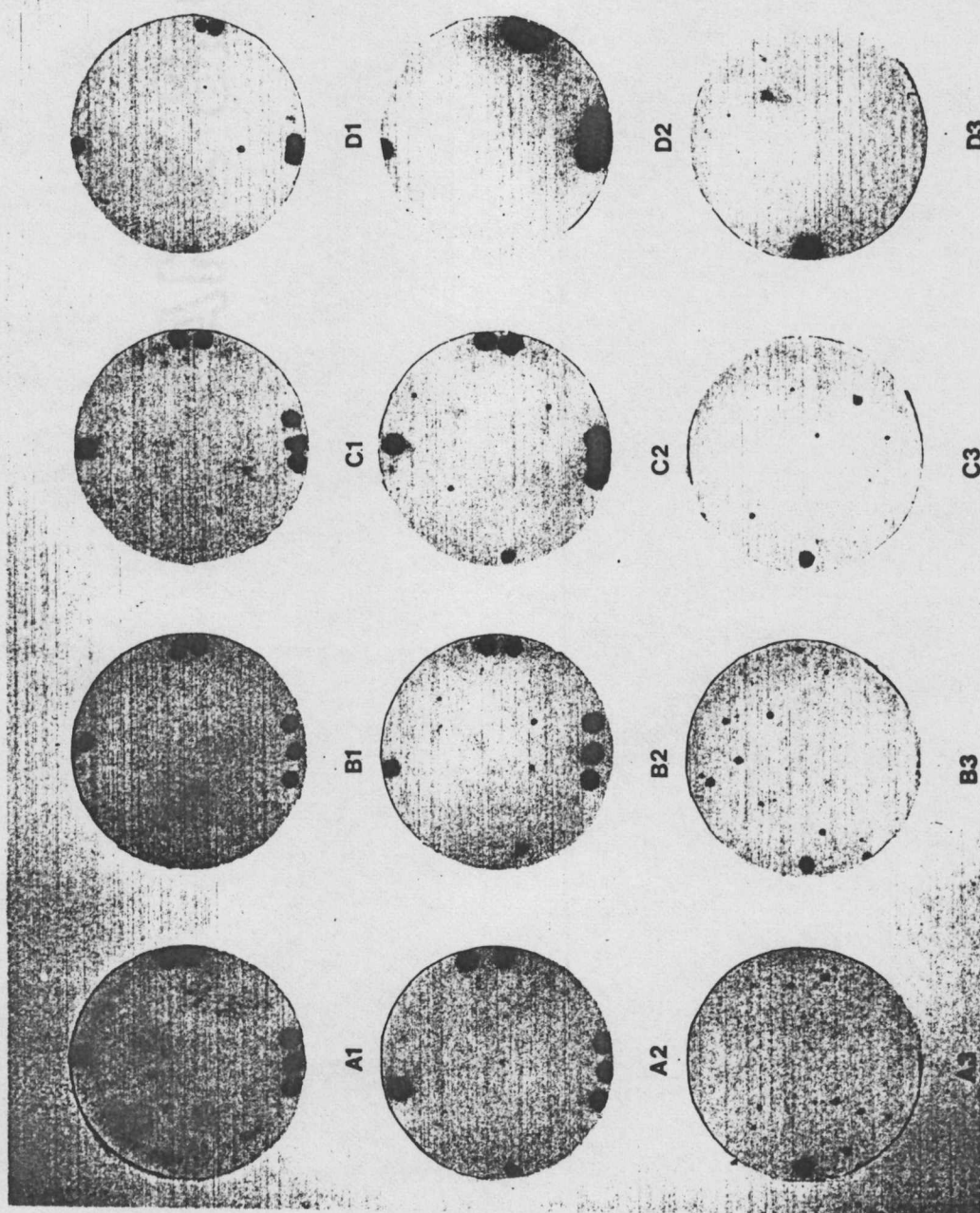


Figure 14

Table 14. Probing of local freshwater and microcosm communities at high colony densities with AHS24 whole genomic probe and p1CA3 subgenomic probe at 70°C hybridization wash.

Sample isolation site	Colony density	Number of signals observed/membrane		
		AHS24 genomic probe	p1CA3 probe	pUC13 probe
Holston River	1.8×10^3	2	3	8
Cherokee Lake	1.2×10^3	10	10	17
Douglas Lake	2.8×10^3	0	3	8
Microcosm	$1.8-4.8 \times 10^2$	0	0	0

Table 14 demonstrates that both the whole genomic AHS24 probe and the subclone probe p1CA3 yield signals statistically within the range of the number observed in the control pUC13 probed filters A3, B3, C3, and D3. Thus, the pUC13 probed communities establish a background level of signals expected using the subgenomic probes due to microorganisms possessing sequences of enteric origin.

This set of experiments was repeated at a lower stringency wash of 65°C in 1X Low buffer. All four environmental communities at colony densities of 10^3 were reprobed using AHS24 and P. putida whole genomic probes as well as all four subgenomic probes p1CA3, p2MLO, p498, p2ML2 and also the control pUC13 parent vector. The autoradiographs of these membranes are shown in Figure 15 and the results are tabulated in Table 15. Generally a higher degree of non-distinct background is observed in the periphery of the membranes at the lower stringency wash. It appears that probe p2MLO yields the lowest amount of background signals compared to the rest of the subgenomic probes and p498 yields the highest level of background signals. This result is consistent with the earlier set of experiments in which individual groundwater isolates were probed with the subgenomic probes and p2MLO appeared to be the most specific AHS24 probe whereas the specificity of the p498 probe was not as narrow as the other subgenomic probes. Again the number of background signals observed for whole genomically and subgenomically-probed membranes is statistically within the range of background signals observed when the communities are probed with the negative control

Figure 15. Probing of local freshwater and microcosm communities at high colony densities with AHS24 and *P. putida* whole genomic probe and p1CA3, p2MLO, p498, and p2ML2 sub-genomic probes at 65°C stringency wash. Membrane set (H) represent samplings from the Holston River, (C) from Cherokee Lake, (D) from Douglas Lake, and (M) from and undosed microcosm. Membranes are labeled with respect to the probes utilized, AHS24 or *P. putida* whole genomic DNA, p1CA3, p2MLO, p498, p2ML2 subgenomic probes, and pUC13 (no AHS24 insert) control probe. Membranes labeled (a) represent a 36-hour exposure, those labeled (b) represent a 96-hour exposure.

