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THE USE OF 16S rRNA GENE CLONES
TO CHARACTERIZE ENRICHED ANAEROBIC COMMUNITIES
WHOSE MEMBERS ARE DIFFICULT TO ISOLATE

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THE USE OF 16S rRNA GENE CLONES TO CHARACTERIZE ENRICHED ANAEROBIC COMMUNITIES WHOSE MEMBERS ARE DIFFICULT TO ISOLATE

By

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ABSTRACT

THE USE OF 16S rRNA GENE CLONES TO CHARACTERIZE ENRICHED ANAEROBIC COMMUNITIES WHOSE MEMBERS ARE DIFFICULT TO ISOLATE

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Enrichment cultures are often used to recover microorganisms that degrade recalcitrant substrates but they may be difficult to isolate and identify. Three anaerobic enrichment cultures were studied: one grew anaerobically on toluene with sulfate as its electron acceptor and the other two grew anaerobically on 3-chlorobenzoate. I determined the structure and composition of these communities by characterizing the 16S rRNA sequences of some of the members. DNA extracted from the enriched cells was used to create 16S rRNA gene libraries following PCR. Clones were sorted into putative phylotypes based on restriction patterns and partially sequenced for further analysis. The major phylotype from the toluene enrichment was identical to a novel toluene-degrading isolate from that enrichment. The 3-chlorobenzoate degrading isolates were not found among the 115 clones obtained from these two enrichments. The dominant 16S rRNA clones from the Florida mangrove swamp and Ohio sewage sludge digester 3-chlorobenzoate enrichments gave identical phylotype patterns but the partial sequence differed by 8%. These results provide evidence that microbial composition determined solely by culturable isolates may not describe the community.

This is dedicated to,
my parents, who have helped make me the person I am today,
my husband, who loves the person I am, and
my son, who continues the circle.

ACKNOWLEDGMENTS

I want to recognize Karl Weber and Dallas Goss for nurturing my love of the biological sciences and providing me with the academic foundation as a young highschool student that was needed to excel in this field. I owe a great deal to James Tiedje for giving me the opportunity to pursue this goal, for his guidance, and for his time in helping me organize my research project . I appreciate the advice and time from my other committee members Tom Schmidt and Pat Oriel. I would like to thank the Microbiology department for accepting me as a Master's student. I also wish to thank Jim Cole for his personal and professional support throughout my stay at the Center for Microbial Ecology. His guidance helped bring me from a dishwasher to a graduate student. I have made many friends during my time at the Center who have given me their guidance and support, especially, Rob and Joanne Sanford, Marcos Fries and Tamara Tsoi. I want to thank Julie Foxworthy for her help with the figures and photographs for my thesis and all my posters and David Foxworthy for unlimited use of his computer and printer.

My 'career' as a graduate student was not one without trial and tribulation. Between research dilemas, class conflicts, and a complicated pregnancy there were many times I wondered if I would ever make it. I owe many "thank you's" to my husband Mark for his encouragement, patience and understanding. My parents, Darwin and Gerri Foxworthy, have instilled in me a drive to succeed and an appreciation for life. I give credit to my father for my love of science and the confidence to attempt my personal goals. Finally, Kyle Albert, thank you for giving me more joy and happiness than I thought possible. You have shown me what is truly important in life.

TABLE OF CONTENTS

LIST OF TABLES

Chapter I

Chapter II

LIST OF FIGURES

Chapter I

Chapter II

Chapter I

THE PARTIAL CHARACTERIZATION OF UNCULTURED COMMUNITY MEMBERS FROM 3-CHLOROBENZOATE MINERALIZING BACTERIAL ENRICHMENT CULTURES

Many natural anaerobic environments have been shown to dechlorinate a variety of halogenated compounds, but isolating organisms enriched from these habitats has proven difficult. It has been estimated that less than 10% of organisms from natural environments can be isolated using traditional cultivation techniques (2), and the percentage may even be less for fastidious anaerobes. Compounding this observation is the experience that few of the organisms from these dechlorinating enrichments resume their activity upon isolation. This can make the description of the composition and function of these communities nearly impossible.

The complete mineralization of recalcitrant substrates in anaerobic environments is completed by multi-step pathways with each step carried out by individual organisms (4). Shelton and Tiedje (16) isolated seven organisms from a 3-chlorobenzoate dechlorinating enrichment. These included a dechlorinating bacterium later identified as *Desulfomonile tiedjei* strain DCB-1, a benzoate-oxidizing bacterium, two hydrogen consuming methanogens, two butyrate-oxidizing bacteria and a sulfate-reducing bacterium. Isolate DCB-1 was found to dechlorinate 3-chlorobenzoate to benzoate. The benzoate-oxidizer, which could only be grown syntrophically with the hydrogen-consuming methane-producer, was found to ferment benzoate with end products of acetate and CH_4. The isolation of these organisms from the enrichment, the characterization of the reactions they carried out (16) and the reconstruction of the consortium activity using three key members (4) provides evidence that mutualistic relationships are necessary in the anaerobic

mineralization of halogenated aromatic compounds. These isolations and reconstruction studies, however, took several years to achieve.

Given the difficulty and inadequacy of isolation as a means of characterizing the composition of such enriched communities, other methods have been explored. Using molecular techniques based upon phylogenetic analysis of 16S rRNA, one can pursue a culture independent approach to reveal further information on organisms that comprise these food chains. Several methods have been explored for analysis of communities using 16S rRNA, including cloning DNA directly and then screening for rRNA encoding genes (10), analysis of cloned 16S rRNA genes from amplification by the polymerase chain reaction (14) and use of reverse transcriptase to obtain 16S rRNA information from isolated ribosomes (19).

The composition of two enrichment cultures capable of reductive dehalogenation of 3-chlorobenzoate was examined using such an isolation independent approach. These enrichments were from two very different inocula, sewage sludge and mangrove sediments. The 16S rRNA genes were amplified from total enrichment DNA and cloned into an *Escherichia coli* vector. The individual 16S rRNA genes were then amplified and digested with *Hin*P1I and *Hae*III and screened using high resolution acrylamide gels to identify putative phylogenetic types or "phylotypes" (13). The phylotype patterns were then further examined by sequence analysis. The dominant phylotypes of both enrichment cultures were found to be members of the delta *Proteobacteria*, but neither phylotype matched the dechlorinating organism isolated from either of the enrichment cultures. This result provides evidence that the composition of successful enrichments are not always

revealed by isolation and raises questions about the functional role of these apparently dominant members.

MATERIALS AND METHODS

Establishment of 3-chlorobenzoate enrichment cultures Sewage sludge from a municipal anaerobic digester in Columbus, Ohio was collected by T. Linkfield in 1986. Estuary sediment from a mangrove swamp on Big Pine Key in Florida was collected in 1987 by T. O. Stevens. Enrichments from these sources were maintained in our laboratory by anaerobic growth on 3-chlorobenzoate since those times. The mangrove enrichment contained marine salts, initially including sulfate but this was eliminated in later years. At the start of the experiments reported here, inocula were transferred into 150 ml serum bottles with 50 ml of enrichment medium. The sewage sludge enrichment medium consisted of RAMM medium (15), 1 mM 3-chlorobenzoate and 1 mg/L resazurin. It was prepared by boiling under N_2-CO_2 (90:10) and adjusting the pH to 7.0 by varying the CO_2 concentration of the headspace. The medium was then dispensed into N_2-CO_2 flushed serum bottles and sterilized by autoclaving. After sterilization the medium was amended with the anaerobic vitamin mix of Wolin et. al. (21). The above medium with the addition of yeast extract 0.1 g/l and a marine salt mixture (NaCl, 12.9 g/l; $MgCl_2$,, 5.3 g/l; KCl, 1.1 g/l and $CaCl_2$,, 0.74 g/l) was used for the mangrove sediment enrichment. Both cultures were monitored using HPLC analysis at 230 nm for the loss of 3-chlorobenzoate and accumulation of benzoate. When the 3-chlorobenzoate was consumed, 1 mM of 3-chlorobenzoate was added from a sterile anaerobic stock solution. Cultures were allowed to mineralize approximately 10 mM 3-chlorobenzoate before a 10% transfer was

performed. At least six serial transfers were performed on both enrichment cultures before subsequent analysis.

