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Major professor

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MICROBIAL COMMUNITIES IN PRISTINE AND TETRACHLOROETHYLENE-CONTAMINATED AQUIFER SEDIMENT

By

Merry Shannon Riley-Buckley

AN ABSTRACT OF A DISSERTATION

Submitted to
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2001

Dr. Terence L. Marsh and Dr. James M. Tiedje

ABSTRACT

MICROBIAL COMMUNITIES IN PRISTINE AND TETRACHLOROETHYLENE-CONTAMINATED AQUIFER SEDIMENT

By

Merry Shannon Riley-Buckley

The microbial communities of the saturated subsurface play an important role in the quality of groundwater, a resource of incalculable value to human and ecological concerns. Further, although the magnitude of the effect that subsurface microbes have on global biogeochemical cycling is not known, estimates suggest that because of the sheer numbers of subsurface bacteria, they likely play a significant role in the cycling of bioactive elements. Despite these facts, subsurface microbial communities have been little studied until recently. In an effort to extend current knowledge in this area, a study of the microbial communities in clean and chemically-contaminated aquifer sediment was undertaken. The diversity and composition of the microbial communities at two sites was assessed using molecular techniques. At a pristine site in Oyster, Virginia, the members of microbial communities in oligotrophic sediment were characterized by phylogenetic methods. The bacterial community profile from oligotrophic sediment was compared to that of the communities in a contrasting geochemical environment in the same formation. Similarities were found to exist between the two communities. Bacterial community diversity in aquifer sediment was markedly lower than that observed in surface soils. A novel group of phylogenetically deeply-branching bacteria was detected at the site and found to exist in eight out of ten sediments tested. The microbial communities at a tetrachloroethylene-contaminated site in Oscoda, Michigan were also studied. Members of communities in clean and contaminated sediment were described phylogenetically, and

found to be closely related to the β, γ, and δ *Proteobacteria*, the *Acidobacteria*, *Leptospirillum / Nitrospira*, and *Green nonsulfur bacteria*. Within the plume, 16S sequences closely related to the species *Syntrophus* were identified, suggesting that interspecies hydrogen or acetate transfer played a role in carbon cycling at the site. Bacterial diversity was found to be significantly higher in contaminated sediment than in sediment from upstream of the plume. Finally, the distribution and abundance of a sulfate-reducing bacterium which was detected within the plume was studied using the emerging method of real-time PCR. The 16S sequence of this organism was found to constitute only a small proportion of total bacterial 16S sequences at the site, and was highly over-represented in the 16S clone library.

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U = units of activity

bp = nucleotide base pairs

ft = feet

DNA = deoxynucleic acid

RNA = ribonucleic acid

dNTP = deoxynucleotide triphosphate

CHAPTER 1

MICROBIAL COMMUNITIES IN THE SATURATED SUBSURFACE

1. INTRODUCTION

The microbial communities of the saturated subsurface are complex, diverse, and, in general, are not well characterized. The human need for quality groundwater resources is expected to increase in the next century (50) even while more and more aquifers are being contaminated by anthropogenic chemicals and human waste. Groundwater is also a key component of aquatic ecosystems like lakes, rivers, and coastal areas, and the chemical properties of this water have a profound effect on ecosystem function. Furthermore, the subsurface is a component of the global cycles of such bioactive elements as carbon, nitrogen, sulfur, and phosphorous, to name a few. The chemical reactions that determine water quality and nutrient cycling in the subsurface are almost exclusively mediated by microbes. In light of this fact, the importance of bridging the gap in our understanding is apparent.

This chapter will review the importance of groundwater to human and ecological concerns, some of the biogeochemical transformations that subsurface microbes carry out, current knowledge about the distribution of different phylogenetic groups found in the subsurface, and methods for analyzing subsurface microbial communities. Finally, the goals and methods of my research into subsurface microbial communities are described.

2. THE IMPORTANCE OF GROUNDWATER

2.1. Human use of groundwater

It is difficult to overestimate the importance of groundwater in the United States. It is estimated that 50% of all Americans and 98% of the population living in rural areas rely on groundwater as their primary source of water (50). The most recent data available indicate that over 77 billion gallons of groundwater is pumped per day in the United States (4). Statistics from the U.S. Geological Survey indicate 19% of groundwater is drawn for public drinking water and 63% is used for irrigation (4).

2.2. Ecological significance of groundwater

Approximately 99.7% of the world's freshwater (not in the form of ice and snow) is held in the subsurface. This groundwater serves as an important component of the hydrologic cycle, particularly in regard to the cycling of freshwater. Precipitation on land eventually becomes surface runoff which, in most geologic environments, infiltrates to the subsurface. Subsurface water has a relatively high residence time, between two weeks and 10,000 years, which may be longer than the residence times calculated for the ocean (about 4000 years). Groundwater which is not held in the primary lithosphere (the rock that forms the core of the earth) eventually exits the subsurface and discharges into lakes, rivers, or wetlands, or it empties directly into the ocean. Each of these environments are complex ecosystems, and groundwater flow rate and water quality have an impact on the function of these ecosystems. The flow of subsurface water into surface ecosystems influences water availability and habitat suitability, among other things, and the quality of subsurface water in these systems often selects for the species that live there. So, the processes that take place in subsurface environments are felt in other environments by virtue of the role of groundwater in the hydrologic cycle.

2.3. Global nutrient cycles and the subsurface

The subsurface serves as a compartment of the global cycles of every known element of biological importance, including carbon, iron, sulfur, and oxygen. Since primary production is thought to be insignificant in the subsurface (27), the dominant processes are likely to be degradative (14, 38). The extent to which subsurface environments effect global nutrient cycles is unknown.

3. BIOGEOCHEMISTRY OF THE SATURATED SUBSURFACE

Madsen and Ghiorse (38) have estimated that the total microbial biomass in subsurface environments worldwide may be 40 times greater than in the top 1 m of soil. Moreover, the subsurface microbiota catalyzes a vast array of chemical transformations that affect global cycles of every bioactive element (27). Here, these processes are classified as aerobic reduction-oxidation processes, anaerobic reduction-oxidation processes, or processes not linked to energy conservation.

3.1. Aerobic reduction-oxidation processes

3.1.1. Degradation of organic compounds

Degradation of organic molecules in groundwater is crucial to efficient global cycling of carbon. Carbon is fixed by green plants and other autotrophic organisms on the surface and incorporated into plant tissues. Upon decomposition of these tissues by surface microbes, soluble organic compounds are carried to the subsurface by infiltrating water. In shallow or oxygenated systems, degradation of this dissolved carbon is largely mediated by aerobic, heterotrophic bacteria. The major carbon end product of aerobic heterotrophic metabolism is CO_2, a pivotal component of the carbon cycle. In this form, carbon may either re-enter the atmosphere or it may be taken up by other microbial processes, including methanogenesis and carbon fixation. Organic carbon concentrations

can vary widely across different formations, but it is thought that most pristine aquifers are carbon-limited (27, 38).

3.1.2. Nitrification

The energy conserving process by which nitrate is created from ammonia is referred to as nitrification. Ammonia is generated by the anaerobic decomposition of plant and animal matter. Generation of nitrate from ammonia is a two step process undertaken by different genera: usually *Nitrosomonas* and *Nitrobacter*. *Nitrosomas* (or *Nitrocystis*) uses ammonia as an electron donor in the overall energy-yielding processes:

$NH_3 + ½O_2 \rightarrow NH_2OH$ (rxn #1)

$NH_2OH + O_2 \rightarrow HNO_2 + H_2O$ (rxn #2)

Nitrite (NO_2^-) is used as an electron donor by *Nitrobacter* (or *Nitrococcus*) species in the following reaction:

$NO_2^- + ½O_2 \rightarrow NO_3^-$ (rxn #3)

The final product, nitrate, can be assimilated by a variety of microorganisms, reduced, and used as NH_2 groups in amino acids and proteins. Alternatively, under anaerobic conditions nitrate can be used as an electron acceptor by denitrifiers. Under the aerobic, oligotrophic conditions observed in shallow local flow systems however, when nitrate is introduced by agriculture or other practices it tends to accumulate (14).

3.1.3. Sulfide or elemental sulfur oxidation

Chemolithotrophic sulfide oxdizing bacteria have been shown to occur in a number of subsurface systems (22). Coal spoil piles, in particular, encourage the growth of these organisms, as they leach elemental sulfur in an aerobic environment, where subsequent bacterial activity creates acid mine drainage. In most systems, however, sulfur is found

in trace quantities and originates from minerals or from the decomposing organic matter in the soil layer. The most widely studied sulfur oxidizer, *Thiobacillus*, uses sulfide or elemental sulfur as an electron donor and molecular oxygen as an electron acceptor for energy conservation:

$2H_2S + O_2 \rightarrow 2H_2O + 2S^0$ (rxn #4)

or

$2S^0 + O_2 + 2H_2O \rightarrow 2H_2SO_4$ (rxn #5)

Reduced sulfur species are typically available in anoxic areas where microbial activity has reduced oxidized sulfur species. By definition, however, oxygen is limiting in these zones, and for sulfur oxidation to proceed, these reduced species need to be transported to an oxic environment. Alternatively, sulfide that is associated with the mineral substratum can be utilized for energy-yielding reactions. For example, Chapelle (15) observed pyrite oxidation in an aerobic subsurface system. The overall reaction is:

$FeS_2 + 15/4\ O_2 + 7/2\ H_2O \rightarrow Fe(OH)_3 + 4H^+ + 2SO_4^{2-}$ (rxn #6)

In aerobic systems with a rich source of sulfide or sulfur, sulfate will tend to accumulate. This is seldom the case, however, as extensive sulfur oxidation will quickly deplete the environment of oxygen and the buildup of sulfuric acid acts as a negative feedback on sulfur oxidation. The end product of sulfide oxidation, sulfate, may accumulate in the subsurface, it may be assimilated by other microorganisms for use in protein production, or it may be used as a terminal electron acceptor for sulfate reducing bacteria in anaerobic zones of the aquifer.