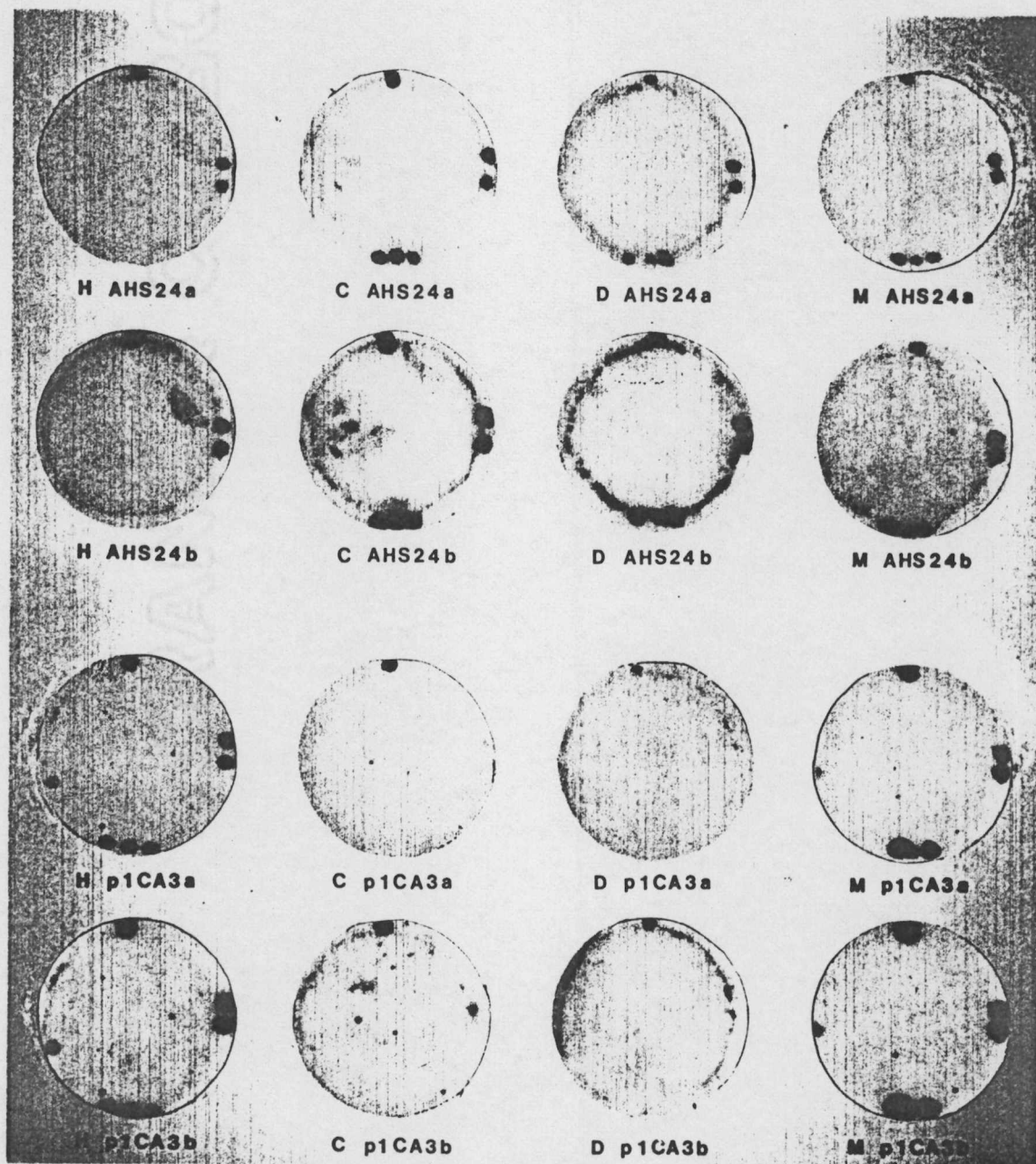


Figure 15

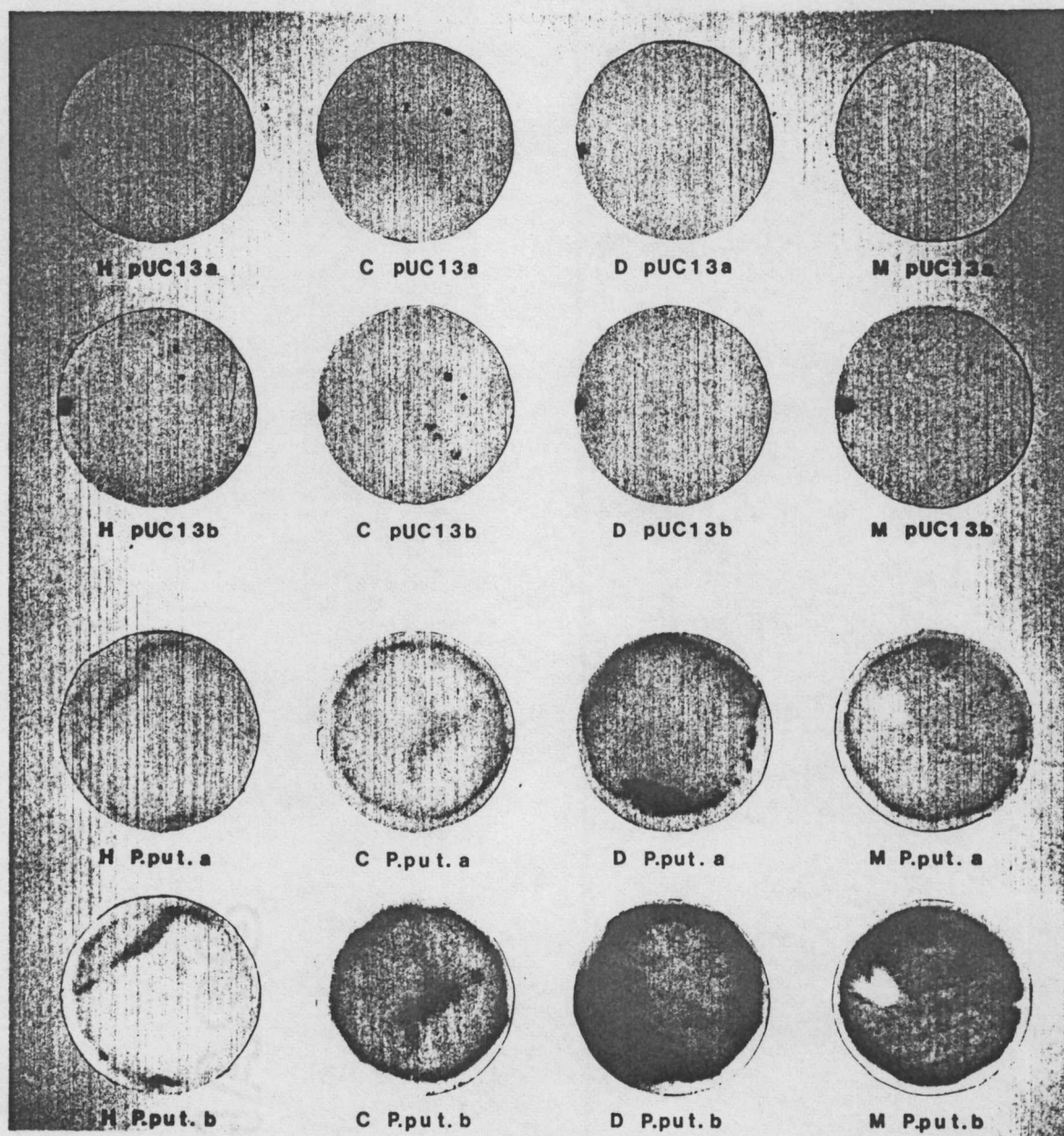


Figure 15 (continued)