Isolation of enrichment culture DNA After addition of HEPES buffer (1 mM final concentration, pH 7.0] to prevent the pH of the culture from becoming too high, cells from enrichment cultures were harvested by centrifugation. Cells were washed with phosphate buffer, pH 7.0, and frozen (-70°C) until use. Two separate DNA isolations were performed from each frozen enrichment sample at an interval of 5 months. Cells were resuspended in 400 µl TEN (Tris, 50 mM pH 8.0; EDTA, 150 mM; and NaCl, 100 mM) and heated to 72°C for 1 h. Cells were lysed using a modified enzymatic method shown to be effective with diverse bacteria (17). Subtilisin (5 mg) was added and incubated 2 h at 37°C followed by Lysozyme (20 mg) at 50°C for 4 h. Protease (1.5 mg) and 20% SDS (50 µl) were then added and incubated at 37°C for 2 h. Cell debris was pelleted using an Eppendorf microfuge (Brinkman Instruments, Inc., Westbury, NY) at 14,000 x g. The supernatant was extracted 3 to 5 times with phenol:chloroform:isoamyl alcohol (50:49:1) to remove protein and polysaccharide debris. Genomic DNA was precipitated on ice for several hours by the addition of 2 volumes of 100% ethanol and 0.2 volume of 10 M ammonium acetate and pelleted at 14,000 x g for 10 min. The pellet was then washed with cold 70% ethanol, dried and resuspended in 50 to 100 µl H_2O.

Southern hybridization. DNA samples isolated by the above method were quantified by gel electrophoresis using a 1.0% agarose gel stained with ethidium bromide. Two samples from successive transfers of the mangrove sediment enrichment (Mangrove A and B) were isolated. The gel was illuminated and the amount of DNA was extrapolated

using 1 μg of a *Hind*III and *Eco*RI cut λ DNA standard. Genomic DNA (1μg) was digested with *Ban*I in a total volume of 50 μl and incubated at 37°C for 1 h. The DNA was then precipitated on ice for 2 h with 0.2 volumes of 10 M ammonium acetate and 2 volumes of ethanol and resuspended in 15 μl Tris EDTA pH 7.5 (10 mM:1 mM). The DNA content of the samples was then requantified using a Hoeffer TKO 100 flourimeter, a TKO 130 capillary cuvette and Hoescht 33258 dye according to the manufacturer's instructions (Hoeffer Scientific Instruments, San Francisco, CA). Equal amounts of each DNA were then run on a 1% agarose gel. The DNA in the gel was then transferred to a Zeta Probe membrane (Biolab Laboratories, Richmond, CA) by capillary action. The membrane was hybridized using the manufacturer's suggestions with a probe we designed to be specific for a large group of the delta sulfate-reducing *Proteobacteria*, which includes the known 3-chlorobenzoate degrader *D. tiedjei* and does not detect the *Desulfovibrio* branch (388GACGCAGCAACGCCG402).

PCR amplification of 16S rRNA genes. The 16S rRNA genes were amplified from the enrichment DNA using oligonucleotide primers which anneal to conserved regions near the 3' and 5' ends of the eubacterial 16S gene. The primers (5'AGAGTTTGATCCTGGCTCAG3' and 5'AAGGAGGTGATCCAGCC3') were modified from the primers FD1 and RD1 of Weisburg et. al. (18) by removing linker sequences present in FD1 and RD1. The 30 μl PCR reaction mixture contained: ca. 0.1, 0.01 or 0.001 μg of enrichment DNA, 1x *taq* DNA polymerase buffer, 1.5 mM Mg Cl_2, 0.2 mM dATP, dTTP, dGTP and dCTP , 0.1 μM of each primer and 0.75 u *taq* polymerase (Promega, Madison, WI). The reactions were amplified in a GeneAmp PCR

system 9600 thermocycler (Perkin Elmer, Norwalk, CT) with a program consisting of an initial denaturation at 92°C for 2 min 10 s, 30 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 2 min 10 s, followed by a final extension at 72°C for 6 min 10 s. The 16S rRNA gene products were then purified by gel electrophoresis using a 1.5% agarose gel. The bands were visualized using 1μg/ml ethidium bromide and then excised and purified using GeneClean purification resin according to the manufacturer's directions (Bio 101, La Jolla, CA). The amount of final product was quantified using a 1.2% agarose gel with a *Hind*III cut λ DNA ladder.

Construction of 16S rRNA gene library. The purified enrichment PCR products were cloned in the vector pCRII, using a TA Cloning kit according to the manufacturer's directions (Invitrogen, San Diego, CA). Competent cells were prepared by inoculating 200 ml of Luria broth with 5 ml of a 15 ml overnight culture and incubating at 37°C. The cells were grown to a density of 0.4 to 0.6 OD_{600} and placed on ice. Cells were then pelleted in plastic centrifuge tubes for 10 min at 3,000 x g and washed three times with 15 to 20 ml cold H_20 and one time with 15 to 20 ml cold 15% glycerol. Cell pellets were then transferred to conical tubes, concentrated by centrifugation as above and resuspended in 1 volume of 15% glycerol (200-300μl). Aliquots of 40 μl were distributed into 1.5 ml Eppendorf tubes for electroporation and the remaining solution was stored at -70°C. Portions of the ligation reaction (1μl) were added to an aliquot of competent cells and set on ice for 5 min. Cells were electrotransformed at 1.75 kV using a Bio Rad Gene Pulsar, and 0.5 ml of cold SOC medium (tryptone, 20 g/l; yeast extract, 5 g/l; NaCl, 0.5 g/l; KCl 0.185 g/l; $MgCl_2$, 1M; $MgSO_4$, 1M and glucose 0.5M) was immediately added. Cells

were transferred to 3 ml plastic tubes and incubated at 37°C for 1 h and then spread on petri plates containing LB medium with ampicillin (50 μg/ml) and X-Gal (40 mg/ml). Subsequent clones were then streaked onto master grid plates for further analysis.

Screening of 16S rRNA clones Individual 16S genes were amplified by using the *E. coli* streak plates from above. Colonies were picked and washed with 50 μl H_2O in 1.5 ml Eppendorf tubes pelleted by centrifugation and resuspended in 10 μl H_2O. The cells were then heated for 10 min in a boiling water bath and pelleted by centrifugation. The supernatant (1 μl) was then used as substrate for the PCR reaction mixture as above. PCR products were digested directly with 12.5 μ each of both *Hae*III and *Hin*P1I (New England Biolabs, Beverly, MA) in a total volume of 50 μl and incubated at least 2 h at 37°C. The DNA was then precipitated with 1 μl glycogen (NEB) by incubating with 2 μl 5 M NaCl and 1 volume of isopropanol on ice. The DNA was pelleted by centrifugation, washed with 70% cold ethanol and resuspended in 10 μl H_2O. The restriction fragments were then analyzed by polyacrylamide gel electrophoresis using 10% polyacrylamide gels. The samples were run using a Hoeffer SE600 system (Hoeffer Scientific Instruments, San Francisco, CA) at 32 V for 30 min, 64 V for 15 min and 184 V for 5.5 h while cooled to 8°C using a Brinkman RM6 Lauda water bath (Brinkman Instruments, Inc., Westbury, NY). The gels were then stained with ethidium bromide (1μg/ml) for 10 min and then destained in TBE for 10 min to visualize the bands.

Rarefaction analysis To evaluate species abundance in samples of unequal size, rarefaction curves were constructed (1). The estimated number of species or different

phylotypes, E(s), in a random sample of n individuals taken from a total population of N individuals was calculated using the equation:

$$E(s) = \sum_{i=1}^{s} \left\{ 1 - \left[\binom{N - n_i}{n} \Big/ \binom{N}{n} \right] \right\}$$

where n_i is the number of individuals of the ith phylotype.

16S sequencing and analysis Plasmid DNA containing the 16S rRNA gene insert was isolated from the clones using the Qiagen plasmid mini kit according to the manufacturers instructions (Qiagen, Chatsworth, CA). The DNA sequence of the insert was determined by automated fluorescent dye terminator sequencing using an ABI Catalyst 800 laboratory robot and ABI 373A Sequencer (Applied Biosystems, Foster City, CA). Primers used corresponded to conserved regions of the 16S sequence (20). Initial phylogenetic placement was obtained using the Ribosomal Database Project (RDP) (8) electronic mail server program SIMILARITY-RANK. Preliminary alignments of related sequences were obtained using the RDP programs SUBALIGNMENT and ALIGN-SEQUENCE. The alignments were completed using the sequence editor GDE obtained from the RDP. A maximum-likelihood phylogenetic tree was created using the program fastDNAml (12), using a weighting mask to include only unambiguously aligned positions with all other program options at their default values. This analysis was repeated on 100 bootstrap samples to obtain confidence estimates on the branching order (6). The program consense from the PHYLIP program package (7) was used to determine the number of times that each group shown in the final tree was monophyletic in the bootstrap analysis. The final tree was then rooted by including the sequence of *Bacillus subtilis*

using the program dnaml from the PHYLIP package and the program TreeTool from the RDP.