3.1.4. FeII oxidation

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Iron oxidizing bacteria, or "iron bacteria" are so common in groundwater that they are considered a microbiological pest by the well water industry. In the saturated subsurface, iron can be derived directly from mineral sources or from the breakdown of organic matter. Ferrous iron is generally found in environments where anaerobic respiration has taken insoluble ferric iron and converted it to soluble ferrous iron. Iron oxidizing bacteria like *Gallionella* use ferrous iron as an electron donor and molecular oxygen as an electron acceptor in the following overall reaction:

$$Fe^{2+} + 4H^{+} + O_2 \rightarrow Fe^{3+} + 2H_2O \qquad \text{(rxn \#7)}$$

This chemolithotrophic activity is observed most often in sediments that are anaerobic but are exposed to the atmosphere either by the intrusion of a well or by natural discharge into an aerobic environment like a river. Microbial iron oxidation is responsible for the thick, slimy coatings of ferric iron observed in some wells or at bottom of a stream or river where an anaerobic aquifer discharges.

3.1.5. CO_2 fixation

Chemolithotrophs, like those that oxidize sulfur, iron, manganese, and hydrogen, can utilize organic compounds as a carbon source in a manner much like chemoheterotrophs do. Utilization of inorganic electron donors and acceptors and an organic carbon source is called mixotrophy. Alternatively, many chemolithotrophs can autotrophically fix CO_2 through use of the Calvin cycle or the TCA cycle; this is known as chemolithoautotrophy.

3.1.6. H_2 oxidation

Hydrogen oxidizing bacteria have been found in a deep subsurface sediment by Fredrickson et al. (22). Hydrogen oxidation is coupled to aerobic respiration in the following overall reaction:

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$2H_2 + O_2 \rightarrow 2H_2O$ (rxn #8)

In surface aquatic environments, where they have been most extensively studied, hydrogen oxidizers are generally found at the interfaces of oxic and anoxic waters. In these areas, H_2 from anaerobic fermentation processes mixes with low concentrations of oxygen, providing the substrates needed for the above energy-conserving reaction.

3.1.7. Oxidation of other reduced species – Mn, P, As, Sb, Mo, U, Se

Other reduced compounds may be used in energy conserving reactions, including manganese, reduced phosphorous, arsenite, antimony, molybdenite, uranium, and selenium. It is not known how important these processes are in the saturated subsurface, but they may be most relevant in systems where high concentrations of these elements are found.

3.2. Anaerobic reduction-oxidation processes

3.2.1. Organics degradation

Organic compounds in anoxic environments may be degraded by fermenters or anerobically respiring microbes. Many fermentation processes degrade high molecular weight organic compounds to organic acids and hydrogen. These fermentation products may be taken up as substrates by anaerobic respirers or methanogens that convert them to carbon dioxide and methane.

3.2.2. Fermentative production of formate, acetate, lactate

Under anaerobic conditions, fermentative processes may play a large role in organics cycling. Fermenters in the subsurface and other environments conserve energy by using an organic compound as an electron donor and either organic compounds or protons as

the electron acceptor. Examples of fermentative processes follow. Here, lactate and ethanol are produced from glucose:

$C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + CH_3CH_2OH + CO_2$ (rxn #9)

In an unrelated reaction, lactate may be transformed to propionate and acetate:

$3CH_3CHOHCOOH \rightarrow 2\ CH_3CH_2COOH + CH_3COOH + CO_2$ (rxn #10)

Protons serve as electron acceptor in the following fermentation of ethanol to acetate:

$CH_3CH_2OH + 2H_2O \rightarrow CH_3COOH + H^+ + 4H_2$ (rxn #11)

Small organic molecules like acetate, propionate, and formate are suitable substrates for anaerobically respiring bacteria. Hydrogen, too, is an extremely important substrate for anaerobic respirers. The passage of these substrates on to anaerobic respirers allows fermenters and respirers to co-exists under oligotrophic conditions.

3.2.3. Iron reduction

Iron may serve as a terminal electron acceptor for bacterial respiration in the absence of oxygen. This process is thought to be relatively important in the saturated subsurface, where mineral iron is often found in high concentrations and oxygen may be depleted. Both autotrophic and heterotrophic iron reducers have been isolated, and potential electron donors include volatile fatty acids, aromatics, and S^0. Ferric iron is reduced in the following overall reaction:

$2Fe^{3+} + 2e \rightarrow 2Fe^{2+}$ (rxn #12)

Iron reduction is responsible for the solubilization of ferric iron in many aquifers, often to such an extent that water treatment facilities must be created specifically for its removal.

3.2.4. Dissimilatory nitrate reduction / denitrification

Due to the paucity of nitrate in pristine groundwater, nitrate reduction is usually considered an important process only in contaminated environments. Contamination may originate from farm runoff or septic leachate or any of a number of different sources. In the absence of the more energetically favorable electron acceptors oxygen and Fe (II), nitrate can be reduced to dinitrogen by a single organism or stepwise by more than one species as some species lack either nitrate reductase or nitrite reductase. Alternatively, nitrate may be reduced to nitrite and then to ammonia in a process referred to as nitrite ammonification. Dissimilatory nitrate reducers use nitrate as an electron acceptor and they may use any of a number of organic metabolites or reduced sulfur such as S^0 or H_2S as an electron donor. The steps of nitrate reduction are as follows.

$NO_3^- + 2H^+ + 2e \rightarrow NO_2^- + H_2O$ (rxn #13)

$NO_2^- + 2H^+ + 2e \rightarrow NO + H_2O$ (rxn #14)

$2NO + 2H^+ + 2e \rightarrow N_2O + H_2O$ (rxn #15)

$N_2O + 2H^+ + 2e \rightarrow N_2 + H_2O$ (rxn #16)

The overall process of nitrite ammonification is:

$NO_2^- + 7H^+ + 6e \rightarrow NH_3 + 2H_2O$ (rxn #17)

It has been thought that denitrification occurs only under anoxic conditions, but Robertson and Kuenen (43) have shown nitrate reduction in *Thiosphaera pantotropha* at atmospheric oxygen concentrations.

3.2.5. Sulfate reduction

Sulfate reduction has been shown to be of great importance to the geochemistry of certain saturated subsurface environments. Sulfate and other oxidized sulfur species are derived from degrading organic matter at the surface or from the microbial process of

sulfur oxidation described previously. Saturated subsurface environments may have high concentrations of sulfate where sulfur minerals like pyrite are present, but most often sulfate is present in small concentrations. When the more energetically favorable electron acceptors oxygen, Fe (III), and nitrate have been depleted from an environment, available sulfate may be used for anaerobic respiration. The overall reaction follows.

$SO_4^{2-} + 5H^+ + 4e^- \rightarrow HS^- + 4H_2O$ (rxn #18)

Sulfate reduction results in the production of sulfide, which is toxic to most organisms, including sulfate reducing bacteria. Dissolved metals like Fe (II), however, will tend to precipitate sulfide, lowering the toxicity.

3.2.6. Methanogenesis

In the absence of any other terminal electron acceptor, CO_2 or acetate may be used for the process of methanogenesis. This can be a significant process in many subsurface systems where electron acceptors have been depleted by high activity of respirative organisms. Stevens and McKinley (54) described the first subsurface ecosystem found in which autotrophic methanogenesis was the dominant metabolism. In methanogenesis, CO_2 produced by respirative organisms is used as a terminal electron acceptor and any of a number of simple organic compounds or H_2 (autotrophic methanogenesis) may be used as en electron donor.

$CO_2 + H_2 \rightarrow CH_4 + 2H_2O$ (rxn #19)

Alternatively, acetate produced by respirers may be cleaved by methanogens in aceticlastic methanogenesis:

$CH_3COOH \rightarrow CH_4 + CO_2$ (rxn #20)

In subsurface systems where methane cannot cycle to an aerobic zone (where it could be aerobically converted to CO_2) the carbon cycle is truncated and natural gas accumulates.

3.3. Other processes not linked to energy conservation

3.3.1. Production of organic and inorganic acids that encourage dissolution

Both heterotrophic and chemolithotrophic microbes can contribute to mineral dissolution by generation of acidic end-products. Heterotrophic metabolism in the subsurface produces organic acids and CO_2 can hydrolyze to form carbonic acid in solution. Some chemolithotrophs form large quantities of sulfuric acid or nitric acid. These acids can react with minerals in the saturated subsurface, bringing about dissolution or alteration of sediments.

3.3.2. Production of siderophores that encourage dissolution

Siderophores, particularly iron-specific siderophores, can cause dissolution of iron minerals. Iron reducers like *Geobacter metallireducens* produce these chelators in order to scavenge insoluble Fe (III) from mineral surfaces. Chelated Fe (III) can be readily utilized for metabolic processes.

3.3.3. Agents of concentration

Microbes may serve as agents of concentration in the subsurface. Certain species actively concentrate inorganic matter intracellularly, as with intracellular sulfur deposition by sulfur bacteria. Passive accumulation of metals may occur on the sheath of bacteria like *Leptothrix* and *Sphaerotilus* and in biofilms. Other species have been found to encourage $CaCO_3$ precipitation outside of the cell (45, 60).

3.3.4. Agents of fractionation

Microbes may act as agents of fractionation by selective uptake of inorganic species. Preferential action may take the form of differential use of metals as terminal electron acceptors, as in the reduction of Mn (IV) before Fe (III). Other activities create stable isotope fractionations. Microbes preferentially utilize light stable isotopes as substrates and electron acceptors. The isotopes which have been shown to fractionate include: ^{32}S and ^{34}S, ^{12}C and ^{13}C, ^{16}C and ^{18}C, ^{14}N and ^{15}N, ^{16}O and ^{18}O.

4. Previously identified aquifer microbes

4.1. Eukarya

4.1.1. Patterns of abundance

Eukaryotes are thought to be present in low numbers in the saturated subsurface. In investigations in which they were observed, eukaryotic populations have been found to be significantly smaller than those of bacteria or archaea (5, 9, 39, 59).