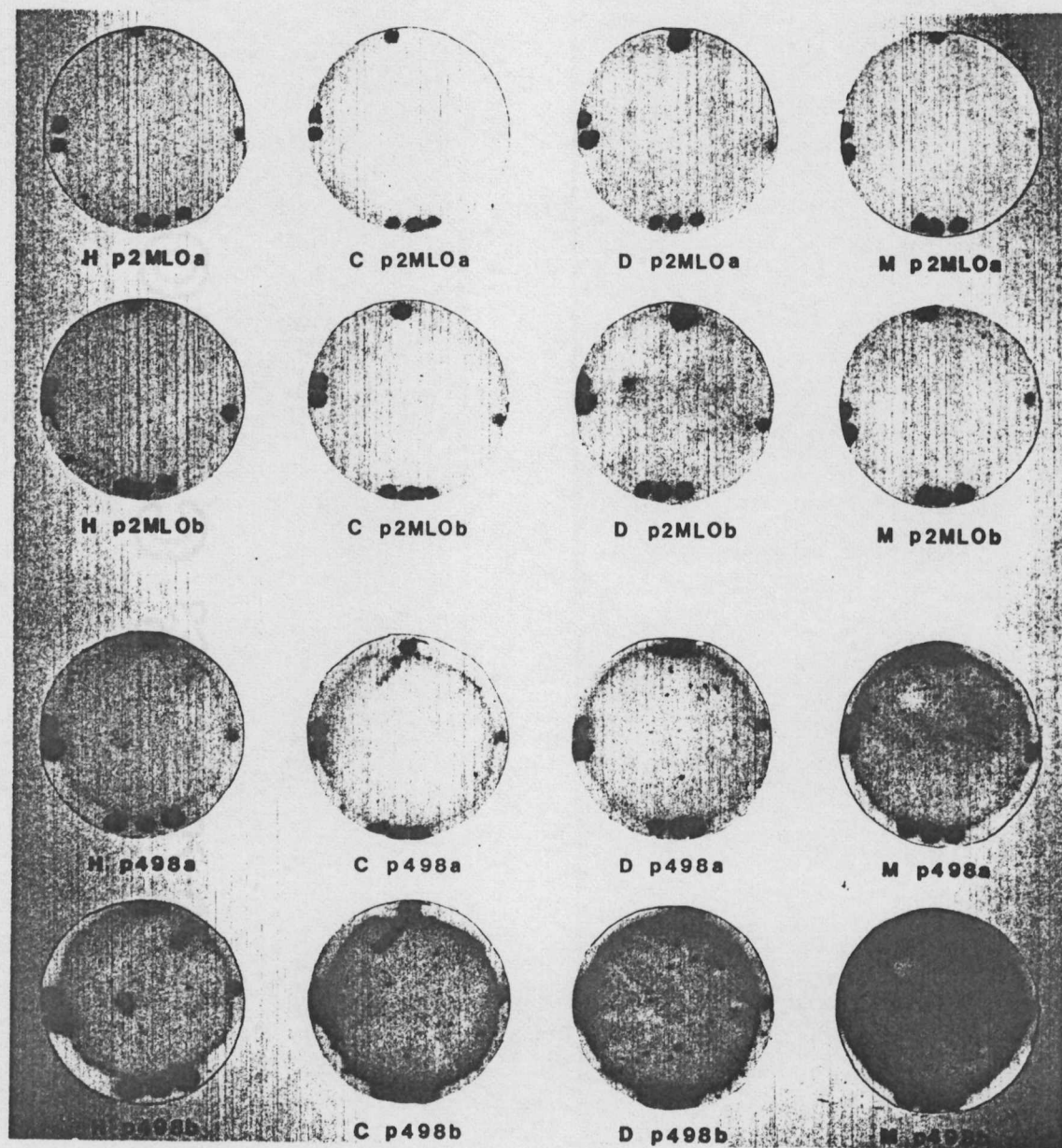


Figure 15 (continued)

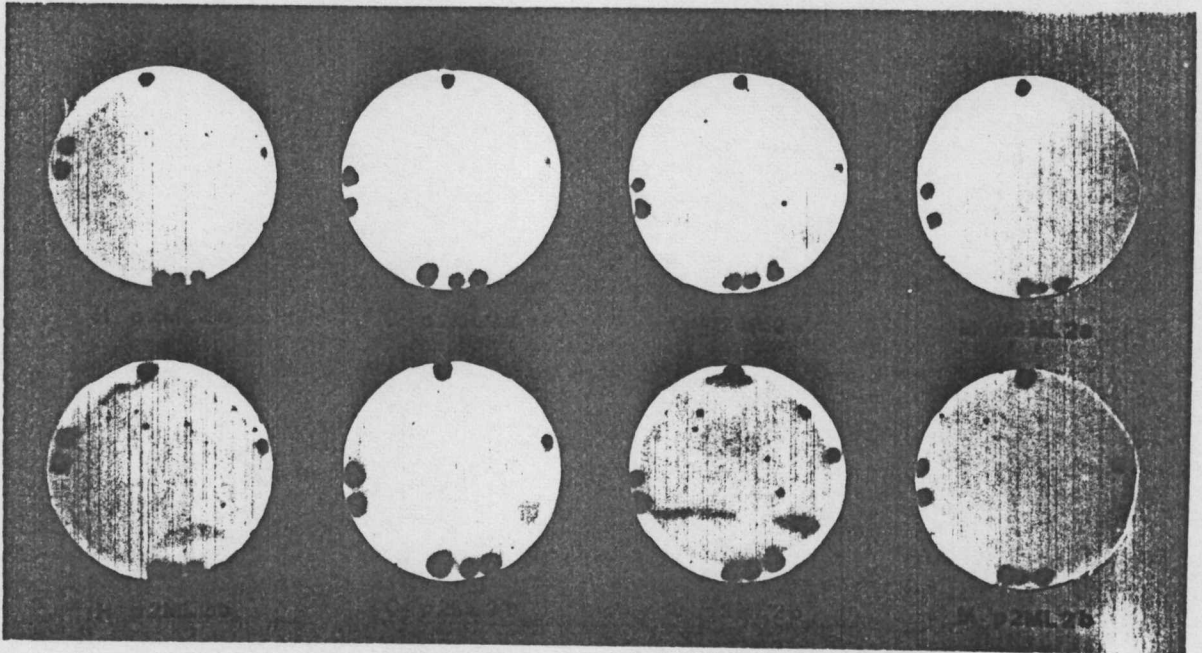


Figure 15 (continued)

Table 15. Comparative specificity of AHS24 chromosomal and AHS24 subclone probes in uninoculated freshwater communities at 65°C hybridization wash.

Sample isolation site	Colony density	Number of signals observed with probes*					
		AHS24	pUC13	p1CA3	p2MLO	p498	p2ML2
Holston River	1.8×10^3	0	6	6	1 ^b	5 ^a	10 ^a
Cherokee Lake	1.2×10^3	0 ^a	6	6	0 ^a	11 ^a	5 ^a
Douglas Lake	2.8×10^3	0 ^a	2	0 ^a	1	13 ^a	7
Microcosm	4.7×10^3	0 ^a	4	2	0	9	2

*Based on 96-hour exp.

^aHigher background observed.

^bAppears to be artifactual.

pUC13 vector. This demonstrates the high sensitivity of both the AHS24 whole genomic probes and subgenomic probes. However, the specificity of the subgenomic probes may be narrowed by removing the AHS24 inserts from the pUC13 plasmid and using these as AHS24 specific probes. This remains to be demonstrated.

6. Detection of AHS24 in an Innoculated Microcosm Community with AHS24 Whole Genomic and Subgenomic Probe p1CA3

In order to demonstrate that AHS24 colonies can be detected quantitatively among a large background of environmental colonies, microcosm communities at a constant cell density of 3×10^2 were inoculated with 1.4×10^3 , 1.4×10^2 , and 1.4×10^1 AHS24 cells immediately before plating. The cells were plated and colonies were allowed to mature for 3 days on 1/10 YEPC agar. The colonies were then transferred to membranes and aliquots AHS24 and pUC13 controls were placed at the periphery of the membrane as previously described. Three sets of membranes corresponding to AHS24 10^{-6} + microcosm 10^{-6} , AHS24 10^{-7} + microcosm 10^{-6} , and AHS24 10^{-8} + microcosm 10^{-6} were each hybridized with the whole genomic AHS24 probe, the p1CA3 subgenomic probe, and the pUC13 control probe and washed at 70°C. The autoradiographs of this experiment are shown in Figure 16 and the results are summarized in Table 16. The hybridization signals observed at 5:1 and 1:2 (AHS24 colonies to microcosm colonies) are too numerous to count, this likely being due to the high densities of AHS24 present on both sets of these membranes (the 5:1 AHS24 to microcosm colonies contained 1.4×10^3 AHS24 colonies, and the 1:2

Figure 16. Representative autoradiographs showing detection of AHS24 in an inoculated microcosm community with AHS24 whole genomic probe and p1CA3 subgenomic probe at 70°C stringency wash. Membranes are labeled M for microcosm community, a-c represent ratios of (a) 5:1 AHS24 to microcosm colonies, (b) 1:2 AHS24 to microcosm colonies, and (c) 1:20 AHS24 to microcosm colonies. Membranes are labeled with respect to probes utilized, AHS24 whole genomic probe, p1CA3 probe and pUC13 (no AHS24 insert) control probe. All autoradiographs were exposed for 44 hours.