RESULTS

The DNA from the two 3-chlorobenzoate degrading enrichments was subject to analysis by Southern transfer and hybridization using an oligonucleotide probe specific for a large group of sulfate-reducing members of the delta *Proteobacteria*. The probe and hybridization conditions were selected to detect the branch containing the known 3-chlorobenzoate degrader, *D. tiedjei*, and not to detect the *Desulfovibrio* branch. Southern hybridization showed delta *Proteobacteria* to be present in both the sewage sludge and mangrove sediment enrichments. These major bands did not match those from the previously isolated 3-chlorobenzoate degrader *Desulfomonile tiedjei* strain DCB-1 (Fig. 1). The RFLP patterns also did not match those of the other 3-chlorobenzoate dechlorinating organisms later isolated from these two enrichments (data not shown). The band differences between serial transfers of the mangrove sediment enrichment (Mangrove A and B) suggests a shift in the delta *Proteobacteria* present during that interval.

The composition of the sewage sludge and mangrove enrichments was further examined by constructing 16S rRNA gene libraries and screening the restriction digests of the various clones. Two separate gene libraries were created for each enrichment using total enrichment DNA extracted five months apart for each library.

A total of 52 clones were examined from the two sewage sludge enrichment libraries. Four different phylotype patterns were identified, none of which matched the phylotype pattern of the isolated dechlorinating organism DCB-O (Fig. 2). Of the clones examined 92% were of the same phylotype, (37 of 40 from the first library and 11 of 12

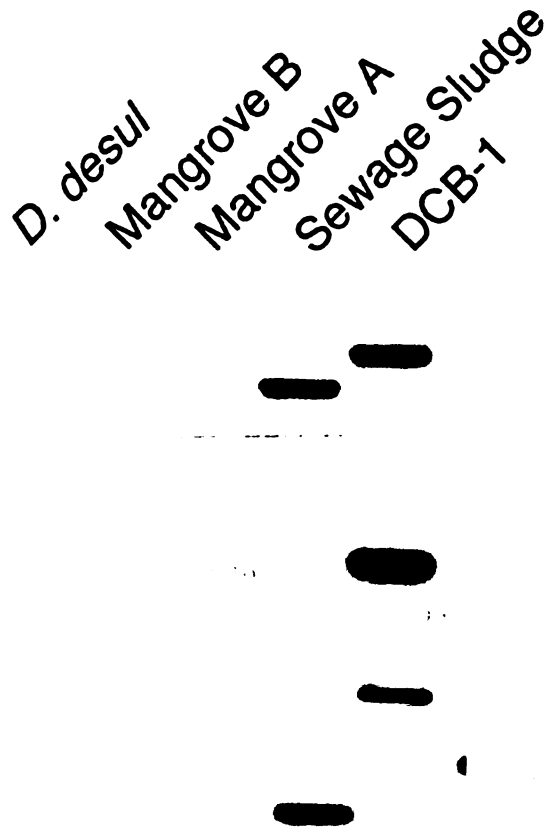
from the second) (Figure 3). Two clones from the first library (B and C) were found to have phylotype patterns differing from the main phylotype (A) by one restriction site and hence are close relatives. Only one other distinct phylotype pattern (D) was observed, and it represented 4% of the total clones (1 of 40 from the first library and 1 of 12 from the second) (Fig. 4).

A total of 63 clones from the two mangrove sediment enrichment libraries were examined with 10 patterns identified (Fig. 5). Again, none of the patterns examined matched the pattern of the isolated dechlorinating organism DCB-F. Of the clones examined, 25 of 30 (83%) from the first enrichment library and 28 of 33 (85%) from the second library (for a total of 53 of 63 (84%)) had the same phylotype pattern. Interestingly, this phylotype pattern was identical to the main phylotype pattern of the sewage sludge enrichment. One other phylotype from the first library was represented by two clones; all other phylotypes were represented by single clones (Fig. 6). Two phylotypes (F and I), each represented by a single clone, differed from the main phylotype (A) by apparently only one restriction site. If grouped with the main phylotype, then only eight distinct phylotypes were observed in the sample examined (Fig. 7).

Rarefaction analysis was used to compare the species richness of the two enrichments (Fig. 8). This analysis predicts that the diversity of both enrichment cultures was not exhausted, but suggested to be increasing at an approximately constant rate.

Sequence analysis of the main phylotype pattern (phylotype A) of both the sewage sludge and mangrove sediment enrichments showed the organism to be a member of the delta *Proteobacteria* (Table 1), but the sequences were not identical differing by 8.39%.

Figure 1. Southern hybridization of genomic DNA from *Desulfovibrio desulfuricans*, two different serial transfers of the mangrove sediment enrichment, the sewage sludge enrichment and *Desulfomonile tiedjei* (DCB-1). DNA samples were digested with *Ban*I and hybridized to a delta *Proteobacteria* specific oligonucleotide probe designed to detect the known 3-chlorobenzoate degrading *D. tiedjei* branch and not to detect the *Desulfovibrio* branch. The probe sequence and sequences of relevant known strains are shown.



	388	402
	GACGCAGCAACGCCG	
D. tiedjei	ACTCTGACGCAGCAACGCCGCGTGGG	
D. acetoxidans	ACCCTGACGCAGCAACGCCGCGTGAG	
D. desulfuricans	AGCCTGACGCAGC**G**ACGCCGCGTGAG	
E. coli	ACCCTGA**T**GCAGC**C**A**T**GCCGCGTGTA	

Figure 1. Southern Hybridization

Figure 2. **Phylotype patterns from the sewage sludge enrichment and the isolated organism DCB-O. STD: *Hae*III digested pBR322 DNA size marker; DCB-O: *D. tiedjei* strain DCB-O; A, B, C, and D: enrichment phylotypes.**

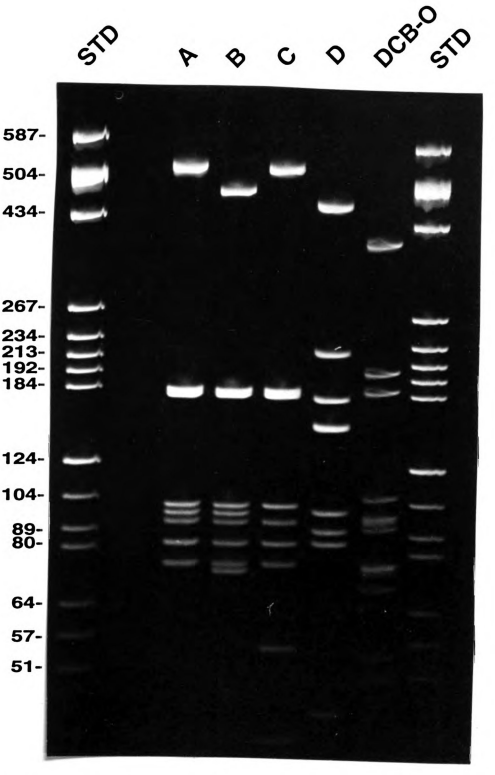


Figure 2. Predominant Phylotypes in the Sewage Sludge Enrichment

Figure 3. **Frequency distribution of the 52 16S rRNA gene phylotypes from the sewage sludge enrichment. The phylotypes shown are ordered by the quantity detected.**

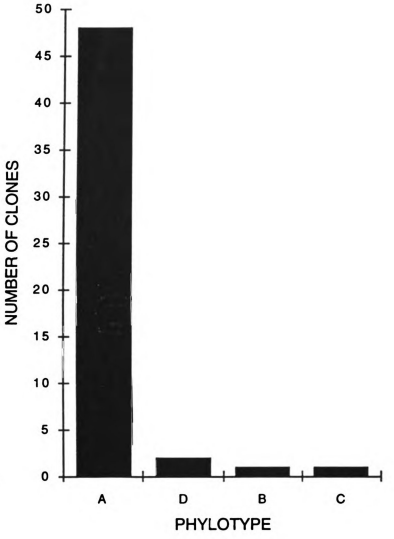


Figure 3. Frequency Distribution from the Sewage Sludge Enrichment

Figure 4. Frequency distribution of the 52 16S rRNA gene phylotypes from the sewage sludge enrichment with similar patterns combined. The phylotypes are ordered by the quantity detected.