It has been asserted that populations of protozoa rely on the availability and numbers of their bacterial prey (20) and from this fact it has been inferred that the low counts of bacteria in the subsurface are the reason for the low observed numbers of protozoa (39). Sinclair et al. (48) detected protozoa in sediment cores only in samples with bacterial counts greater than 10^4 CFU/gdw. Madsen et al. (39) cultivated relatively high numbers of protozoa in aquifer sediments contaminated with polyaromatic hydrocarbons (PAH's) compared to the counts observed in uncontaminated sediments from the same site (400 protozoa per gram versus <50 per gram). The authors assert that higher bacterial growth rates observed in the contaminated sediments are the cause of the high protozoa counts. Sinclair et al. (47) detected high numbers of protozoa in the unsaturated zone of a fuel-contaminated site. Numbers were also high in the saturated zone where contaminated

sediment met oxygenated groundwater. The authors argue that these observations are the result of high oxygen sensitivity in protozoa. Using MPN techniques to examine protozoa in sediments taken from within and outside of a plume of monoaromatic hydrocarbons, Zarda et al. (61) noted high counts in surface soil, a decrease in numbers in the vadose zone, and an increase in the saturated sediments. Strauss and Dodds (55) observed a steep decrease in protozoa numbers with depth in a profile of a pristine site; counts went from 10^5 protozoa/g dw in surface soils to 392 protozoa/g dw in saturated sediments at a depth of 10.3 meters. Sinclair and Ghiorse (48) observed two patterns in a pristine aquifer in Oklahoma. They noted both a decrease in protozoa counts with depth and that deep layers with high permeability present an exception to this rule in that higher protozoa counts were observed in coarser sediments. Using these observations, the authors speculate that protozoa abundance is affected by pore neck size and forces that carry these organisms from the more heavily populated surface soils.

Only a few investigations have sought to quantify fungal populations in the subsurface. Madsen et al. (39) observed on the order of 10 fungal CFU per gram in contaminated and uncontaminated sediment from up to 10 m below the soil surface. Bone and Balkwill (10) failed to observe yeast and other "eukaryotic forms" in sediment samples from below 0.2 m deep. White et al. (59), failed to detect long-chain polyenoic fatty acids, indicators of eukaryotes, in samples of aquifer clay from 400 meters below the surface. Ludvigsen et al. (36) detected fatty acid esters which they attribute to microfungi or algae in extracts of landfill-leachate contaminated sediment samples taken from 12 m below the surface.

4.1.2. Identity of eukaryotic organisms

The specific identity of subsurface eukaryotes has seldom been investigated beyond rough estimates of identity by morphological examination. Sinclair and Ghiorse (48) noted, using most-probable-number estimates, that ciliates decreased to extinction within 0.5 m of the surface in an uncontaminated site in Oklahoma. Flagellates and amoeba were detected throughout the profile. In examining the culturable protozoa from a hydrocarbon-contaminated plume near Hunxe, Germany, Zarda et al. (61) observed ciliates and naked amoebae, but the dominant protozoa were flagellates. Studies employing molecular techniques to phylogenetically identify subsurface microeukaryoates have yet to be reported in the literature.

4.2. Archaea

4.2.1. Patterns of abundance

While archaea have been isolated from enrichments of subsurface inoculum, few studies have undertaken a description of the *in situ* abundance and activities of archaeal populations in the subsurface. Bekins et al. (8) studied the distribution of several microbial groups, including methanogens, in a contaminated aquifer in Bemidji, Minnesota with most probable number (MPN) techniques. They found that methanogen populations, like the other groups, had a greater affinity for sediment surfaces (85%) than they did for the interstitial space. Furthermore, they detected 10-100 times more methanogens per gram of sediment in the unsaturated zone than in the saturated zone, possibly due to the presence of higher concentrations of nutrients in shallow sediments. It is expected that, in most cases, the forces that determine the distribution of bacteria in aquifers also determine the distribution of archaeal species.

4.2.2. Identity of subsurface archaea

In their study of a contaminated aquifer at the former Wurtsmith Air Force Base near Oscoda, Michigan, Dojka et al. (18) cloned archaeal 16S rRNA sequences from anaerobic, jet-fuel contaminated sediments using both archaeal and universal primer sets. They found archaeal sequences to be related to known methanogens and to an unknown Crenarchaeote. They propose that aceticlastic methanogens detected at the site and a known H_2-producer, *Syntrophus*, also detected, may be involved in a syntrophic association in which H_2 is passed from *Syntrophus* to the methanogens. Chandler et al. (11) derived an archaeal 16S rRNA clone library from a deep subsurface sediment extracted from beneath the Hanford Site in Washington state. The sediment had a relatively high E_h (300 mV) and a high concentration of organic carbon (0.7% wt:wt). The sequences were all most closely related to thermophilic Crenarchaea. The authors speculate that these archaea may oxidize sulfur as their primary mode of energy-conservation. Using taxon-specific oligonuceotide probes, Fry et al. (26) determined that archaeal rRNA comprised 1.8% and 2.5% of the total rRNA in two deep, anaerobic, alkaline aquifer sediments. They assert that these populations are likely to be methanogenic since previous enrichments at the site uncovered methanogenic archaea. Finally, Stevens and McKinley (54) have uncovered a subsurface environment that may be dominated by lithoautotrophic methanogenesis. Using stable carbon isotope ratio measurements, they determined that hydrogen of lithological origins was being incorporated into methane, most likely through the metabolism of archaeal methanogens. It is worth noting that two studies exist (40, 46) in which researchers have used specific tools to detect Archaea in the subsurface (cultivation and rRNA analysis, respectively) but failed to detect the presence of this group.

4.3. Bacteria

4.3.1. Patterns of abundance

Data on the distribution of bacteria in the saturated subsurface is somewhat more extensive than data on *Eukarya* or *Archaea*, but the picture is still incomplete. In studies that measured direct microscopic counts and plate counts in pristine subsurface sediment (9, 10, 40, 49, 59), bacterial numbers have been found to be lower than those observed in the overlying soil. It is largely agreed that the relatively lower concentrations of organic matter and nutrients in sediments below the soil horizons is the largest determining factor for this observation (27). In sediment from a buried coal tar site, Madsen et al. (39) found that chemical contamination resulted in increased numbers of viable bacteria, a phenomenon observed in microbial communities from other environments as well (16, 19). There is conflicting data on the relative numbers of bacteria in unsaturated and saturated subsurface sediments and the results are apparently not correlated to any measured parameter. For example, Beloin et al. (9) found higher levels of microbial biomass indicators in saturated sediment than in the upper zones but Kieft et al. (34) found greater relative numbers of the same or similar indicators in unsaturated sediment at their study site in Washington state. Other studies have failed to uncover a relationship between saturation and bacterial counts (5, 10).

Sediment texture (and hence, permeability) may be a more important influence on relative numbers of bacteria than degree of saturation. Albrechtsen (2) investigated the relative numbers of viable bacteria in the various size fractions of a single aquifer sediment. It was found that although viable numbers are inversely proportional to grain size, a size cut-off exists below which numbers of viable bacteria drop off: the size

fraction 0.2-1.2 um held only 0.01-0.04% of the total numbers of bacteria found in the whole sediment. The highest numbers of viable bacteria per gram of sediment were found in the 1.2-100 um fraction. Fredrickson et al. (23) uncovered the same phenomenon in interleaved layers of sandstone and shale, but expressed their findings in terms of the pore-throat size of the sediments examined. They observed a total elimination of metabolic activity (as measured by ^{14}C-labeled substrate uptake) in layers with pore throat sizes below 0.2 um. They suggest that while inactive cells may be maintained within sediment with low pore sizes, pore throats above 0.2 um are required for sufficient nutrient flux to maintain active metabolism.

It is generally accepted in the field that the vast majority of bacteria in the saturated subsurface are attached to the substratum, rather than suspended in the interstitial space (3, 8), and further investigation has shown that sediment communities are distinct from those of the surrounding groundwater (3, 35). These data suggests that groundwater sampling does not serve as an appropriate surrogate for the more expensive and labor-intensive sampling of subsurface sediment.

4.3.2. Identity of bacteria

Bacteria and 16S sequences previously isolated from the subsurface fall into these phylogenetic divisions: *Proteobacteria, High G+C Gram positive bacteria, Low G+C Gram positive bacteria,* candidate divisions OP5, OP8, OP10, and OP11, *Cytophaga/Flexibacter/Bacterioides, Acidobacteria, Spirochetes,* Termite group I, *Verrucomicrobium,* and *Green nonsulfur.* Figure 1 represents these groups in the context of the currently accepted bacterial divisions and proposed divisions (32) and Table 1 is a lists of these organisms and the most closely related genera, where determined.