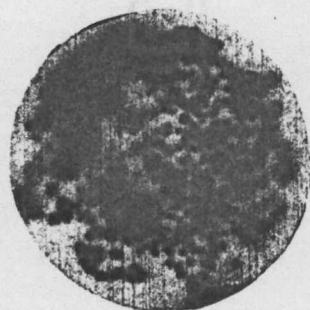
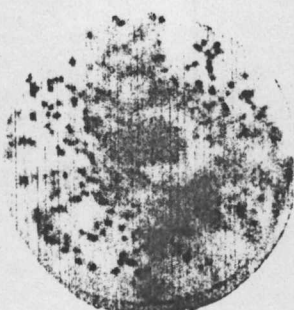
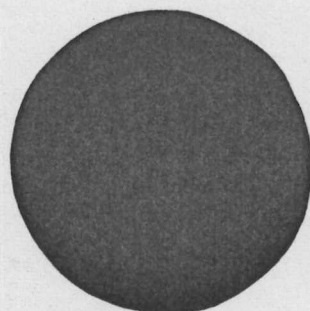
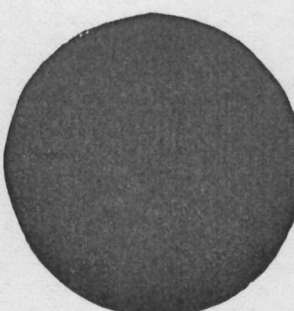
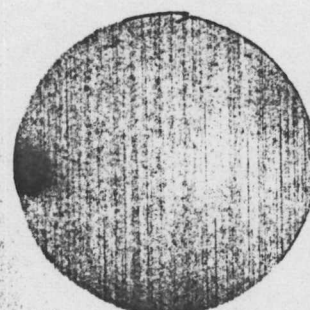
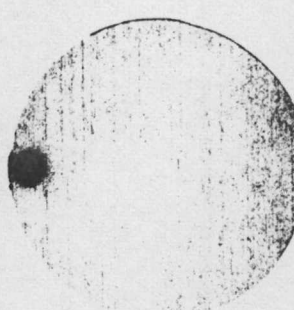
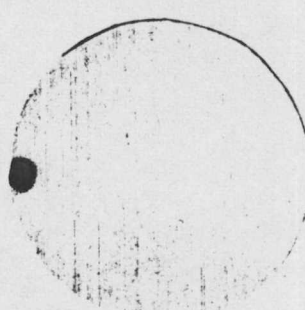
**M AHS24 a****M AHS24 b****M AHS24 c****M p1CA3a****M p1CA3b****M p1CA3c****M pUC13a****M pUC13b****M pUC13c**

Figure 16

Table 16. Detection of AHS24 in an inoculated microcosm community with AHS24 whole genomic probe and AHS24 subgenomic probe at 70°C stringency wash.

Ratio of AHS24 to microcosm colonies	Number of signals/membrane		
	AHS24 Genomic probe	p1CA3 probe	pUC13 probe
AHS24 10^{-6} + microcosm 10^{-6} 5:1	TNC ^a	TNC ^a	0 ^b
AHS24 10^{-7} + microcosm 10^{-6} 1:2	TNC ^{a,c}	TNC ^{a,c}	0
AHS24 10^{-8} + microcosm 10^{-6} 1:20	29	19	0

^aToo numerous to count.

^bHigh background observed.

^cBased upon signal intensity.

membrane contained 1.4×10^2 AHS24 colonies). The final set of membranes at ratios of 1:20 AHS24 colonies to microcosm colonies yields distinct detectable signals when probed with AHS24 whole genomic probes in a countable range. Twenty-nine signals were observed when utilizing the AHS24 whole genomic probe which differs by 107% from the expected 14 signals from AHS24 that would arise from colonies on control plates of AHS24 alone at the 10^{-8} dilution. Nineteen signals were observed when utilizing the p1CA3 subgenomic probe which differs by 36% from the expected 14 signals. This result does not necessarily demonstrate that the p1CA3 subgenomic probe is more specific for AHS24 than the whole genomic AHS24 probe because the number of colonies of AHS24 is low enough to generate large significant differences due to sampling error alone. The experiment does, however, demonstrate the sensitivity of both AHS24 and p1CA3 probes. The differences in signal intensity of AHS24-probed membranes as compared to p1CA3 probed membranes can be explained by the hundred fold increase of specific activity of the p1CA3 probe ($1.3\text{--}1.7 \times 10^8$ cpm/ μg) over the specific activity of the AHS24 whole genomic probe ($2.4\text{--}3.0 \times 10^6$ cpm/ μg). This experiment was repeated again by plating a ratio of 1:195 AHS24 colonies to microcosm colonies. The expected number of colonies of AHS24 to microcosm colonies on a plate was 3.0×10^1 AHS24 colonies to 6.2×10^3 microcosm colonies. These expected numbers were determined by independent quantitative platings of serial dilutions of AHS24 and microcosm samples. The colonies were transferred to membranes and processed as previously described. The AHS24 whole genomic probe and all subgenomic probes p1CA3,

p2ML2, p498, and p2MLO were hybridized to this set of membranes and washed at 65°C. The autoradiograms of this experiment are shown in Figure 17 and the results are summarized in Table 17. In Figure 17, membrane "a" contained only AHS24 colonies as a control. One half of the membrane was probed with pUC13 and a single intense signal due to hybridization of enteric sequences of pUC13 to JM107 are visible. The other half of the membrane was probed with AHS24. Membrane "b" contained only microcosm colonies as a control. One half of the membrane was probed with pUC13 and the other half was probed with AHS24 whole genomic DNA. The results of these controls demonstrate the sensitivity of the AHS24 whole genomic probe for AHS24 with low background due to hybridization with DNA from members of the microcosm community. These results of membrane b demonstrate the slight background due to cross-hybridization of the plasmid pUC13 DNA with DNA from the microcosm colonies. This background could also result from contaminating *E. coli* genomic DNA in the midiplasmid preparation. From the estimated percent difference from the number of signals expected from AHS24 colonies compared to the actual number of strong signals observed, the p498 probe gives the closest value to that expected; however, the background observed on this membrane is high. The next closest value is from that obtained with the p2MLO probe at 40% difference and the background observed with this probe is visibly low under these hybridization conditions. In all cases, except when probe p2ML2 was used, the number of signals observed is greater than the 32 predicted at this dilution of AHS24 in support of