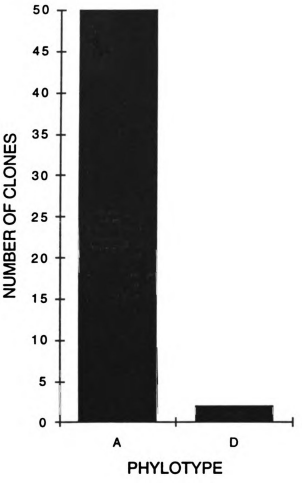


Figure 4. Frequency Distribution from the Sewage Sludge Enrichment

Figure 5. Computer generated representation of the different phylotypes from the mangrove sediment and the isolated organism DCB-F compiled from photographs taken as they were detected. STD: *Hae*III digested pBR322 DNA size marker; DCB-F: the dechlorinating isolate; A-J: enrichment phylotypes.

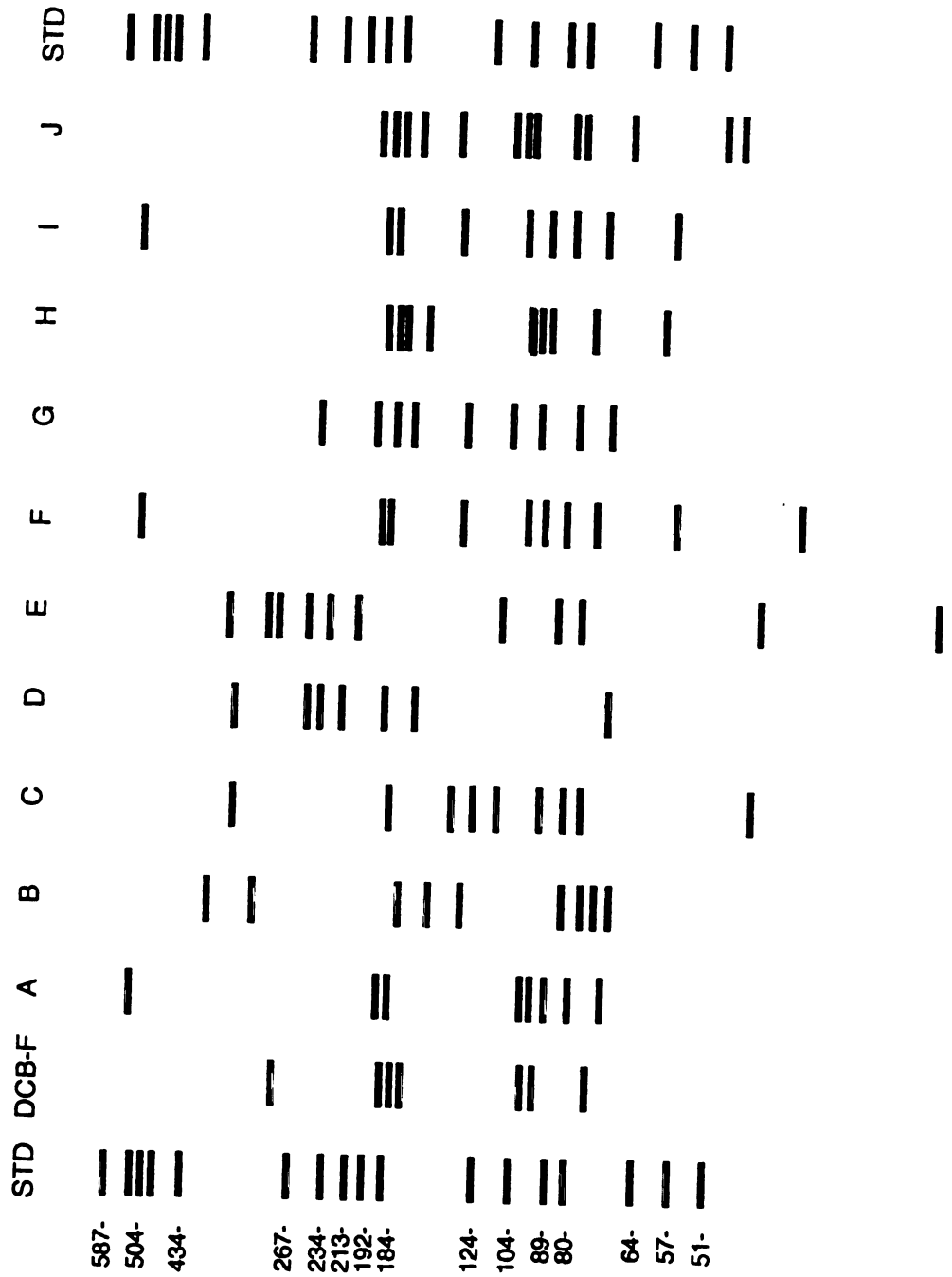


Figure 5. Predominant Phylotypes in the Mangrove Sediment Enrichment

Figure 6. Frequency distribution of the 63 16S rRNA gene phylotypes from the mangrove sediment enrichment. The phylotypes are ordered by the quantity detected.

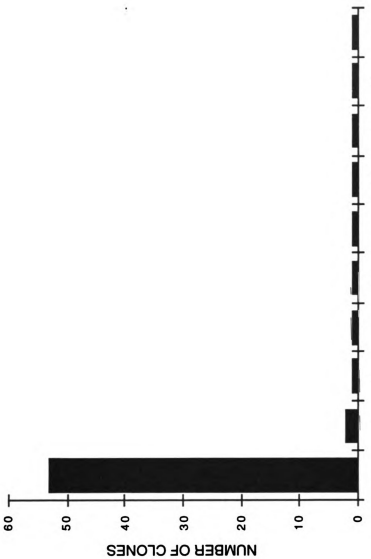


Figure 6. Frequency Distribution from the Mangrove Sediment Enrichment

Figure 7. Frequency distribution of the 63 16S rRNA gene phylotypes from the mangrove sediment enrichment with similar patterns combined. The phylotypes are ordered by the quantity detected.

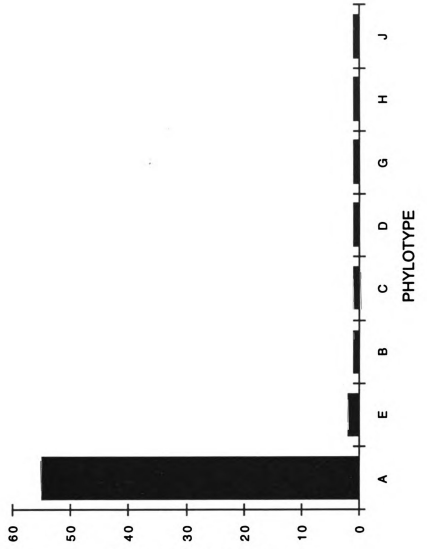


Figure 7. Frequency Distribution from the Mangrove Sediment Enrichments

Figure 8. Rarefaction curves for the phylotypes observed in the sewage sludge enrichment and the mangrove sediment enrichment. Values from the X-axis represent the expected number of phylotypes in a random sample of n individuals taken from the total population of phylotypes from each enrichment.