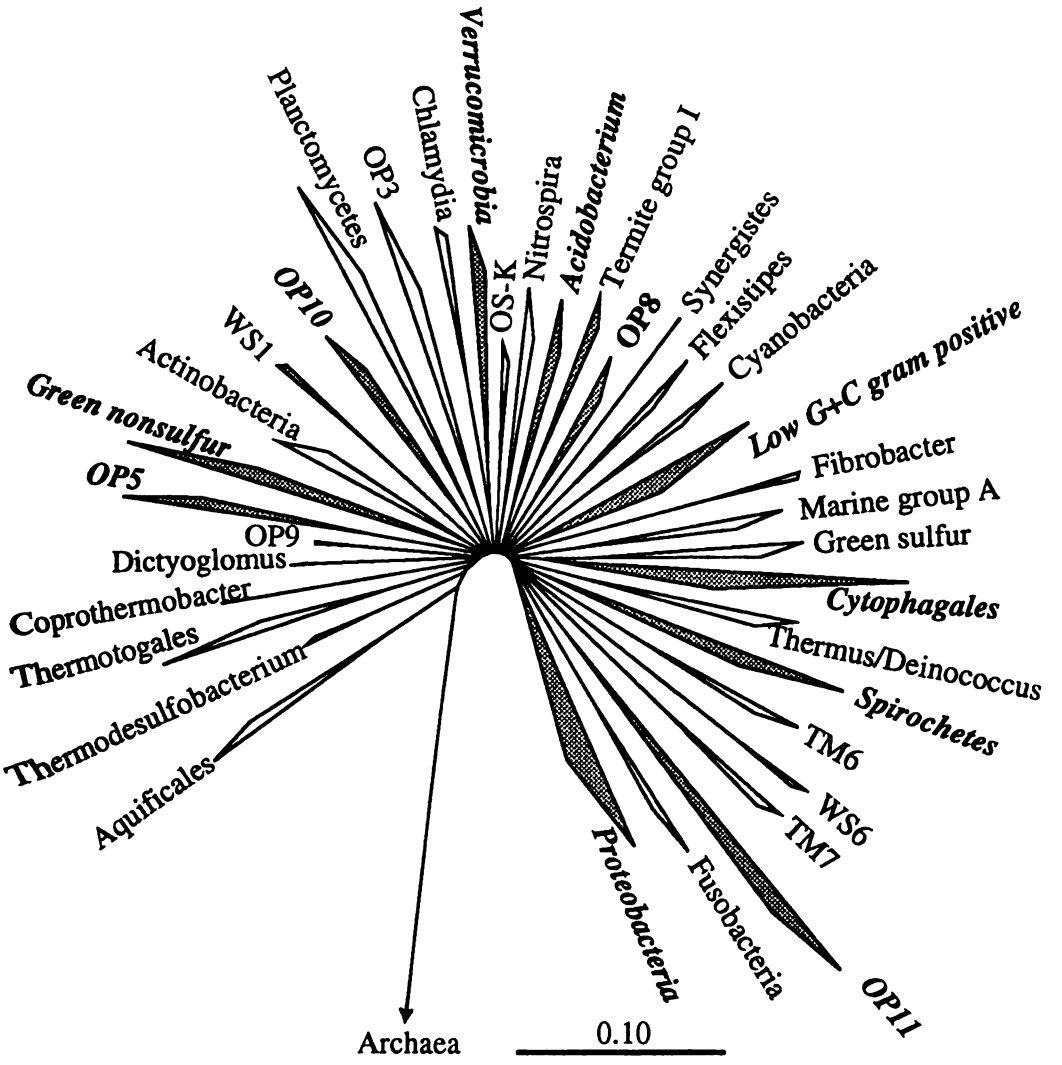

Figure 1 Radial phylogram of selected bacterial divisions. Shaded wedges indicate divisions for which representative isolates or 16S sequences have been recovered from the subsurface. Adapted from Hugenholtz et al. (32).

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Table 1 Bacterial isolates and 16S clones previously derived from the subsurface. The division (or putative division) and genera are listed if determined for a given isolate or sequence. All isolates and clones were phylogenetically identified by sequencing of the 16S gene, except isolates developed by Fries et al (24), which were identified through fatty acid methyl ester (FAME) analysis.

Division or putative division	Genera (if determined)	isolate or clone	Method of analysis	reference
α Proteobacteria	*Erythromicrobium*	16S clone	sequencing	(13)
α Proteobacteria	*Methylobacterium*	isolate	FAME	(24)
α Proteobacteria	*Sphingomonas*	isolate	sequencing	(6)
α Proteobacteria	*Sphingomonas*	isolate	sequencing	(21)
α Proteobacteria	*Sphingomonas*	16S clone	sequencing	(53)
α Proteobacteria		16S clone	sequencing	(18)
α Proteobacteria		16S clone	sequencing	(18)
β Proteobacteria	*Azoarcus*	16S clone	sequencing	(18)
β Proteobacteria	*Burkholderia*	16S clone	sequencing	(13)
β Proteobacteria	*Burkholderia*	16S clone	sequencing	(53)
β Proteobacteria	*Burkholderia*	isolate	sequencing	(62)
β Proteobacteria	*Comamonas*	isolate	sequencing	(6)
β Proteobacteria	*Comamonas*	16S clone	sequencing	(13)
β Proteobacteria	*Comamonas*	isolate	FAME	(24)
β Proteobacteria	*Duganella*	16S clone	sequencing	(18)
β Proteobacteria	*Hydrogenophaga*	isolate	FAME	(24)
β Proteobacteria	*Janthinobacterium*	isolate	FAME	(24)
β Proteobacteria	*Ralstonia*	16S clone	sequencing	(53)
β Proteobacteria	*Variovorax*	isolate	sequencing	(6)
β Proteobacteria	*Variovorax*	isolate	FAME	(24)
β Proteobacteria		16S clone	sequencing	(18)
δ Proteobacteria	*Desulfovibrio*	16S clone	sequencing	(41)
δ Proteobacteria	*Syntrophus*	16S clone	sequencing	(18)
δ Proteobacteria		16S clone	sequencing	(18)
γ Proteobacteria	*Acinetobacter*	isolate	sequencing	(6)
γ Proteobacteria	*Acinetobacter*	isolate	FAME	(24)
γ Proteobacteria	*Acinetobacter*	16S clone	sequencing	(41)
γ Proteobacteria	*Actinobacillus*	isolate	FAME	(24)
γ Proteobacteria	*Legionella*	16S clone	sequencing	(18)
γ Proteobacteria	*Methylomonas*	isolate	sequencing	(33)

Table 1 *Continued*

Division or putative division	Genera (if determined)	isolate or clone	Method of analysis	reference
γ Proteobacteria	*Shewanella*	16S clone	sequencing	(41)
γ Proteobacteria	*Thiomicrospira*	16S clone	sequencing	(41)
Proteobacteria	*Pseudomonas*	isolate	sequencing	(6)
Proteobacteria	*Pseudomonas*	16S clone	sequencing	(13)
Proteobacteria	*Pseudomonas*	isolate	FAME	(24)
Proteobacteria	*Pseudomonas*	16S clone	sequencing	(53)
Hi G+C gram positive	*Arthrobacter*	isolate	sequencing	(6)
Hi G+C gram positive	*Clavibacter*	16S clone	sequencing	(11)
Hi G+C gram positive	*Corynebacterium*	isolate	FAME	(24)
Hi G+C gram positive	*Micrococcus*	16S clone	sequencing	(13)
Hi G+C gram positive	*Micrococcus*	isolate	FAME	(24)
Hi G+C gram positive	*Nocardia*	16S clone	sequencing	(13)
Hi G+C gram positive	*Nocardia*	isolate	FAME	(24)
Low G+C gram positive	*Bacillus*	isolate	sequencing	(6)
Low G+C gram positive	*Bacillus*	16S clone	sequencing	(13)
Low G+C gram positive	*Bacillus*	isolate	FAME	(24)
Low G+C gram positive	*Bacillus*	16S clone	sequencing	(41)
Low G+C gram positive	*Bacillus*	16S clone	sequencing	(53)
Low G+C gram positive	*Desulfosporosinus*	isolate	sequencing	(44)
Low G+C gram positive	*Desulfotomaculum*	16S clone	sequencing	(18)
Low G+C gram positive	*Eubacterium*	16S clone	sequencing	(41)
Low G+C gram positive	*Exiguobacterium*	16S clone	sequencing	(18)
Low G+C gram positive	*Staphylococcus*	isolate	FAME	(24)
Low G+C gram positive	*Streptococcus*	isolate	sequencing	(6)
Low G+C gram positive		16S clone	sequencing	(18)
OP10		16S clone	sequencing	(18)
OP11		16S clone	sequencing	(18)
OP5		16S clone	sequencing	(18)
OP8		16S clone	sequencing	(18)
Cytophaga/Flexibacter/ Bacterioides	*Flavobacter*	16S clone	sequencing	(53)
Cytophaga/Flexibacter/ Bacterioides		16S clone	sequencing	(18)
Cytophaga/Flexibacter/ Bacterioides	*Chryseobacterium*	16S clone	sequencing	(18)

Table 1 *Continued*

Division or putative division	Genera (if determined)	isolate or clone	Method of analysis	reference
Acidobacteria		16S clone	sequencing	(7)
Acidobacteria		16S clone	sequencing	(18)
Spirochetes		16S clone	sequencing	(18)
Termite group I		16S clone	sequencing	(18)
Verrucomicrobium		16S clone	sequencing	(18)
Green nonsulfur		16S clone	sequencing	(18)

5. Methods for analysis of microbial communities

There are a number of methods available to study the microbial communities of the saturated subsurface. Methods of community analysis are generally divided between those which require cultivation and those which do not. Each method has its strong points and its limits. In deciding which method to use, a researcher has to balance these pros and cons and arrive at a choice that targets his or her objective the best. Some of the more commonly used approaches are listed here.

5.1. Cultivation and characterization of isolates

The cultivation of subsurface microbes offers one route to describing the diversity of the community. Isolates can be metabolically characterized to determine what biogeochemical processes may be important in the ecosystem. Alternatively, the substrate range of isolates may be determined to elucidate possible pathways present in the system. Drawbacks include the possibility that isolates are poorly representative of the metabolically active members of the community, since it has been reported that only 1-10% of environmental strains can be cultivated (52) and the fraction of culturable bacteria in aquifer sediment can vary from sample to sample within the same formation (5). Hence, isolates derived from the subsurface may, in fact, be representative of the dominant metabolic groups present, but this has not been tested to date.

5.2. Analysis of functional genes

Testing for the presence of certain functional genes in the subsurface, whether they are extracted in community DNA or recovered from the genomes of isolated organisms, is another approach to understanding the metabolic features of the subsurface microbial

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community. Functional genes can be used as indicators of certain processes, like denitrification or toluene degradation for example, and the presence of these genes can be tested in samples taken directly from the environment or from enrichment cultures (30). One drawback to using this method is that in order to detect a given functional gene it must have been previously identified and characterized, which can be laborious. Another point of concern lies in the fact that detection of a given gene does not necessarily mean that it is expressed under the current conditions. Furthermore, metabolic processes that are not specifically targeted will not be detected, so pathways of importance may be missed in this type of analysis.