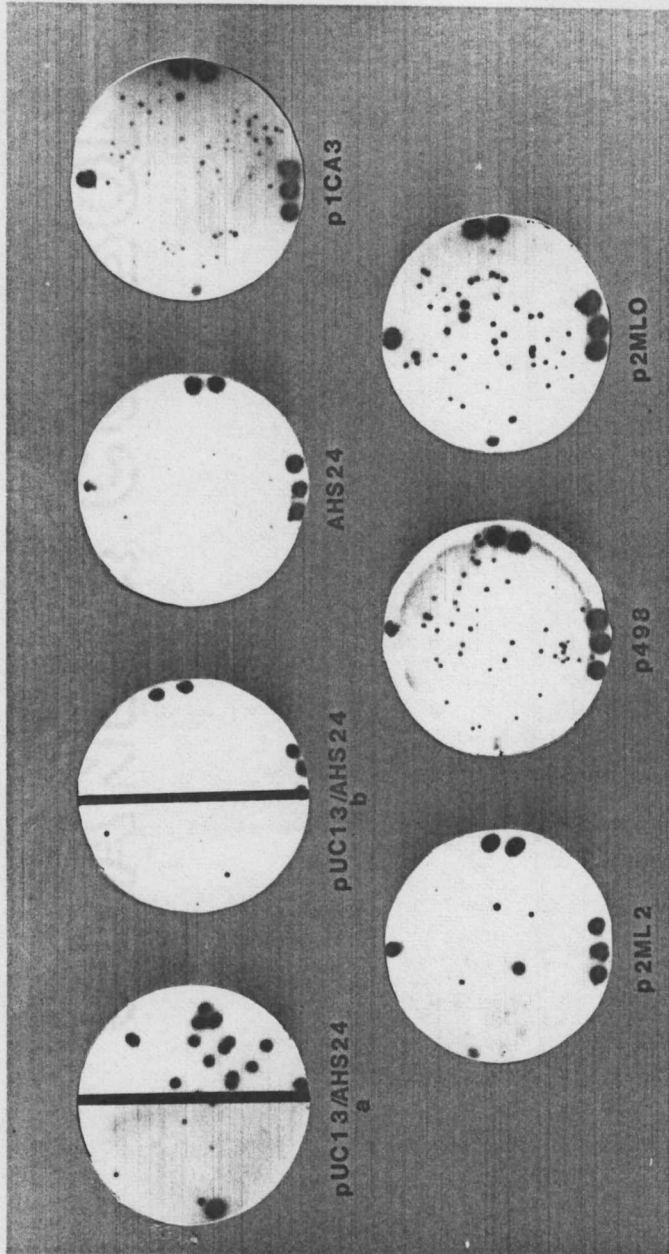


Figure 17. Representative autoradiographs of comparative specificity of AHS24 chromosomal versus AHS24 subgenomic probes in AHS24 inoculated microcosm communities at 65°C stringency wash. Membranes are labeled according to probes used, AHS24 whole genomic DNA, p1CA3, p2ML2, p498, or p2MLO subgenomic probes, and pUC13 (no AHS24 insert) control probe. Membrane (a) serves as a positive control of AHS24 colonies only on the membrane. Membrane (b) serves as a negative control of microcosm colonies only on the membrane.

Table 17. Comparative specificity of AHS24 chromosomal versus AHS24 subgenomic probes in AHS24-dosed microcosm communities at 65° stringency wash.

Probe used	Number of observed signals	% Difference from expected
AHS24	52	62
p1CA3	55	72
p2ML2	4 ^a	88
p498	40 ^a	25
p2MLO	45	41

^aHigh background observed.

the cross-hybridization of probe sequences with homologous sequences in other genomes. The observation of only 4 strong signals on the p2ML2 plate is not explainable, although more distinct weak signals are observable in the background. This set of observations demonstrates the sensitivity of AHS24 whole genomic and subgenomic probes for AHS24 in relatively low ratios of AHS24 to other microorganisms in a microcosm community at high colony densities.

V. DISCUSSION AND CONCLUSIONS

This study has demonstrated that the novel application of DNA-DNA hybridization is of high utility in screening microbial communities for designated subpopulations of microorganisms or specific subpopulations of genes in the environment. In general, the study has shown:

1. Colony hybridization can be utilized to detect the presence of particular microorganisms from environmental samplings with high sensitivity.
2. Subgenomic probes, although highly sensitive, have to be carefully chosen in order to be more discriminatory than whole genomic probes. Only one of four subgenomic probes, p2MLO, appears to demonstrate consistent narrowed specificity.

In reference to the second observation, screening of AHS24 inoculated and uninoculated communities with AHS24 genomic and subgenomic probes has proven the high sensitivity of these probes for their homologous sequences in colony hybridizations; however, it has not necessarily demonstrated high specificity for AHS24 in every case [Tables 14, 15, and 16 (pp. 78, 85, and 89); Figures 14, 15, 16, and 17 (pp. 75, 80, 87, and 92)]. The set of experiments in which AHS24 genomic and subgenomic probes were hybridized to individual groundwater isolates (Tables 10 and 11, pp. 63 and 65; Figures 11 and 12, pp. 62 and 66) clearly illustrates this point. All subgenomic probes tested demonstrate equal sensitivity to AHS24 target DNA comparable to the sensitivity of the AHS24 whole genomic probe. This can be

explained by the short hybridization time used which would cause the more complex genomic probe to hybridize to less of its potential target DNA.

Most subgenomic probes, with the exception of p2MLO and p1CA3, hybridize to a wider range of groundwater isolates than the AHS24 whole genomic probe. This could be explained by the presence of enteric sequences in the pUC13 parent vector of the subgenomic probes or possible contamination of the subgenomic probe preparation with E. coli.

The observation that probe p2MLO demonstrates a more narrow specificity in hybridization to various groundwater strains is also supported by the screening of AHS24 dosed and undosed environmental communities (Tables 15 and 17, pp. 85 and 93; Figures 15 and 17, pp. 80 and 92). The p2MLO subgenomic probe may carry a sequence of a gene or genes that is unique for AHS24 and thus will provide a means by which it may be specifically tracked in the environment. Probe p1CA3 also demonstrates a more narrow specificity, but only under some of the stringency conditions and probe concentrations used in this study (Table 13, p. 77). This observation emphasizes the need for careful design and monitoring of many experimental parameters such as the concentration of probe DNA, the stringencies of the hybridization and wash and also the amount of target DNA on the membrane as is the case in any application of colony hybridization. After the groundwater isolates were initially screened for cross-hybridization (Tables 8 and 9, pp. 55 and 58), it became clear that

there was a need to have internal hybridization controls. To do this, the amount of target DNA in positive control spots was standardized in all subsequent experiments. This was done by simply placing equal aliquots of AHS24 cells directly to the membrane border. Since this internal standardization was done with positive controls only, some variability in the amount of groundwater isolate target DNA and variability in transfer of the DNA to the membrane could possibly obscure the accurate assessment of the sensitivity of the subgenomic probes. Thus, the use of more direct and quantitative methodologies such as the placement of standardized aliquots of cells or DNA from each groundwater isolate on the membrane will likely render more reproducible and reliable estimations of subgenomic probe specificities; however, a point is reached where the development of increasing precision compromises the speed and convenience of this application.

Another parameter to be carefully monitored is the time of exposure series of the autoradiograph for each hybridization. In the hybridization in which 1 μ g of AHS24 whole genomic or p1CA3 subgenomic probe was utilized to probe the individual groundwater isolates (Figure 13, p. 71; Table 12, p. 70) an exposure series of 6 hours, 18 hours, and 96 hours was made. At this particular probe concentration of ten times that previously used, the AHS24 whole genomic probe yields intense hybridization signals not only to AHS24, but to P. putida and P. aeruginosa in the 18 and 96 hour exposures. A less intense signal is observed for P. aeruginosa in the 6 hour exposure. The p1CA3 subgenomic probe also yields an intense hybridization signal in the 96 hour exposure for P. aeruginosa and P. putida target

DNAs; however, its more pronounced discriminatory capability as compared to the discriminatory ability of the AHS24 whole genomic probe is demonstrated in the 6 and 18 hour exposures. The p1CA3 probe clearly yields an intense hybridization signal to P. putida, but only a slightly positive signal to P. aeruginosa. This example of narrowed discriminatory ability only under certain experimental conditions illustrates need for carefully followed exposure series (as well as probe concentration series) in determining subgenomic probe specificities for applications in microbial community screening by colony hybridization.

In the course of this study it was also shown that two Arthrobacter strains, AHS24 and ABS10, appear to share detectable DNA cross-hybridizations with two members of the Pseudomonas fluorescens DNA homology Group I of Pseudomonas, P. putida and P. aeruginosa. Since these hybridization experiments can not be directly compared with those generally used to determine the percent homology between members of different genera or species, only speculation with regard to the significance of this observation can be made. Since significant DNA homologies have never been observed between Gram positive and Gram negative bacteria (39), the observed hybridization between the Gram variable AHS24 and Pseudomonas species is rather puzzling. The mole percent G+C content determined by the buoyant density method for all reference Arthrobacter species are within the range of 62-73 mole percent (31). The mole G+C content for P. putida A is 62.5 ± 0.9 (31). Strains sharing a G+C content within a 10% range could share DNA homologies, but outside of this range, strains are

likely to have very little DNA homology (19). Therefore, it is feasible that the putative Arthrobacter AHS24 could share DNA homology with P. putida, but according to a compilation of 16s RNA analysis by Fox (17), the genus Arthrobacter is believed to be highly divergent from the genus Pseudomonas. There is, however, significant taxonomic variability in both genera as more information concerning the phylogenetic relatedness based on nucleic acid hybridization methods is acquired. An investigation by Stackebrandt (45) revealed that very few Arthrobacter species are closely related based on DNA-DNA homology experiments and that most Arthrobacter species are indeed distantly or insignificantly related to each other based on DNA-DNA hybridization methods. It must be stressed that DNA-DNA hybridizations are generally used only to detect homology between very closely related microorganisms (30) and that very little if any information on the genetic relationships of microorganisms which share homologies of less than 20% can be acquired from this type of analysis (45). Only the more conserved rRNA sequences used in competition experiments with the DNA have been shown to be helpful in the elucidation of taxonomic relationships. Palleroni demonstrated significant heterogeneity in the genus Pseudomonas (40) and recent studies have supported this work. In fact, it is widely accepted now that the genus Pseudomonas as defined today is rather superficial and that the Pseudomonads actually fall into six different phylogenetic groups which are closely to moderately related to various genera of phototrophic and nonphototrophic microorganisms (45).