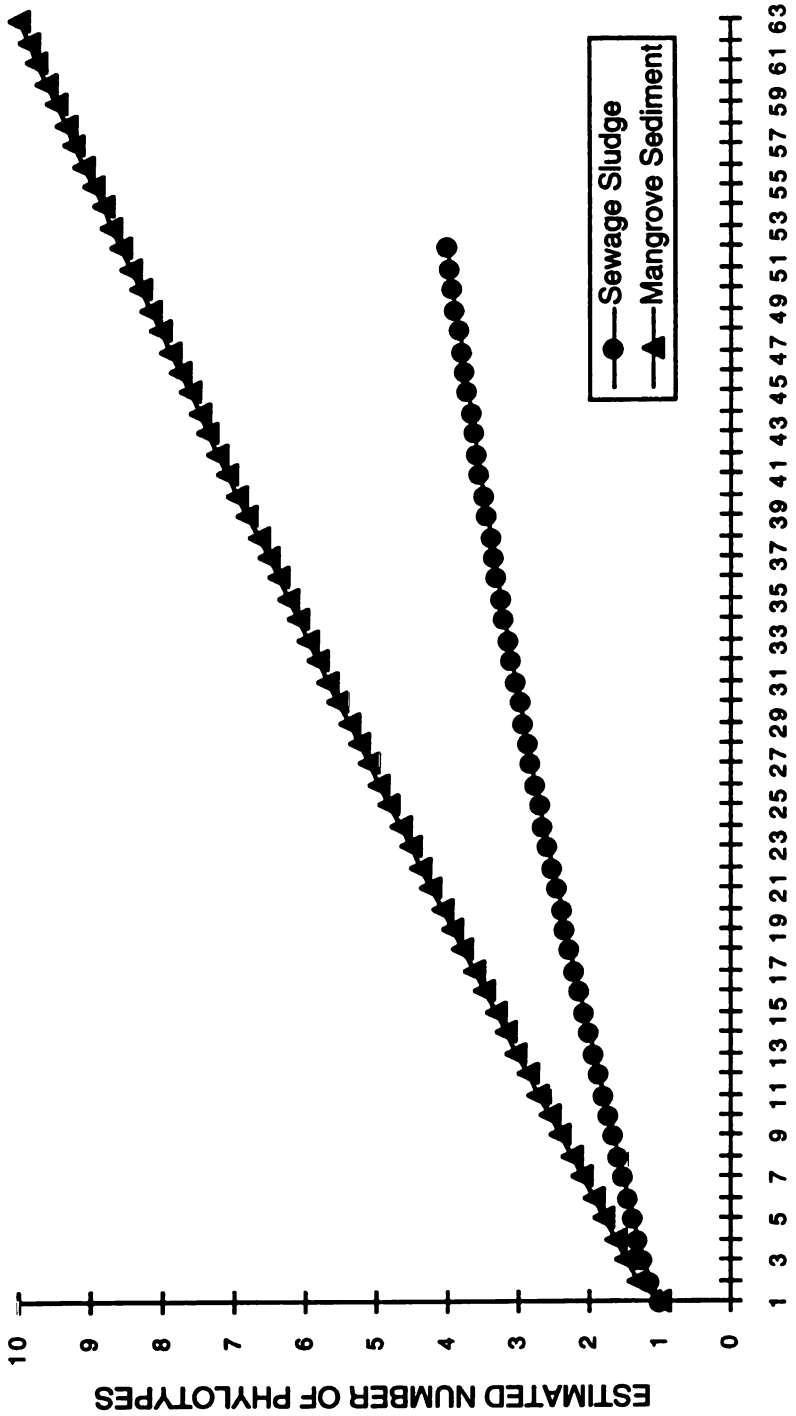


Figure 8. Rarefaction Analysis of the Sewage Sludge and Mangrove Sediment Enrichments

Table 1. **Sequence analysis of the sewage sludge and mangrove sediment phylotypes using an approximate 300 bp variable region corresponding to approximately bases 29-324 on the *E. coli* 16S rRNA gene map.**

TABLE 1. Sequence analysis of the sewage sludge and mangrove sediment enrichments

Phylotype	S_{ab}[a]	Highest Similarity
Sewage sludge A	0.478	*Pelobacter propionicus*
B	0.478	*Pelobacter propionicus*
Mangrove A	0.498	*Desulfomicrobium escambium*
B	0.568	*Eubacterium plautii*
C	0.380	*Thermoanaerobacter ethanolicus* str. 39E
D	0.310	*Micrococcus luteus* str. Hucker S66
E	0.370	*Gloebacter violaceus*
F	0.483	*Desulfomicrobium escambium*
G	0.379	*Magnetospirillum magnetotacticum*
H	0.457	*Pelobacter propionicus*
I	0.498	*Desulfomicrobium escambium*

[a] similarity rank used in the RDP database which equals the number of shared oligomers divided by either the number of unique oligomers in the submitted sequence or the database sequence whichever is lower

DISCUSSION

When examining the composition of microbial food webs one can not simply rely on cultivation dependent studies. Many organisms in natural environments are thought to exist in a dormant or injured state capable of reproducing only under the proper stimulus, or are thought to carry out certain metabolic reactions only at specific times in the growth cycle. Still other organisms depend on a multiplicity of special conditions, often created by other members of the community. These conditions can not be reproduced in the laboratory causing isolation results to give a skewed representation of the true community. Organisms isolated from these niches may also loose enzymatic capabilities exhibited in their natural environments further obscuring the true function of the organism in its natural environment. Molecular methods have proved useful for revealing additional members of these communities and their biochemical potential. But as with culture dependent approaches, these methods can also exhibit bias which must be evaluated before a full interpretation of community composition is possible.

The enzymatic method of Visuvanathan et. al. (17) was used for DNA extraction since this method has proven very effective for recovering DNA from a broad range of microorganisms present in small amounts (3×10^7 bacterial cells). This method was shown to lyse 68 bacterial strains including gram positive, gram negative and many mycobacterial strains, whose cell walls are resistant to other enzymatic lysis methods, and yielded high molecular weight DNA that was digestible with most restriction endonucleases (17). By using a lysis method capable of extracting high yields of good quality DNA, bias introduced in the DNA extraction and recovery step should be reduced. Other bias can

occur due to preferential amplification of DNA from one organism over another during the PCR reaction due to primer mismatches and from preferential ligation in the cloning system used. Another problem with these approaches is that they may not give a true representation of the community if the diversity of the sample is not exhausted in the clone population. The sequence analysis step can also obscure true composition if the region being sequenced doesn't reveal the diversity present. Finally, the interpretation of composition is currently limited by the incompleteness of databases. There are also inherent types of bias in these methods such as the difference in the number of 16S rRNA operons per organism which would cause some organisms with a low copy number to be underestimated. Also, the size of the organism can determine the extent of enzymatic function in the community, a smaller organism may be represented more often but the larger organism may be carrying out the majority of the activity causing a misinterpretation of its dominance in the existing food web. Although these biases may exist, in the present study I have at least found the results to be reproducible since two separate isolations of DNA from each enrichment and the subsequent manipulations of each yielded frequencies of the dominant organisms that varied by only 0.0 to 2.0%.

Only a few dechlorinating bacteria have been successfully isolated from anaerobic environments shown to dechlorinate a wide variety of recalcitrant substrates, although these organisms are believed to be quite ubiquitous in nature (3, 9, 10, 16). Since isolation has often proven ineffective, little is known about the extent of diversity and physiology of organisms carrying out these reactions. In this study the predominant organism in both the sewage sludge and brackish mangrove sediment enrichments was

identical in phylotype pattern but differed in 16S rRNA sequence by ca. 8.39 % in the 300 bp region chosen for sequencing .

The major phylotype found in both enrichment gene libraries did not match the phylotypes of the isolated organisms, strains DCB-O and DCB-F. Although both enrichments were methanogenic, the primers used were eubacterial and do not amplify archaeabacteria, eliminating the possibility that this organism is a methanogen. The partial 16S rRNA sequences of the two dominant phylotypes indicate that they are members of the delta *Proteobacteria* and confirm that they are not DCB-O, DCB-F or DCB-1. This phylotype could be another 3-chlorobenzoate degrader since our enrichment and isolation conditions limited the types of organisms which could grow. Since 3-chlorobenzoate was the only substrate provided in the isolation medium, it might be expected that only organisms that could use benzoate as an electron donor in order to obtain energy from the dechlorination reaction would be selected. The main phylotype may represent an organism unable to utilize benzoate as an electron donor but capable of growth using an intermediate of benzoate degradation as the electron donor in the dechlorination reaction.

An alternate hypothesis is that the major phylotype detected is a benzoate fermentor. It has been observed that sulfate reducing bacteria can grow syntrophically with methanogens fermenting benzoate to acetate and CH_4 (16). Both enrichment cultures completely mineralized 3-chlorobenzoate to CH_4 and CO_2 with benzoate observed as a transient intermediate. This hypothesis is consistent with the structure of the food chain described in the 3-chlorobenzoate enrichment from which strain DCB-1 was isolated (16). Microscopic examination of the predominant morphotypes in the

reconstructed consortium described by Dolfing and Tiedje (4) showed the benzoate fermentor was the most numerous cell type (65%) with the dechlorinator (DCB-1) making up 31% of the cell types (5). The sewage sludge and mangrove sediment enrichment cultures were maintained similarly to the DCB-1 dechlorinating enrichment providing further evidence of the possible existence of a similar 'food chain'.