5.3. Analysis of the metabolism of radio-labelled substrates or electron acceptors

The use of radioactively-labelled substrates or electron acceptors can be used to investigate the metabolism of subsurface microbial communities on a bench scale or a field scale. For example, Fredrickson et al. (23) used [^{14}C] acetate, [^{14}C] glucose, and $^{35}SO_4^{2-}$ in sediment microcosm studies to determine the viability and the sulfate reduction capacity of the subsurface community. One drawback to using labeled substrates in microcosm experiments is that one can only confirm the *potential* for a given metabolic process, since *in situ* rates of a given reaction, given ambient concentrations of the substance of interest, are not measured.

5.4. Analysis of membrane components – PLFA and FAME

Fatty acids and phospholipids found in the membranes of all microbes can be employed in analyzing the composition of the subsurface community. In phospholipid fatty acid (PLFA) analysis and fatty acid methyl ester (FAME) analysis, the lipids in a given sediment are extracted and the polar lipids are separated from the mixture and analyzed

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(PLFA) or the lipids are esterified and analyzed (FAME). Signature molecules have been identified that can indicate the presence of certain microbial groups (25). However, this method provides only a low degree of resolution and allows the researcher to determine the relative abundances of only those groups that have been cultivated, characterized, and have distinct membrane lipids. Also, sample size considerations may be prohibiting, as cell counts in subsurface sediment are often so low that large quantities of sediment are needed for the extraction (>75 g) which may be expensive to acquire.

5.5. Analysis of small subunit rRNA or small subunit rDNA

The small subunit (SSU) rRNA gene and its transcripts, 16S rRNA in bacteria and archaea and 18S rRNA in eukaryotes, are employed in many different methods of community analysis. The SSU rRNA molecule serves in the ribosome as part of the machinery of translation and its function has been found to be highly conserved among all living organisms. There are a number of advantages to using 16S and 18S over other molecular markers. Firstly, the SSU gene is present in all organisms. This means that having the 16S sequence of a given organism allows researchers to place that organism within the context of the universal tree of life. Also, some parts of the molecule are highly conserved, so large evolutionary distances between kingdoms can be resolved, and other parts are less conserved, allowing for finer resolution of genera and species. Furthermore, the 16S molecule holds a great deal of phylogenetic information: each of the 1500+ nucleotide bases has four possible identities (A, G, C, U). The 16S gene is not translated into protein, so its sequence is not subject to the restrictions and complications which translation into amino acids imposes. Finally, there are extensive databases of known 16S sequences available (including the Ribosomal Database Project

http://www.cme.msu.edu/RDP/html/, and GenBank, available through the National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/) which can be used for comparing or identifying newly discovered 16S sequences.

There are drawbacks to the use of the 16S gene in community analysis. Biases may be introduced by the polymerase chain reaction (PCR), which is often used to amplify the community 16S sequences. Artifacts of the reaction may include preferential amplification of minority sequences, fidelity problems in polymerization, chimeric sequences, and founder effects (12, 51, 56, 57). Furthermore, 16S results may be difficult to extrapolate to the environment because of lack of agreement between phylogenetic identity and the metabolism of the organism (1). This is certainly a consideration when employing 16S methods, but many phylogenetic groups (species, genera, and divisions) have been shown to have consistent metabolic traits, allowing conjecture about the processes that closely related organisms undertake (18). Also, 16S rDNA extracted from environmental samples (soil,sediment, or water, for example) may have been derived from dead or inactive cells, as it is unclear exactly how long DNA remains stable in the environment. Each of these limitations must be acknowledged when using the 16S gene in community analysis.

5.5.1. Amplified ribosomal DNA restriction analysis

In amplified ribosomal DNA restriction analysis (ARDRA) of microbial communities, 16S genes in community DNA are amplified through the PCR and digested with a restriction enzyme. The resulting mixture of long and short fragments are separated by electrophoresis to produce a profile of the 16S genes from the sample. ARDRA allows the researcher to compare microbial communities from sample to sample, possibly to

monitor the presence or absence of certain ribotypes or to observe whether the microbial community at large is affected by a particular event (25). One drawback of this method is the difficulty in identifying individual members of the community in a given community profile. A single 16S type creates an unknown number of bands, so a band cannot be correlated, with certainty, with a given ribotype.

5.5.2. Denaturing gradient gel elctrophoresis analysis

In denaturing gradient gel electrophoresis, or DGGE, 16S genes from the community are amplified through PCR and separated on an acrylamide gel. A chemical denaturant in the gel causes the 16S genes to be separated according to their base pair sequence. This method allows many of the same analyses afforded by ARDRA analysis and provides a less complicated community profile with a one-band-to-one-ribotype ratio. However, DGGE is subject to poor reproducibility due to the vagaries of assebling the gradient gel.

5.5.3. Fluorescent in situ hybridization analysis

Fluorescent in situ hybridization microscopy, or FISH, is a method of analysis in which cells are washed from the sample (sediment, in this case) and are stained with fluorescently-tagged rRNA probes. The stained cells are viewed with an epifluorescence microscope which allows the user to apply varying wavelengths of incident light to cause fluorescence of the chemical tags attached to the rRNA probes. The result is a series of images in which cells that are tagged with each probe used are highlighted. The specificity of the probes is the key: they can be designed to target groups from the Kingdom level to the strain level. Using this method, one can determine the numbers and relative proportions of different phylogenetic groups within a sample. One drawback of FISH is that the only groups that are identified and enumerated in a given sample are

those which the researcher has specifically targeted. For example, a particular division which comprises a large part of the community will not be enumerated if the researcher doesn't know in advance to target it and design a specific probe for it. Furthermore, some groups lack a consistent signature sequence in the 16S gene, preventing the design of a probe to specifically target them.

5.5.4. rRNA hybridization analysis

The hybridization techniques in FISH were derived from earlier work with rRNA hybridization in which whole rRNA from the microbial community is extracted and attached to a membrane. The community rRNA is exposed to radioactively labeled phylogenetic rRNA probes, and the amount of hybridization of a probe to the community RNA is quantified. The concentration of these probes that attach to a given community sample is proportional to the number of target sequences in the sample. Using rRNA hybridization, the relative proportions of different phylogenetic groups in the community can be established. This method has the same drawbacks as FISH, namely, that untargeted phylogenetic groups and groups for which a probe cannot be designed are not detected.

5.5.5. Cloning the 16S gene

The 16S DNA from a sediment sample can be amplified (with the PCR), cloned into *Escherichia coli*, and analyzed to determine the identities of individual community members. This method has proven to be a powerful tool in the discovery of previously unknown bacteria and archaea and in assessing the distribution of known species. The drawbacks to using 16S in community analysis (as described above) clearly apply to the

use of cloning, but it has allowed many researchers to explore the variety of community members in the subsurface.

5.5.6. Terminal restriction fragment length polymorphism analysis

In terminal restriction fragment length polymorphism (T-RFLP) analysis, the 16S genes from a microbial community are amplified using a primer that is fluorescently labeled at the 5' end. The amplified genes are then digested with a restriction enzyme and size-separated by acrylamide gel electrophoresis. The terminal 16S fragments are fluorescently tagged and can be detected and compiled to create a profile of the microbial community. The length of any particular terminal fragment is dependent upon the sequence of the original 16S gene, and the 16S sequence is dependent upon the identity of the organism from which it was derived. So, in addition to the comparative analysis that ARDRA allows, T-RFLP profiles can be analyzed using databases of 16S sequences. The 16S sequences in these databases can be digested with restriction enzymes *in silico* to determine the terminal 16S restriction fragment lengths of known species. Peaks in T-RFLP profiles can be correlated to the restriction fragment lengths of known species or phylogenetic groups. T-RFLP analysis is subject to the limitations inherent to all 16S methods, but it can be a useful tool for comparative community analysis.

5.5.7. Real-time PCR of SSU genes or functional genes

Real-time PCR is a molecular method in which the numbers of a targeted DNA sequence are quantified by comparing the progress of a reaction containing an unknown number of target sequences with the progress of standard reactions. The method has been used to quantify a range of DNA sequences, including nitrite reductase (28), glycoprotein D of human immunodefficiency virus (31), meningococcal-specific genes IS1106, ctrA, and

siaD (29), and large (37) and small subunit rRNA (17, 37, 42, 58). The method has great potential for investigating microbial community structure through quantifying SSU genes and functional genes in environmental samples, but it is not yet widely used for this purpose. The drawbacks of analyzing the distribution of functional genes and SSU genes apply to real-time PCR.

6. Specific aims of this work

To achieve a better understanding of subsurface microbial communities, we have undertaken a comparative analysis of the communities in pristine and chemically-contaminated aquifer sediment by dissecting the communities of two different sites using molecular, cultivation-independent approaches.

The specific aims of these projects were as follows:

1. Determine the community structure in several samples of pristine aquifer sediment using T-RFLP of PCR amplified 16S rRNA genes.
2. Describe members of the bacterial community in a pristine subsurface sediment by cloning and sequencing selected 16S sequences.
3. To use T-RFLP to assess the community-level differences between contaminated and uncontaminated sediment sediments derived from the Bachman aquifer. Intrinsic bioremediation of tetrachloroethylene (TCE) has been shown to occur within the plume at this site.
4. To describe members of bacterial communities in these clean and contaminated sediments by cloning 16S genes from the site.
5. To elucidate the relative abundance of a 16S clone which has been recovered from contaminated sediment at the Bachman site using real-time PCR.

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CHAPTER 2

A CULTIVATION-INDEPENDENT ANALYSIS OF BACTERIAL COMMUNITIES IN PRISTINE AQUIFER SEDIMENT

INTRODUCTION

Groundwater is an important resource both economically and ecologically, and the microbial communities that reside in the saturated subsurface play an integral role in the quality of this resource. As groundwater filters through subsurface sediment, microbes metabolize soluble organic and inorganic compounds, immobilizing or mineralizing them and improving water purity (36). Subsurface microbes are also responsible for the degradation of anthropogenic contaminants in groundwater (3, 31, 41).