There are at least three possible hypotheses for this apparent DNA-DNA cross-hybridization between a putative member of the genus Arthrobacter and the genus Pseudomonas:

1. AHS24 should be classified as a Pseudomonad despite its Arthrobacter-like morphology.
2. The apparent cross hybridization is not relevant to taxonomic classification.
3. The cross hybridization is due to sequences common to both Pseudomonas and this particular Arthrobacter isolate.

In spite of the fact that AHS24 undergoes the classic Arthrobacter morphological switch from Gram positive cocci in older cultures to Gram negative rods in exponentially growing cultures and then back to Gram positive cocci in older cultures, it is possible that it should be classified as a Pseudomonad. AHS24 was tentatively classified as an Arthrobacter species based upon this characteristic as well as other more traditional phenotypic and biochemical characteristics. It was not analyzed for its mol G+C content, genome size, or its cell wall peptidoglycan type (a character often used to distinguish different members of coryneform bacteria). AHS24 did not demonstrate cross-hybridizations to known reference Arthrobacter strains, A. globiformis ATCC 8010, A. crystalpoietes ATCC 15481, and Arthrobacter TM1 in hybridizations performed at 65°C with 0.1 µg of labeled genomic AHS24 DNA and washed at 70°C. Only when the hybridizations were performed with 1 µg of AHS24 genomic probe DNA at the lower stringency wash of 65°C did cross-hybridization occur with

A. globiformis, Arthrobacter TM1, but not with A. crystalpoietes. The cross-hybridization of AHS24 to P. putida and P. aeruginosa is evident, however, even at the higher stringency wash of 70°C (Table 13, p. 77) suggesting that AHS24 may be more closely related to a Pseudomonas strain in DNA sequence, yet still exhibits the classic morphology switch of an Arthrobacter species.

Another feasible explanation for these observations is that the lower 65° stringency washes resulted in signals after hybridizations between different genera. From Table 13 (Figures 11, 12, and 13, pp. 62, 66, and 71) it is evident that when AHS24 whole genomic probe is used to probe P. putida and P. aeruginosa, both strains yield equally intense signals in all hybridization conditions ranging from very intense with a 1 µg probe concentration 1X Low 65°C wash to less intense under the 0.1 µg probe concentration 1X Low 65°C and 70°C washes. This could superficially suggest that the stringencies of all of the hybridizations were not sufficient to even allow the AHS24 whole genomic probe to discriminate between AHS24 and the distantly related genus of Pseudomonas (if AHS24 does indeed represent a strain of Arthrobacter). However, in comparing the hybridization of P. putida whole genomic probe with P. putida and P. aeruginosa target DNAs (Table 13, p. 77), it can be noted that under 0.1 µg probe concentration 1X Low 65°C and 70°C hybridization washes that the P. putida probe yields a less intense signal with P. aeruginosa than with P. putida target DNA. This suggests that the hybridizations were at least stringent enough to distinguish between these two members of the

P. fluorescens DNA homology group I. It is important to note, however, that these two species share only 13-16% DNA-DNA homology based on competition experiments (40), but share 80-85% homology based upon ribosomal RNA-DNA competition experiments. This lowered DNA-DNA sequence homology could account for the differential signals observed when P. putida genomic DNA was used to probe itself and P. aeruginosa. E. coli target DNA yields a signal as intense as the signal from P. aeruginosa target DNA under 0.1 µg P. putida probe concentration 1X Low 65°C hybridization conditions (Tables 11 and 13, pp. 65 and 77). E. coli shares only 46-48% ribosomal RNA homology with Pseudomonas species of Group I and no significant DNA homology based upon competition experiments (45), therefore a signal arising from E. coli target DNA when probed with P. putida DNA would not be anticipated at high stringencies. A signal arising from E. coli when probed with P. putida DNA is not seen under the highest stringency wash of 70°C, suggesting that this stringency is sufficiently strong to eliminate hybridizations between less distantly related genera than Arthrobacter and Pseudomonas (17). Under the 1X probe concentration 70°C hybridization conditions with a probe of AHS24 whole genomic DNA, P. putida and P. aeruginosa target DNAs do show a signal, but P. putida whole genomic probe with E. coli JM107 target DNA does not (Table 10, p. 63). So AHS24 seems to have, by these experiments, sequences more homologous to some sequences in the Pseudomonads, more so than any sequence in P. putida is homologous to any sequence

in E. coli. It is not understood why the reciprocal hybridization of P. putida whole genomic DNA to AHS24 target DNA did not yield a signal, but it is important to note that the other putative Arthrobacter species, ABS10, from the same environmental sampling, did yield a signal when probed with P. putida DNA. This positive hybridization was observed independently by Alex Breen in the laboratory of Dr. Gary Sayler at the University of Tennessee, Knoxville. It is apparent from these data that more careful homology studies will have to be performed upon these putative Arthrobacter species in order to determine their taxonomic classification.

Another possible hypothesis for the cross-hybridization observed between the putative Arthrobacter isolates and P. putida and P. aeruginosa is that AHS24 and ABS10 carry common sequences perhaps as mobile elements in their genome such as transposon-like sequences or lysogenized phages. Although no plasmids have ever been isolated from AHS24 or ABS10, in spite of repeated attempts (data not shown), it is possible that these strains may harbor very large plasmids or other genomic sequences that carry catabolic genes similar to those found on a ubiquitous catabolic plasmid such as the TOL plasmid which could explain the observed "lack" of plasmids in these strains, yet account for the DNA-DNA homologies observed. There is a precedent for this plausible explanation in that Arthrobacter TM1 has the ability to degrade 4-chlorobenzoate by the same pathway reported in a Pseudomonas sp. strain CBS 3 (29) and a Norcardia species (28). ABS24 has not been analyzed for catabolic

capacities; however, preliminary evidence has shown ABS10 to utilize styrene as its sole carbon source (Alex Breen, personal communication).

If AHS24, which expresses gram positive characteristics, should be classified as a Pseudomonad, this could be an example of blending the characteristics of two disparate groups. Even if the cross-hybridization is due to some mobile element, this would not alter this conclusion, but would instead explain how it could happen. The question is whether it has closest relatives in the acceptable taxonomic sense which demands a single line of descent, or whether its closest relatives cannot be described with a single line of descent. This latter alternative would fit AHS24 if it indeed has genes conferring gram positive morphology and genes normally occurring uniquely in the Pseudomonads. To confirm this, of course, the genes in question would have to be isolated and studied.

Although interesting, the taxonomic questions that arise from these observations are still somewhat beyond the focus of the development of new ways to detach certain genes or subpopulations of microorganisms in the environment. This work, using AHS24 as a representative groundwater microorganism, has demonstrated the promising utility of colony hybridization as a means for tracking genetically engineered microorganisms in the future. There are many advantages to this technology already:

1. The technique is efficient and easy, many colonies can be screened per filter.

2. The technique allows for detection within one week. This time frame varies with the growth rate of the isolates and depends on the availability of probes.
3. Detection of homology between probe and target DNA does not rely on phenotypic expression of the isolate or abundance of growth on the filter. If probes are sufficiently labeled to a high specific activity (10^8 CPM/UG), signals can be detected by autoradiography amidst 1-12 μ g of target DNA, assuming there are 10^9 cells/1 mm³ colony and an average genome size of $1-8 \times 10^9$ daltons.
4. The technique can be used as a preliminary or initial screening procedure to detect the presence or proliferation of certain genes or microorganisms in freshly isolated samplings of groundwater or other freshwater communities.