One would expect to see the phylotype of the isolated organisms DCB-O and DCB-F matching a predominant phylotype found in their respective enrichment culture, as found in the toluene oxidizing enrichment (see Chapter 2). Although the results of this study were reproducible, the phylotype of the isolated organisms was not detected. Phylotypes of the two pure cultures were detected by the same lysis, amplification and cloning procedures so that any methodological bias against them was not apparent . The isolates DCB-O and DCB-F are large cells measuring 2 to 3 μ by 10 to 20 μ, respectively, with a low copy number [2 to 4, (Jim Cole, personal communication)] of the 16S rRNA gene. This may have decreased the amount of 16S rRNA gene amplification beyond our level of detection. Rarefaction analysis of the enrichments supports the hypothesis that there are still a large number of phylotypes yet undetected but of similar frequency as the detected minor phylotypes. Since the number of new phylotypes was increasing at a constant rate, the phylotype of the dechlorinators could have been detectable with analysis of additional clones. Even if found as a very rare clone, this would not confirm that they had a major role in dechlorination in the enrichment.

Using the described methodology, information about the structure and composition of the enrichment communities was determined. This allowed additional speculation on the functional roles of the detected organism. The results obtained provide

evidence that the bias introduced in culture independent means of exploring community structure and function can be minimized and that the results obtained can be reproduced with only minimal variability.

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Chapter II

THE CHARACTERIZATION OF UNCULTURED BACTERIAL COMMUNITY MEMBERS FROM A SULFATE-REDUCING, TOLUENE-OXIDIZING ENRICHMENT CULTURE

Soils, sediments and groundwater are frequently contaminated with the monoaromatic hydrocarbons known as BTEX (benzene, toluene, ethylbenzene and xylene). Due to their high solubility in water these compounds can move in groundwater thereby contaminating drinking water far from the original site of contamination. Contamination occurs from leaks in underground storage tanks, surface spill accidents, as well as from improper disposal techniques. Clean-up of these compounds is of great interest because of their carcinogenic potential even at very low concentrations (5, 11).

Aerobic degradation of toluene and other alkylbenzenes has been well documented. Oxygen is the rate-limiting reactant since it's vital to ring activation and cleavage (12). It is also used as the electron acceptor in the complete mineralization of these compounds. Anaerobic conditions, however, develop quickly due to depletion of oxygen by aerobic substrate utilization, low oxygen solubility in water and low rates of diffusion through porous soils and sediments. These factors then limit the amount of aerobic degradation that can occur in most contaminated sites making the anaerobic mineralization of BTEX of great importance.

In the absence of oxygen, other electron acceptors may potentially support the degradation of BTEX. To date, the degradation of toluene by bacterial isolates and consortia has been shown to occur under denitrifying (4, 6, 9, 10, 15, 16, 20), fermentative methanogenic (13, 22), sulfate-reducing (1, 8, 19) and ferric iron-reducing

(17, 18) conditions. Further studies of the community structure of these environments should increase our understanding of role microorganisms play in the complete mineralization of petroleum contaminated sites.

In this study, a sulfate-reducing enrichment culture from a contaminated subsurface soil from an aviation fuel storage facility in Maryland (1) was examined to determine the community structure. At the onset of the study, isolation of the organism responsible for toluene degradation had proven elusive. A culture-independent molecular approach based on 16S rRNA phylogeny was used to determine the dominant organisms in the enrichment. DNA was isolated from the enrichment and the 16S rRNA genes were amplified and cloned into an *Escherichia coli* vector. The individual 16S rRNA genes were then amplified, digested and screened using high resolution acrylamide gels to obtain respective phylotypes. The unique phylotypes were further characterized by sequence analysis.

The diversity of this community was not exhausted after 15 phylotypes had been identified. The most dominant phylotype was found to be related to the H_2-producing genus *Syntrophobacter* (S_{ab} 0.464). A pure isolate (PRTOL1) from the enrichment capable of oxidation of toluene was obtained during the course of the study (3) and its 16S rRNA sequence was identical to that of dominant phylotype found. Surprisingly, the second most dominant phylotype was found to be closely related to the sulfate reducing 3-chlorobenzoate degrader *Desulfomonile tiedjei* (S_{ab} 0.583). The results of this study provide evidence that culture independent approaches can be used in examining the structure and possible function of unculturable members of enrichments.

MATERIALS AND METHODS

The bacterial cells were obtained from a previously described sulfate-reducing, toluene-degrading enrichment (1). The enrichment was inoculated in June of 1990 and has been metabolizing toluene with sulfate as the electron acceptor since that time. The suspension analyzed was a 0.2 % dilution of the original microcosm. Bacterial cells were concentrated by centrifugation and then sent on dry ice from Stanford University where they were then placed at -70°C until analyzed. DNA was isolated from the cells by the method of Visuvanathan et. al. (23) as described in Chapter 1. A black precipitate was mixed with the bacterial cell matter making several more phenol:chloroform:isoamyl alcohol extractions necessary. The amplification of the 16S rRNA genes, construction of the 16S rRNA gene libraries and analysis of resulting phylotype patterns were carried out as previously described (Chapter 1).

RESULTS

A total of 44 16S rRNA clones were examined from the sulfate-reducing, toluene-oxidizing enrichment. Fifteen different phylotype patterns were detected (Fig. 1). Of the 44 clones, 70% had the same pattern and this phylotype pattern was identical to that of the toluene-degrading organism isolated from the enrichment. One other phylotype (B) was represented by three clones with all other phylotypes being represented by a single clone (Fig. 2). Four phylotype patterns were found to differ from the dominant phylotype by only one restriction site (D, F, G and H). If grouped with the dominant phylotype, then 11 distinct types were observed (Fig. 3).

To analyze the extent of diversity recovered from the sample, a rarefaction curve was constructed (Figure 4). The analysis predicts that the diversity of this community was not exhausted but was increasing at a constant rate.

The phylotypes were then subject to sequence analysis for further characterization (Table 1). The dominant phylotype had a sequence identical to that of the toluene-reducing organisms isolated from the enrichment. Surprisingly, the second most dominant phylotype was 93.5% similar to the 3-chlorobenzoate degrader *Desulfomonile tiedjei* suggesting these two organisms are very closely related (Fig. 5).

Figure 1. **Phylotypes from the toluene oxidizing enrichment. STD: *Hae*III digested pBR322 DNA size marker; A-O are all clones with different band patterns.**

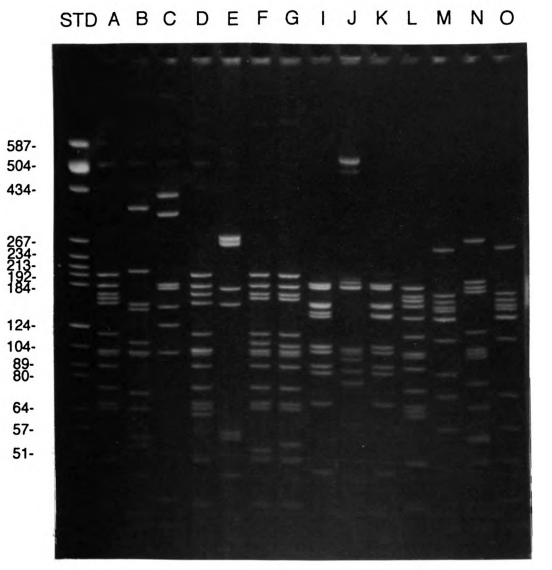

Figure 1. Predominant Phylotypes in the Toluene Enrichment

Figure 2. **Frequency distribution of the 44 16S rRNA gene clones from the toluene oxidizing enrichment. The phylotypes are ordered by the quantity detected.**