To date, numerous studies of subsurface microbial communities have been carried out, but by no means do we have a complete description of life in the saturated subsurface. Early work has shown that life in saturated sediment is strictly microbial, prokaryotic, and adapted to oligotrophic conditions. Most studies have examined the cultivable fraction of the microbial community (6-8, 17, 19, 20, 23, 26, 27, 30, 38). While these studies have provided a degree of insight into the physiology and numbers of certain subsurface organisms, they are severely limited in the wider significance of their findings because the culturable members do not necessarily reflect the entire community or even a substantial fraction of the community (11, 16, 34, 39). These studies have shown, however, that subsurface microbes represent a high degree of physiological and morphological diversity (6, 7, 17, 23, 30, 38). Other investigations have contributed

information about abundance patterns in the subsurface, and they have begun to correlate aquifer geochemistry and population abundance (6, 7, 17, 19, 20, 23, 30, 38). Specific information on the phylogenetic groups and metabolic types present in uncontaminated subsurface sediments is severley limited, however.

We have undertaken an analysis of the microbial communities in a pristine aquifer on the eastern shore of Virginia, where sediments range from aerobic and oligotrophic to microoxic and relatively organic-rich. We have employed two nucleic acid-based techniques, 16S rRNA T-RFLP and cloning, in an effort to compare and describe these communities. While we observe similarities between the bacterial communities from the two sites, there are also strong differences. Clones from the aerobic zone are phylogenetically diverse and most show little homology with known species. We have also detected two rDNA clones that show little relatedness to known phylogenetic divisions and are deeply rooted in the bacterial phylogenetic tree.

MATERIALS AND METHODS

Sample collection The study site is located in Oyster, VA. Two areas of the aquifer were sampled: the narrow channel focus area (NCFA) and the south Oyster focus area (SOFA) (Figure 1). A single intact core was aseptically collected from each sampling point indicated in the diagram from the NCFA in October 1998 and from the SOFA in April 1999. Cores were placed in plastic liners, divided, placed in gamma-irradiated polypropylene tubes, and held at 4° C for seven days, after which they were stored at -80°C. The sediment samples from the NCFA and SOFA were collected from 6.5 – 8.0 and 4.9 – 7.0 meters below the ground surface, respectively.

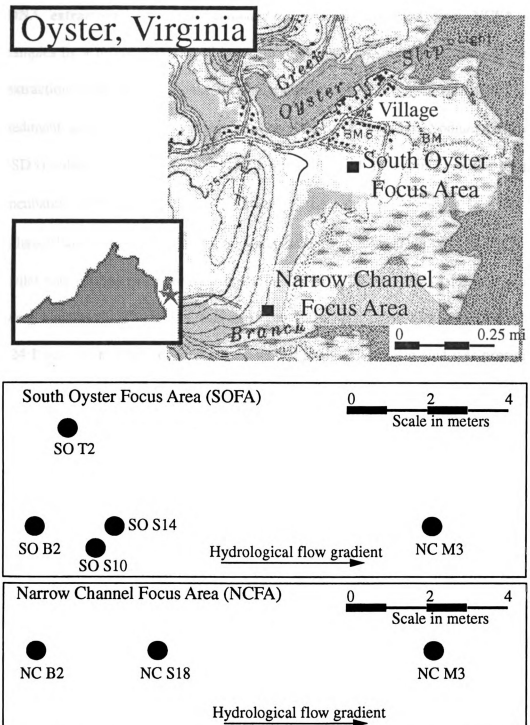


Figure 1 Map of Oyster, Virginia, indicating the locations of the south Oyster focus area (SOFA) and the narrow channel focus area (NCFA). Below, the areas of the SOFA and the NCFA have been expanded to indicate the positions of sampling points.

DNA extraction Microbial community DNA was extracted from NCFA sediment samples by a protocol described by Zhou et al. (44) with modifications. Briefly, 15 ml extraction buffer and proteinase K (final concentration 0.04 mg/ml) was added to 15 g of sediment, and the mixture was incubated at 37°C for 30 minutes. Sodium dodecyl sulfate (SDS) solution was added for a final concentration of 18 mg/ml, and the mixture was incubated at 65°C for 2 hours, followed by three cycles of freezing in a dry ice and ethanol bath and thawing in a 65°C water bath. The supernatant was collected and the pellet was washed twice with extraction buffer and SDS and the washes and supernatant were combined. This liquid was extracted twice with chloroform and isoamyl alcohol (24:1 vol:vol) and the aqueous phases were combined. Nucleic acid was precipitated by the addition of 0.6 volume of isopropanol, then resuspended in water and precipitated by the addition of 2 volumes of ethanol. The pellet was resuspended in modified TE buffer (10 mM Tris-HCL, 0.1 mM EDTA, pH 8) and used in polymerase chain reactions.

DNA was extracted from SOFA sediment samples with a Soil DNA Kit Mega Prep (MoBio) according to the manufacturer's instructions. We used 10 g of sediment for each extraction. Isopropanol (0.7 volume) was added to the final DNA elution, and the solution was incubated at 4°C overnight. The solution was centrifuged at 12,000xg for 30 min, the supernatant was decanted, and the pellet was resuspended in 200 ul of water. The DNA was reprecipitated by addition of 0.15 volume of 3 M sodium acetate and 2 volumes of ethanol. The solution was stored at -20°C overnight. Community DNA was pelleted by centrifugation at 12,000xg for 30 min. The pellet was dried and resuspended in 50 µl of water, and stored at -20°C until needed.

16S rDNA T-RFLP As sediment samples from the two sites were extracted by different methods, the amount and purity of the genomic DNA in the final extraction volume differed as well. Accordingly, the T-RFLP amplification mixture for each sample set was adjusted independently.

NCFA sediment samples NC B2 (8 m), NC S18 (6 m), NC S18 (8 m), and NC M3 (6 m) were used for T-RFLP analyses. Bacterial 16S ribosomal genes were amplified from bulk DNA in reaction mixtures that contained 1X PCR buffer (Perkin Elmer), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mM of the reverse primer, 0.5 mM of the forward primer, 8 ng bovine serum albumin per ul, approximately 0.08 ng/ul of template DNA, and 0.02 U of AmpliTaq (Perkin Elmer) per ul.

SOFA sediment samples SO B2 (5 m), SO B2 (6 m), SO T2 (5 m), SO T2 (6 m), SO S14 (5 m), SO S14 (6 m), SO M3 (6 m), and SO S10 (4 m). For T-RFLP of the SOFA DNA, bacterial 16S genes were amplified in the same manner as described for the NCFA samples, with the following exceptions: the forward and reverse primer concentrations were both 0.25 uM, the template concentration was 0.8 ng/ul and the AmpliTaq concentration was 0.05 U/ul.

The forward primer for all reactions, 27F, which is specific for bacteria (5'-AGA GTT TGA TCC TGG CTC AG-3') (24), was labeled at the 5' end with the phosphoramidite dye 5-hexachlorofluorescein ("hex-labeled"). The reverse primer used in SOFA amplifications was the universal primer 1392R (5'-ACG GGC GGT GTG TRC-3')(32). Amplifications of NCFA DNA contained either 1392R or the universal primer 1525R (5'-AAG GAG GTG ATC CAG CC-3') (5).

Reaction mixtures were incubated in a GeneAmp 2400 PCR System thermal cycler (Perkin Elmer) at 94°C for 3 minutes, followed by 35 cycles at 94°C for 45 seconds, 60°C for 30 seconds, and 72°C for 90 seconds and a final extension step of 72°C for 10 minutes.

We prepared three replicate amplifications of each NCFA DNA sample and two replicates of each SOFA DNA sample. Replicate reaction mixtures were combined and purified using Wizard® PCR purification columns (Promega) and eluted with a final volume of 50 ul of modified TE buffer (0.1 mM EDTA, 10mM Tris, pH 8.0). For restriction digests, 200 ng of purified PCR product were digested with 15 U of *Hha*I, *Msp*I, or *Rsa*I (BMB) at 37°C for 3.5 hours. For electrophoresis, 1 ul of this mixture was loaded on an acrylamide slab gel. The lengths of the terminal restriction fragments from the amplified rDNA products were determined using an ABI Prism 377 DNA sequencer and ABI software (PE Applied Biosystems) as described by Liu et al. (25). In order to eliminate primer fragments from detection and to avoid inaccurate sizing of long fragments, we excluded all fragments smaller than 30 bp and larger than 600 bp from the analysis.

TRFLP analyses T-RFLP profiles were analyzed using GeneScan 3.1 software (PE Applied Biosystems). For enumerating the restriction fragments in each profile, a fluorescence intensity threshhold was set at 50 so that only fragments greater than this intensity were included in further analysis. Comparisons between T-RFLP profiles were conducted using the Genotyper software package (version 2.5, Perkin Elmer). Fragments shorter than 30 base pairs (bp) or larger than 600 bp were excluded from analysis in order to eliminate primer artifacts and to avoid the problems associated with identifying the

length of large fragments. The *Hha* I profiles were compared using the T-RFLP Profile Analysis tool through the Ribosomal Database website (http://www.cme.msu.edu/RDP/cgis/trflp.cgi?su=SSU), which determines the percent similarity between two profiles by dividing the the number of terminal fragments present in both profiles by the number of fragments in the profile with fewer fragments.

Cloning of 16S rRNA genes Sediment from the narrow channel site was selected for 16S rDNA cloning as this site represented a typical pristine, oligotrophic, aquifer. Three sediments were used for cloning experiments: S18 (6 m), S18 (8 m), and B2 (8 m). Bacterial 16S ribosomal genes were amplified from bulk DNA in reactions that contained 1X PCR buffer (Perkin Elmer), 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 mM of each primer, 8 ng bovine serum albumin per ul, and 0.02 U of AmpliTaq (Perkin Elmer) per ul. The forward primer was 27F and the reverse primer was 1392R. The amount of template in each amplification and the cycling conditions were the same as those used for T-RFLP reactions. The PCR products were cloned using a TOPO TA Cloning Kit (Invitrogen Corp.) in accordance with the manufacturers instructions. Plasmid DNA was extracted and purified with a Qiagen Mini Plasmid-prep kit (Qiagen).