Despite these obvious benefits, environmental application of colony hybridization still has a few uncertain parameters to be more carefully controlled in order to increase the sensitivity, specificity, and reproducibility of the technique. One of the potential problems with this application is nonuniformity in the amount of target DNA between groundwater isolates grown or transferred onto the same filter. This problem can easily be corrected when the number of isolates per filter is controlled. Rather than growing or transferring colonies onto the filter, it would be more reliable to place the same quantity of DNA from each isolate directly onto the filter. This would allow for a more accurate competition between target DNAs for the probe. As stated

before, however, this extra step of DNA isolation from each isolate clearly hinders the efficiency of the technique, especially when optimum conditions for DNA isolation from environmental isolates is not known. DNA isolation is simply not practical especially when screening a whole representative population of microorganisms from a freshwater or groundwater community on one filter. Another occasional uncertainty with the technology is that a few isolates can grow or can be transferred in excess onto the filter such that after lysis and fixing, some aggregates of cells or cellular debris containing DNA can wash off the filter and compete in solution for probe DNA. This may or may not be significant depending upon the amount of labeled probe DNA present in the hybridization. There is also the potential for false positive signals arising from residual proteins left from the lysis step on the filter which nonspecifically bind probe DNA. It may be possible to reduce these nonspecific binding proteins by including a SDS wash (0.1%) after the DNA has been fixed by baking or UV light to the filter. Another way to exclude false positive signals from the final results is to include a control of a replica filter which has been probed with a known nonhomologous DNA sequence such as hemoglobin DNA. Those signals arising from nonspecific binding of probe DNA could thus be more readily determined.

One other problem encountered specifically in this study was the presence of parent vector sequences and possible E. coli chromosomal DNA contamination of all subgenomic probes. Although appropriate "vector-minus-insert" controls were performed, some of the subgenomic

probes may have had more narrow ranges of specificity as compared to AHS24 whole genomic probe if AHS24 had been removed from the parent vector and used as a uniformly labeled probe at a very high specific activity (10^9 CPM/ μ g).

The AHS24 inserts used to make all subgenomic probes are currently being excised from the parent pUC13 vector to determine the specificity of these probes without enteric sequences present in the hope that indeed the specificity of these probes will be narrowed further. The development of the new AHS24 probes represents an ongoing effort to further refine the precision by which subpopulations of specific microorganisms or subsets of genes can be monitored in the environment through colony hybridization. In this study AHS24 has served as the model groundwater microorganism for sensitive and specific detection in screening experiments with individual groundwater isolates and in screening experiments with whole environmental communities of microorganisms. The technology of specific subgenomic probe development and colony hybridization clearly has further promise for application to other potentially important natural isolates from groundwater, freshwater, or soil communities of interest.

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APPENDICES

APPENDIX A

SAMPLE CALCULATIONS OF INDEPENDENT TRANSFORMANTS
AND ESTIMATED GENOME EQUIVALENTS FOR
AHS24 LIBRARIES

Assumption 1: The putative Arthrobacter AHS24 genome is approx.
5,000 kb.

Assumption 2: The average length of an insert is about 1 kb.

a. Calculations for 2ML2 library

After transformation, 10 μ l of the 22.0 ml of cells were plated on ampicillin selection media; 17 transformants were scored.

$$\frac{22.0 \text{ ml}}{0.010 \text{ ml}} \times 17 \text{ transformants} = 3.7 \times 10^4 \text{ independent transformants in the total volume of cells}$$

Genome equivalent = $\frac{\text{fraction of plasmids containing inserts} \times \text{number of independent transformants} \times \text{average length of insert}}{\text{Arthrobacter genome size}}$

$$\text{Genome equivalent} = \frac{5}{16} \times 3.7 \times 10^4 \times \frac{1 \text{ kb}}{5,000 \text{ kb}}$$

$$\text{Genome equivalent} = 2.3$$

b. Calculations for ICA library

After transformation, 10 μ l of cells were plated on ampicillin selection media; 5 transformants were scored.

$$\frac{1.0 \text{ ml}}{0.010 \text{ ml}} \times 5 \text{ transformants} = 5.0 \times 10^2 \text{ independent transformants in the total volume of cells}$$

$$\text{Genome equivalent} = 9/11 \times 5.0 \times 10^2 \times \frac{1 \text{ kb}}{5,000 \text{ kb}}$$

$$\text{Genome equivalent} = 0.08$$

c. Calculations for 49CA library

After transformation, 10 μ l of the 1.45 ml of cells were plated on ampicillin selection media; 7 transformants were scored.

$$\frac{1.45 \text{ ml}}{0.010 \text{ ml}} \times 7 \text{ transformants} = 1.0 \times 10^3 \text{ independent transformants in the total volume of cells}$$

$$\text{Genome equivalent} = 8/12 \times 1.0 \times 10^3 \times \frac{1 \text{ kb}}{5,000 \text{ kb}}$$

$$\text{Genome equivalent} = 0.13$$

d. Calculations for 1H2 library

After transformation, 10 μ l of the 1.0 ml of cells were plated on ampicillin selection media; 80 transformants were scored.

$$\frac{1.0 \text{ ml}}{0.010 \text{ ml}} \times 80 \text{ transformants} = 8.0 \times 10^3 \text{ independent transformants in the total volume of cells}$$

$$\text{Genome equivalent} = 5/9 \times 1.0 \times 10^3 \times \frac{1 \text{ kb}}{5,000 \text{ kb}}$$

$$\text{Genome equivalent} = 0.89$$

APPENDIX B

TABLE

Table B-1. Record of specific activity and total amount of probe DNA utilized for all hybridization experiments.

Figure number	Autoradiograph label	Specific activity of (CPM/ μ g DNA)	Probe total counts in hybridization bag (CPM)	Total amount of probe DNA added (μ g)
7	AHS24	$3.5-5.4 \times 10^7$	$4.4-6.8 \times 10^6$	0.13
7	pUC13	$3.5-5.4 \times 10^7$	$4.4-6.8 \times 10^6$	0.13
7	^{49}Ca a	$3.5-5.5 \times 10^7$	$4.4-6.8 \times 10^6$	0.13
7	2MLa	$3.5-5.4 \times 10^7$	$4.4-6.8 \times 10^6$	0.13
7	1H2*	$2.3-6.5 \times 10^7$	$1.1-3.3 \times 10^7$	0.50
7	1CA*	$2.3-6.5 \times 10^7$	$1.1-3.3 \times 10^7$	0.50
7	$^{49}\text{CAB}^*$	$2.3-6.5 \times 10^7$	$1.1-3.3 \times 10^7$	0.50
7	2MLb*	$2.3-6.5 \times 10^7$	$1.1-3.3 \times 10^7$	0.50
9	AHS24 1	4.3×10^7	5.4×10^6	0.13
9	AHS24 2	4.3×10^7	5.4×10^6	0.13
9	AHS24 3	4.3×10^7	5.4×10^6	0.13
9	AHS24 4	4.3×10^7	5.4×10^6	0.13
9	P. put. 1	3.7×10^7	4.6×10^6	0.13
9	P. put. 2	3.7×10^7	4.6×10^6	0.13
9	P. put. 3	3.7×10^7	4.6×10^6	0.13
9	P. put. 4	3.7×10^7	4.6×10^6	0.13
9	AHS23 1	4.6×10^7	5.8×10^6	0.13
9	AHS23 2	4.6×10^7	5.8×10^6	0.13
9	AHS23 3	4.6×10^7	5.8×10^6	0.13
9	AHS24 4	4.6×10^7	5.8×10^6	0.13
10	AHS24e,f ⁺	4.2×10^7	1.1×10^7	0.25
10	AHS24g,h ⁺	4.2×10^7	1.1×10^7	0.25
10	ABS10e,f ⁺	-**	4.4×10^7	-**
10	ABS10g,h ⁺	-**	4.4×10^7	-**
10	P. put. e,f ⁺	1.8×10^7	4.5×10^6	0.25
10	P. put. g,h ⁺	1.8×10^7	4.5×10^6	0.25
10	AHS23e,f ⁺	2.1×10^7	5.3×10^6	0.25
10	AHS23g,h ⁺	2.1×10^7	5.3×10^6	0.25
11	CW1	3.9×10^7	3.9×10^6	0.10
11	CW2	5.2×10^7	5.2×10^6	0.10
11	CW3	9.0×10^7	9.0×10^6	0.10
11	CW4	1.7×10^7	1.7×10^6	0.10
11	CW5	3.9×10^7	3.9×10^6	0.10
11	CW6	5.1×10^7	5.1×10^6	0.10
12	AHS24a,b*	1.9×10^7	1.9×10^6	0.10
12	p1CA3a,b*	7.3×10^7	7.3×10^6	0.10
12	pUC13a,b*	2.6×10^7	2.6×10^6	0.10
12	P. put. a,b*	1.5×10^7	1.5×10^6	0.10
12	p2MLOa,b*	2.6×10^7	2.6×10^6	0.10
12	p498a,b*	3.1×10^7	3.1×10^6	0.10
12	p2ML2a,b*	2.9×10^7	2.9×10^6	0.10
13	AHS24 a,b,c ⁺⁺	5.0×10^7	5.0×10^7	1.0
13	p1CA3 a,b,c ⁺⁺	5.3×10^7	5.3×10^7	1.0