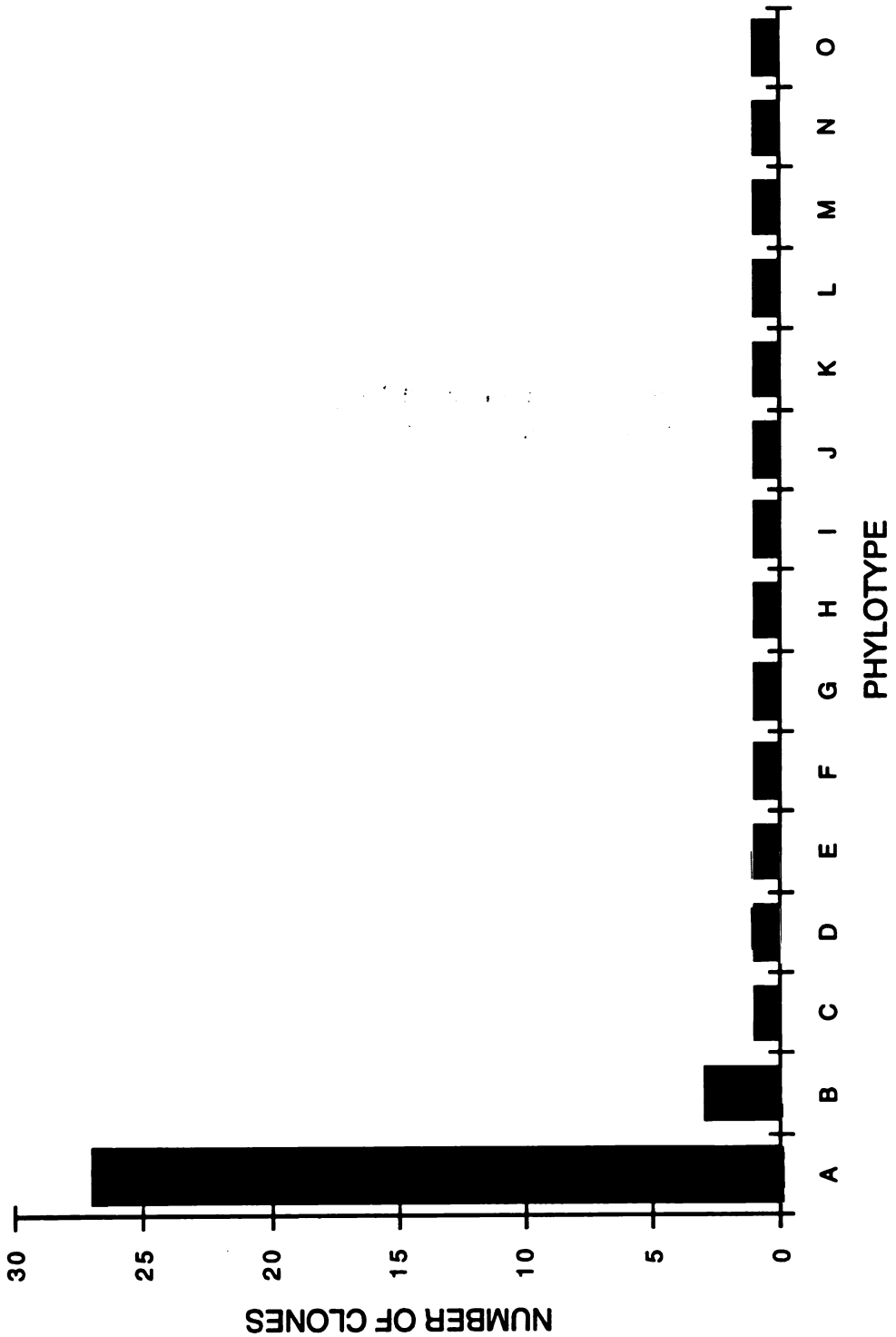

Figure 2. Frequency Distributin from the Toluene Oxidizing Enrichment

Figure 3. Frequency distribution of the 44 16S rRNA gene clones from the toluene oxidizing enrichment with similar patterns combined. The phylotypes are ordered by the quantity detected

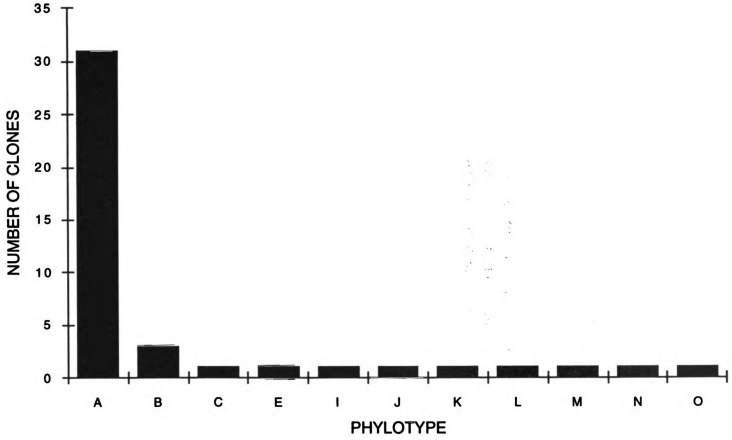

Figure 3. Frequency Distribution from the Toluene Oxidizing Enrichment

Figure 4. Rarefaction curve for the phylotypes from the toluene oxidizing enrichment. Values from the X-axis represent the expected number of phylotypes in a random sample of n individuals from the total number of phylotypes from the enrichment.

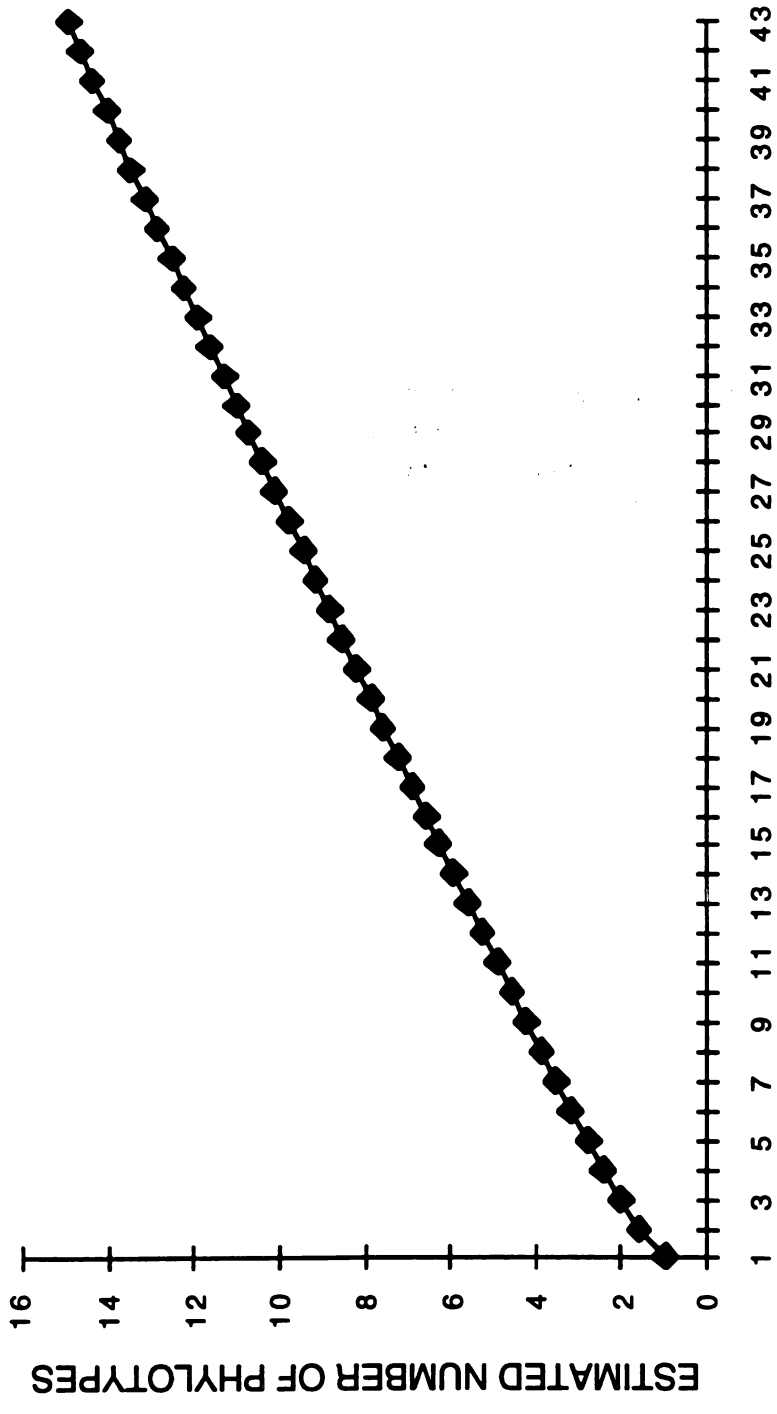

Figure 4. Rarefaction Analysis of the Toluene Oxidizing Enrichment

Table 1. Sequence analysis of the toluene oxidizing enrichment phylotypes using an approximate 300 bp variable region corresponding to approximately bases 29-324 on the *E. coli* 16S rRNA gene map.