Screening of rDNA clones by ARDRA The plasmid inserts of approximately 40 clones from each library were amplified using the PCR conditions described for cloning, with roughly 30 ng of purified plasmid DNA template per 25 ul reaction. Five ul of rDNA PCR products were digested with 10 U of the 4-base specific restriction enzyme *Cfo* I in 1X NEB buffer (New England Biolabs) in a final volume of 15 ul for 3 hrs and 30 min at 37°C. Digested DNA fragments were separated by acrylamide gel (10% acrylamide) electrophoresis and visualized by silver staining. Restriction fragment lengths were

estimated by comparison with molecular weight standards which ranged from eight to 587 bp (DNA molecular weight marker V2, Roche Molecular Biochemicals), and plasmids with unique patterns were selected for partial sequencing of the rDNA insert.

Sequencing of rDNA clones Plasmid inserts from selected rDNA clones were amplified using the same conditions described above for cloning. Amplified rDNA inserts were purified by using Ultrafree MC Millipore 30,000 NMWL filter units (Millipore) according to the manufacturer's instructions. Sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Reaction Kit and an ABI Prism 377 DNA sequencer (PE Applied Biosystems) according to the manufacturer's directions. Primers for sequencing included 27F, 355F (5'-ACT CCT ACG GGR SGC AGC-3') (4), 536R (5'- GWA TTA CCG CGG CKG CT –3') (33), 1100R (5'-AGG GTT GCG GTG GTT G-3') (ref. TLM?), and 1392R. Fifty-two (52) clones were partially sequenced using 27F and five clones that were of particular phylogenetic interest were fully sequenced.

Phylogenetic analyses All sequenced clones were checked for chimeric sequences using the Ribosomal Database Project II CHIMERA_CHECK version 2.7 (http://www.cme.msu.edu/RDP/html/index.html). Seven chimeras were detected among the sequences and were eliminated from further analysis. Sequences were aligned against close relatives in the Ribosomal Database Project II release 8.0 (28) using Arb software (40). Alignments were refined by visual inspection and percent similarity to known isolates and previously cloned sequences was determined. For analysis, a mask was generated to exclude highly variable regions where the alignment was uncertain or where one or more of the sequences had an alignment gap. All presented dendrograms were

constructed using the fastDNAml (maximum likelihood) function in the Arb software package (40). The robustness of tree topologies was tested through 100-replicate bootstrap resampling of sequences using evolutionary distance and the optimality criteria of maximum likelihood and maximum parsimony (PAUP* version 4.0b8, written by David L. Swofford). Evolutionary distance variables and corrections were selected through the use of Modeltest (35).

RESULTS

Description of the Study Site The site is located in the village of Oyster, Virginia, on the Delmarva Peninsula. The aquifer underlying Oyster is a shallow, unconfined formation and is currently the focus of a study on bacterial transport by the U.S. Department of Energy's Natural and Accelerated Bioremediation Research Program. Two areas were sampled for this study: the narrow channel focus area (NCFA) and the south Oyster focus area (SOFA) (Figure 1). The NCFA lies to the south-southwest of the village of Oyster. The water in this part of the formation was oxic (5.5 mg O_2/l) and low in organic carbon (1000 ppb). The SOFA lies directly to the south of Oyster. Here, the groundwater was hypoxic (0.52 mg O_2/l) due to upwelling suboxic groundwater and was comparatively high in organic carbon (36,000 ppb). The water table at both sites was roughly 1.5 to 2.0 meters below the ground surface.

16S rDNA T-RFLP We were able to identify up to 39 terminal fragments per T-RFLP profile of Oyster sediment bacterial communities. On average, *Hha* I and *Msp* I digestions produced more bands per profile (20 and 21, respectively), than did *Rsa* I (16). See Table 1 for a list of the number of terminal fragments in each *Hha* I T-RFLP profile.

Table 1 Total number of terminal fragments detected in *Hha* I bacterial T-RFLP profiles from Oyster, Virginia

South Oyster sediment	number of terminal fragments
SO B2 (5 m)	21
SO T2 (4 m)	18
SO T2 (6 m)	15
SO S14 (5 m)	16
SO S14 (6 m)	23
SO S10 (4 m)	16
SO M3 (6 m)	8
Narrow channel sediment, primer set	
NC B2 (6 m), 8F/1392R	29
NC S18 (6 m), 8F/1392R	18
NC S18 (8 m), 8F/1392R	25
NC M3 (6 m), 8F/1392R	20
NC B2 (6 m), 8F/1525R	22
NC S18 (6 m), 8F/1525R	39
NC S18 (8 m), 8F/1525R	13
NC M3 (6 m), 8F/1525R	14

Within the SOFA samples we can identify five profiles which are highly similar. Each of the eight terminal fragments present in the SO M3 (6 m) profile are present among the fragments in four other profiles: SO B2 (5 m), SO S14 (5 m), SO T2 (4 m) and SO T2 (6 m). The *Msp* 1 and *Rsa* 1 digestions of these samples also display a high degree of similarity (data not shown). Profiles SO T2 (6 m) and SO S14 (5 m) are also highly analogous (93% identical fragments). Overall, each of the south Oyster profiles showed no less than 40% identity with any of the other profiles.

Within the narrow channel samples, we note that the NC M3 (6 m) profiles are markedly divergent from the other profiles. The 27F/1525R NC M3 (6 m) profile, in particular, has a maximum of 28% similarity with other narrow channel profiles.

There was a degree of similarity between the SOFA and NCFA community profiles. Terminal restriction fragments common to both sites included: *Hha* I 206 bp, 369 bp, 567 bp, and 573 bp, *Msp* I 494 bp, *Rsa* I 119 bp and 475 bp. Profiles from the two sites shared as many as 50% of the same fragments.

Phylogenetic analysis The phylogenetic diversity of the bacterial community in the narrow channel study site was determined through the analysis of three clone libraries of 16S ribosomal RNA genes. Figure 1 is a schematic diagram of the focus area and the points from which sediment was extracted for use in the clone study. Bulk DNA was extracted from aseptically-collected sediment samples and primers specific for bacterial 16S sequences were employed to amplify the sequences of interest. In order to select unique 16S clones for sequencing, 130 clones were screened using ARDRA (10). The number of unique ARDRA patterns from each library was quite high: 26 out of 40 screened in the NC S18 (6 m) library (65%), 39 / 45 in the NC S18 (8 m) library (87%),

and 31 / 35 in the NC B2 (8 m) library (89%). There was a degree of overlap between libraries, and we were able to discern 89 different ARDRA patterns among the 120 clones screened. We sequenced 15 clones from NC S18 (6 m), 4 clones from NC S18 (8 m), and 12 clones from NC B2 (8 m).

Table 2 lists the phylogenetic affiliations of the narrow channel focus area clones as determined using the Arb software package with the RDP II 8.0 database and lists the closest 16S sequence match in the GenBank database as determined by a BLAST search. Figure 2 (a-e) is a series of maximum likelihood dendrograms depicting the phylogenetic placement of 18 of these SSU rDNA clones.

There are a number of clones that may be representatives of novel phylogenetic divisions. Two clones, in particular, cluster together but fail to show a distinct association with any known phylogenetic group (Figure 2e). These clones are apparently deeply rooted in the bacterial tree and in Figure 2e they are shown in the context of other deeply rooted genera and several later-diverging species. We will refer to these clones as "NCFA group I". None of the cloned sequences in this study are identical to known species or previously discovered sequences.

DISCUSSION

Many studies of bacterial communities in uncontaminated subsurface environments have failed to evaluate the phylogenetic diversity of these communities as they have been limited by the bottleneck of cultivation, which prevents detection of species that resist isolation. This can be a significant limitation when one considers that 90-99% of environmental species may be uncultivable (39) and that this number may be

Table 2 Phylogenetic placement of bacterial 16S sequences from the narrow channel four mat in Octopus Spring

Table 2 Phylogenetic placement of bacterial 16S sequences from the narrow channel focus area in Oyster, Virginia

Clone	Occurrence of ARDRA pattern in clone libraries (%age) S18 (6 m)	S18 (8 m)	B2 (8 m)	Division, subdivision	Closest relative in the RDP 8 database	closest GenBank relative	Score
NCFA 1	3			β proteobacteria	92% str. 1090	Uncultured beta proteobacterium SBR1021	624
NCFA 2	8			β proteobacteria	93% Nitrosomonas marina3	clone WR1100	862
NCFA 3	13	7		Clone T78 group		strain BD3-16	642
NCFA 4	3			δ Proteobacteria		Uncultured bacterium GR-WP33-30	420
NCFA 5			3	δ Proteobacteria		Uncultured eubacterium TRA1-10	297
NCFA 6		2	3	δ Proteobacteria		Unidentified bacterium wb1_A12	553
NCFA 7			3	Fibrobacter / Acidobacteria		uncultured hydrocarbon seep bacterium BPC102	285
NCFA 8			3	Fibrobacter / Acidobacteria		Geobacter arculus	377
NCFA 9			3	Fibrobacter / Acidobacteria, Acidobacteria subdivision, Mt Coot-tha clone III group		Arthrobacter oxidans	1172
NCFA 10	3			Hi G+C gram positive		Unidentified Actinomycete OPB41	377
NCFA 11	3			Hi G+C gram positive		Frankia sp. FrCth	315
NCFA 12			3	Hi G+C gram positive		Unidentified bacterium clone 1959	482

Table 2 (Continued)

Table 2 (Continued)