Table B-1 (continued).

Figure number	Autoradiograph label	Specific activity of (CPM/ μ g DNA)	Probe total counts in hybridization bag (CPM)	Total amount of probe DNA added (μ g)
14	A1	3.9×10^7	3.9×10^6	0.10
14	B1	3.9×10^7	3.9×10^6	0.10
14	C1	3.9×10^7	3.9×10^6	0.10
14	D1	3.9×10^7	3.9×10^6	0.10
14	A2	5.2×10^7	5.2×10^6	0.10
14	B2	5.2×10^7	5.2×10^6	0.10
14	C2	5.2×10^7	5.2×10^6	0.10
14	D2	5.2×10^7	5.2×10^6	0.10
14	A3	9.0×10^7	9.0×10^6	0.10
14	B3	9.0×10^7	9.0×10^6	0.10
14	C3	9.0×10^7	9.0×10^6	0.10
14	D3	9.0×10^7	9.0×10^6	0.10
15	H AHS24a,b	1.9×10^7	1.9×10^6	0.10
15	C AHS24a,b	1.9×10^7	1.9×10^6	0.10
15	D AHS24a,b	1.9×10^7	1.9×10^6	0.10
15	M AHS24a,b	1.9×10^7	1.9×10^6	0.10
15	H p1CA3a,b	7.3×10^7	7.3×10^6	0.10
15	C p1CA3a,b	7.3×10^7	7.3×10^6	0.10
15	D p1CA3a,b	7.3×10^7	7.3×10^6	0.10
15	M p1CA3a,b	7.3×10^7	7.3×10^6	0.10
15	H pUC13a,b	2.6×10^7	2.6×10^6	0.10
15	C pUC13a,b	2.6×10^7	2.6×10^6	0.10
15	D pUC13a,b	2.6×10^7	2.6×10^6	0.10
15	M pUC13a,b	2.6×10^7	2.6×10^6	0.10
15	H P. put. a,b	1.5×10^7	1.5×10^6	0.10
15	C P. put. a,b	1.5×10^7	1.5×10^6	0.10
15	D P. put. a,b	1.5×10^7	1.5×10^6	0.10
15	M P. put. a,b	1.5×10^7	1.5×10^6	0.10
15	H p2MLOa,b	2.6×10^7	2.6×10^6	0.10
15	C p2MLOa,b	2.6×10^7	2.6×10^6	0.10
15	D p2MLOa,b	2.6×10^7	2.6×10^6	0.10
15	M p2MLOa,b	2.6×10^7	2.6×10^6	0.10
15	H p498a,b	3.1×10^7	7.3×10^6	0.10
15	C p498a,b	3.1×10^7	7.3×10^6	0.10
15	D p498a,b	3.1×10^7	7.3×10^6	0.10
15	M p498a,b	3.1×10^7	7.3×10^6	0.10
15	H p2ML2a,b	2.9×10^7	2.9×10^6	0.10
15	C p2ML2a,b	2.9×10^7	2.9×10^6	0.10
15	D p2ML2a,b	2.9×10^7	2.9×10^6	0.10
15	M p2ML2a,b	2.9×10^7	2.9×10^6	0.10
16	M AHS24a	$2.4-3.0 \times 10^6$	$2.4-3.0 \times 10^5$	0.10
16	M AHS24b	$2.4-3.0 \times 10^6$	$2.4-3.0 \times 10^5$	0.10
16	M AHS24c	$2.4-3.0 \times 10^6$	$2.4-3.0 \times 10^5$	0.10
16	M p1CA3a	$1.3-1.7 \times 10^8$	$1.3-1.7 \times 10^6$	0.01
16	M p1CA3b	$1.3-1.7 \times 10^8$	$1.3-1.7 \times 10^6$	0.01
16	M p1CA3c	$1.3-1.7 \times 10^8$	$1.3-1.7 \times 10^5$	0.01
16	M pUC13a	$2.2-8.2 \times 10^7$	$2.2-8.2 \times 10^5$	0.01
16	M pUC13b	$2.2-8.2 \times 10^7$	$2.2-8.2 \times 10^5$	0.01
16	M pUC13c	$2.2-8.2 \times 10^7$	$2.2-8.2 \times 10^5$	0.01

Table B-1 (continued).

Figure number	Autoradiograph label	Specific activity of (CPM/ μ g DNA)	Probe total counts in hybridization bag (CPM)	Total amount of probe DNA added (μ g)
17	pUC13a ¹	2.6×10^7	2.6×10^6	0.10
17	AHS24a ₁	1.9×10^7	1.9×10^6	0.10
17	pUC13b ₂	2.6×10^7	2.6×10^6	0.10
17	AHS24b ²	1.9×10^7	1.9×10^6	0.10
17	AHS24	1.9×10^7	1.9×10^6	0.10
17	p1CA3	7.3×10^7	7.3×10^6	0.10
17	p2ML2	2.9×10^7	2.9×10^6	0.10
17	p498	3.1×10^7	3.1×10^6	0.10
17	p2MLO	3.1×10^7	3.1×10^6	0.10

*2 filters/bag.

**Sample was from crude lysis preparation and DNA conc. was not determined.

¹Indicates same filters, different time of exposure.

²Indicates same filters, different time of exposure.

APPENDIX C

LIST OF ABBREVIATIONS

p	a notation for plasmid
kb	one kilobase
μ l	microliter
μ g	microgram

VITA

Carole Lynne Harris was born in Elwood, Indiana, on February 26, 1961. She attended Shawnee Elementary, Middle, and High School in Lima, Ohio. She graduated in June of 1979 from Findlay High School in Findlay, Ohio. The following September she entered Texas Lutheran College, majoring in biology. She finished her junior and senior years at the University of Tennessee, Knoxville. In June of 1983, she received her Bachelor of Arts degree in biology and chemistry with a minor in physical sciences. In the Fall of 1983 she accepted a teaching assistantship in the Department of Microbiology at the University of Tennessee, Knoxville, and began study toward a Master's degree. She entered the doctoral program in the Department of Biological Sciences at the University of Pittsburgh in September of 1985.

Carole Harris is a member of Alpha Epsilon Delta and The Golden Key Honor Society. She is a Marathon Oil Scholar and twice the recipient of the Hugo Leissner Scholarship.