TABLE 1. Sequence analysis of the toluene oxidizing enrichment

Phylotype	S_{ab} [a]	Percent Identity[c]	Highest Similarity
Pure culture	0.464[b]	92.23	*Syntrophobacter wolinii*
Toluene A	0.464		*S. wolinii*
B	0.583	93.51	*Desulfomonile tiedjei*
C	0.492		*Thyasira flexuosa*
D	0.464		*S. wolinii*
E	0.965		*Lactobacillus delbrueckii*
F	0.464		*S. wolinii*
G	0.464		*S. wolinii*
H	0.464		*S. wolinii*
I	0.544		*Treponema sp.* str. H1
J			
K	0.599		*Treponema sp.* str. H1
L	0.464		*S. wolinii*
M	0.678		*Clostridium thermoautotrophicum*
N	0.437		*Pelobacter acetylenicus*
O	0.677		*Clostridium thermoautotrophicum*

[a] similarity rank used in the RDP database which is the number of shared oligomers divided by either the number of unique oligomers in the submitted sequence or the data base sequence whichever is lower

[b] S_{ab} value is low due to absence of the initial 70 bp of sequence in the RDP database

[c] Nearly complete 16S rRNA sequences were analyzed for these clones

Figure 5. Maximum likelihood phylogenetic tree of Phylotype A/PRTOL 1 (isolate from the anaerobic toluene oxidizing enrichment) and representative delta *Proteobacteria*. Numbers at internal nodes are the percent of 100 bootstrap samples in which the group to the right of the node was monophyletic. The scale bar indicates the expected number of changes per position.

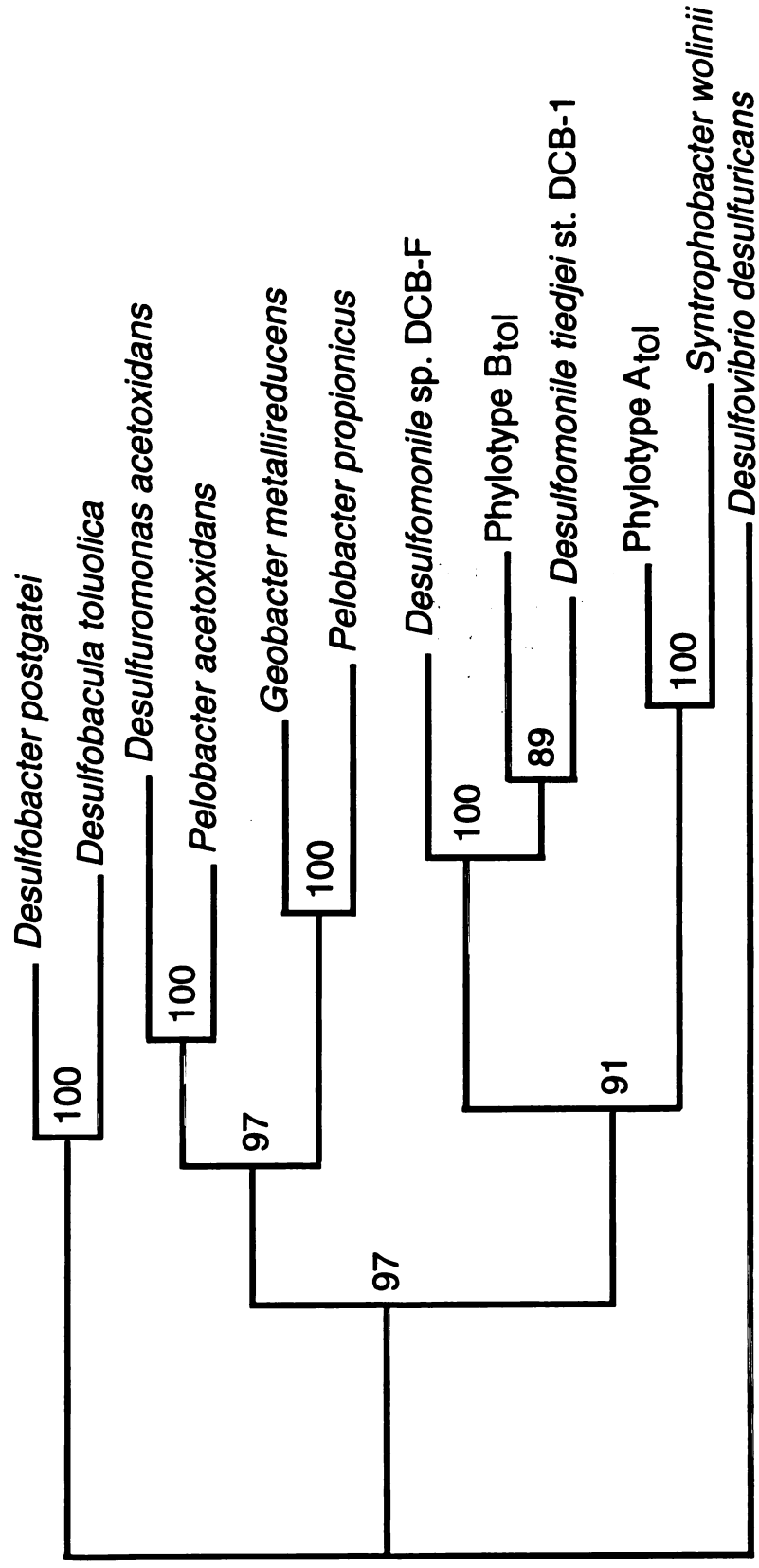

Figure 5. Maximum Likelihood Phylogenetic Tree

DISCUSSION

The dominant phylotype, which is identical in 16S rRNA sequence to the pure culture later identified, is a member of the delta *Proteobacteria*. It's a close relative to the hydrogen-producing genus *Syntrophobacter*. Although phylogenetically similar, the isolate and its nearest relative do not seem to occupy similar positions in their respective communities. *Syntrophobacter*, grown as a syntroph with sulfate reducers or methanogenic bacteria which lower the $[H_2]$, obtains energy from using H^+ as its electron acceptor, but the isolate obtained from the enrichment culture uses sulfate as its electron acceptor. However, *Syntrophobacter wolinii* has also been shown to grow as a sulfate-reducer (14). Given the close relationship between *S. wolinii* and the toluene isolate, perhaps the new isolate can also use H^+ as an electron acceptor.

Of the phylotypes analyzed a wide variety of organisms were detected. Most of the phylotypes which were sequenced did not have 16S rRNA sequences close to the isolates whose 16S rRNA sequences are available in the RDP database. The limited ribosomal database makes it difficult to speculate about the types of organisms present and their function in the community.

Phylotype B, the second most dominant member, was identified as being a close relative to *D. tiedjei*, a delta *Proteobacteria* capable of degrading 3-chlorobenzoate. *D. tiedjei* is also capable of growth using benzoate (7) and thiosulfate (21) as an electron donor. Beller et. al. (2) described benzoic acid as one of the intermediate products observed in the toluene-degrading, sulfate-reducing enrichment cultures. It is possible that this *D. tiedjei*-like organism could have grown on the benzoate produced in the mineralization pathway.

A second phylotype of interest was phylotype E which was almost identical in sequence to *Lactobacillus delbrueckii*. This organism, which is used in the production of yogurt, would not be expected in the described enrichment. The possibility of contamination during the maintenance of the enrichment or during the PCR amplification of the 16S rRNA genes seems unlikely since this organism is not cultivated in either laboratory that had this enrichment. It is likely that this and the other organisms in the enrichment exist in low numbers using the minor fermentation products and other cell products produced by the major member(s). The frequency distribution profile is suggestive of this type of role for the diverse and more rare members.

At the onset of this study, the description of organisms responsible for the anaerobic oxidation of toluene under sulfate reducing conditions had proven elusive. By using a culture independent approach, information about the community structure and composition was obtained with some hypotheses as to the function of organisms present. Not only did the described methodology identify the major member of the enrichment, which was later isolated and shown to anaerobically oxidize toluene, but several other organisms were sufficiently characterized to reveal a very diverse community in spite of the very selective environment. The methodology may prove valuable in examining other enrichment and reactor communities.

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