Clone	S18 (6 m)	S18 (8 m)	B2 (8 m)	Division, subdivision	Closest relative in the RDP 8 database	closest GenBank relative	Score
NCFA 13			14	Hi G+C Gram Positive, Arthrobacter globiformis subgroup	99% Arthrobacter oxidans	Arthrobacter oxidans	1128
NCFA 14			3	Hi G+C, Propionibacter group, Nocardioides albus subgroup		Nocardioides sp.	837
NCFA 15	3		6	Leptospirillum / Nitrospira		Uncultivated soil bacterium clone C002	716
NCFA 16	3			Nitrospira, Nitrospira moscoviensis group	95% Nitrospira moscoviensis	Nitrospira moscoviensis	712
NCFA 17			6	Nitrospina		Unknown Actinomycete (MC 13)	309
NCFA 18		2		γ Proteobacteria, Pseudomonas stutzeri subgroup	97% P. putida, 96% P. stutzeri	Pseudomonas sp. (strain BKME-9)	975
NCFA 19		2		g Proteobacteria, Pseudomonas and relatives	98.2% P. azotoformans	Pseudomonas brennerii	1092
NCFA 20		2		undefined		Actinobacteria; Rubrobacteridae	821
NCFA 21	8	9	11	undefined		Actinobacteria; Rubrobacteridae	557
NCFA 22	3			undefined		Actinobacteria; Actinobacteridae	291

Table 2 (Continued)

Table 2 (Continued)

Clone	S18 (6 m)	S18 (8 m)	B2 (8 m)	Division, subdivision	Closest relative in the RDP 8 database	closest GenBank relative	Score
NCFA 23	3			undefined		Green non-sulfur bacteria; Chloroflexaceae; environmental samples	442
NCFA 24	3			undefined		Green non-sulfur bacteria; Chloroflexaceae; environmental samples	446
NCFA 25	3			undefined		clone TBS10	448
NCFA 26	3			undefined		Grassland soil clone sl3_806	278
NCFA 27	3			undefined		Leptospirillum sp. DSM2391	311
NCFA 28			3	undefined		candidate division OP3	220
NCFA 29			3	undefined		Metal-contaminated soil clone K20-51 Bacteria; CFB group	361
NCFA 30			11	NCFA group I			
NCFA 31	8	11		NCFA group I			

Figure 2 a

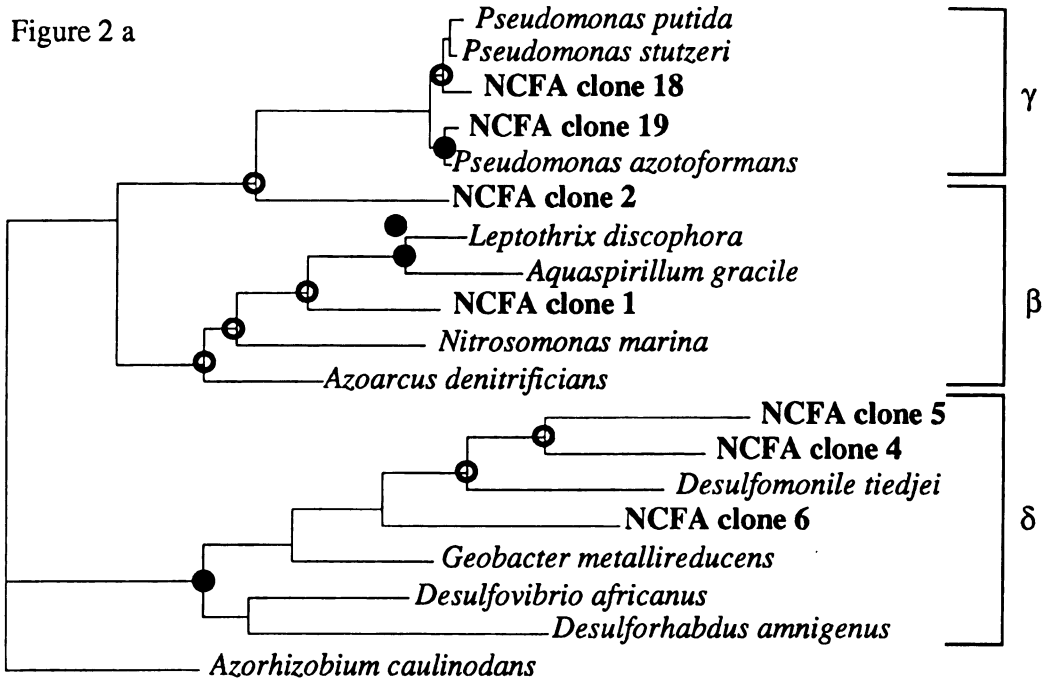
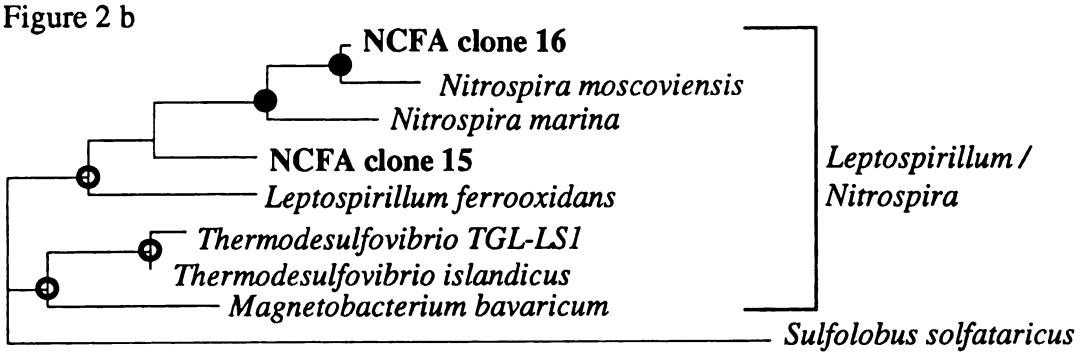


Figure 2 a-e Maximum likelihood dendrograms of bacterial 16S sequences obtained from the narrow channel focus area in Oyster, Virginia. Divisions are listed out side the brackets for panels b through d and subdivisions are listed in panel a (*Proteobacteria).* Optimality criteria used in bootstrap analysis of the sequences were: maximum likelihood, maximum parsimony, and neighbor joining. Bifurcations supported (bootstrap values >75%) by one optimality criteria but are only marginally supported (50-75%) or not supported (<50%) by the other criteria are indicated with open circles. Bifurcations supported by two or three of the criteria are indicated with closed circles. The number of characters in each analysis and the range of the mask (*E. coli* numbering) were as follows: a) 330 characters, bases 216-484, b) 443 characters. bases 134-602, c) 332 characters, bases 116-527, d) 432 characters, bases 134-537, and e) 1165 characters, bases 92-1390.

Figure 2 b



0.10

Figure 2 c

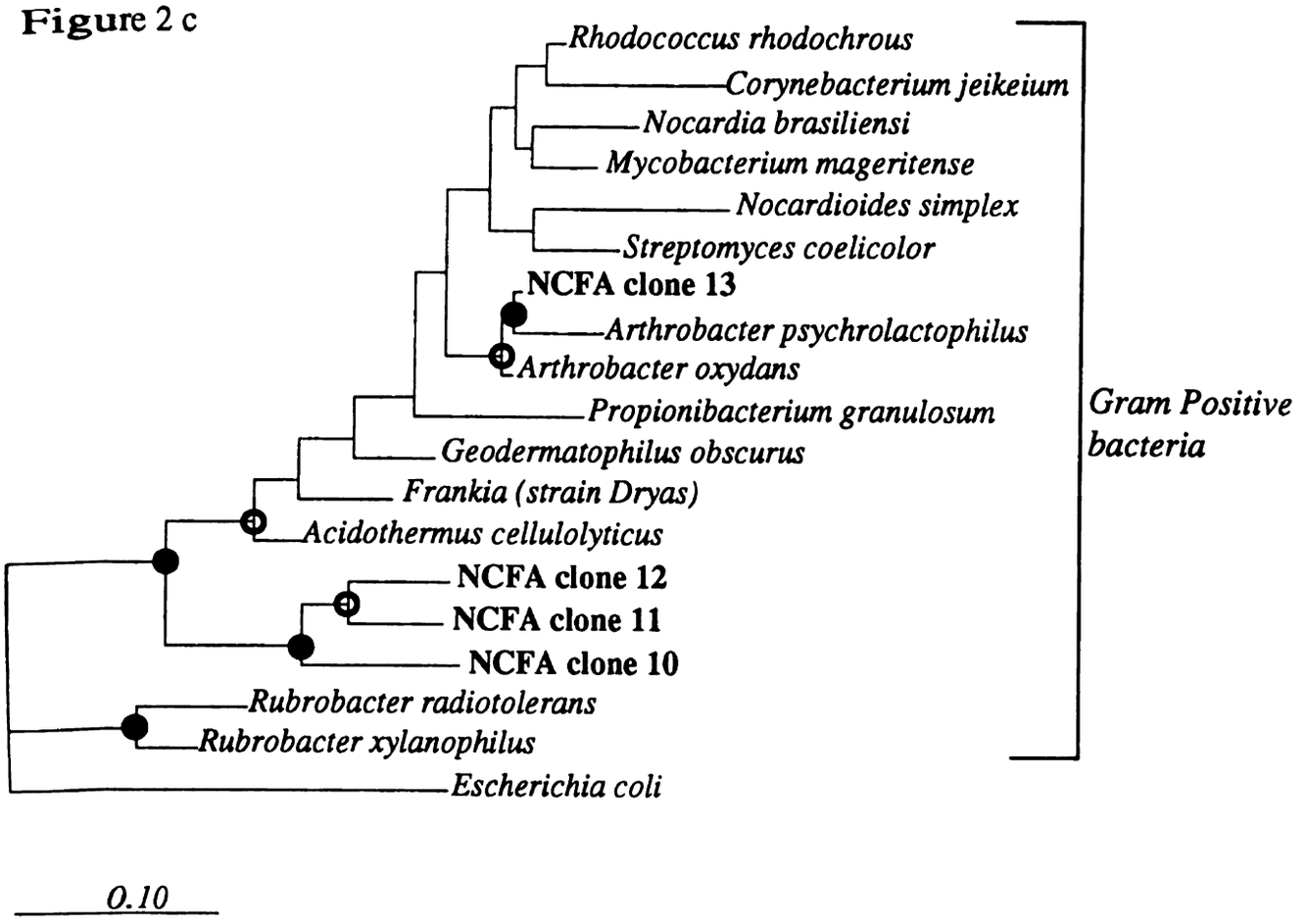
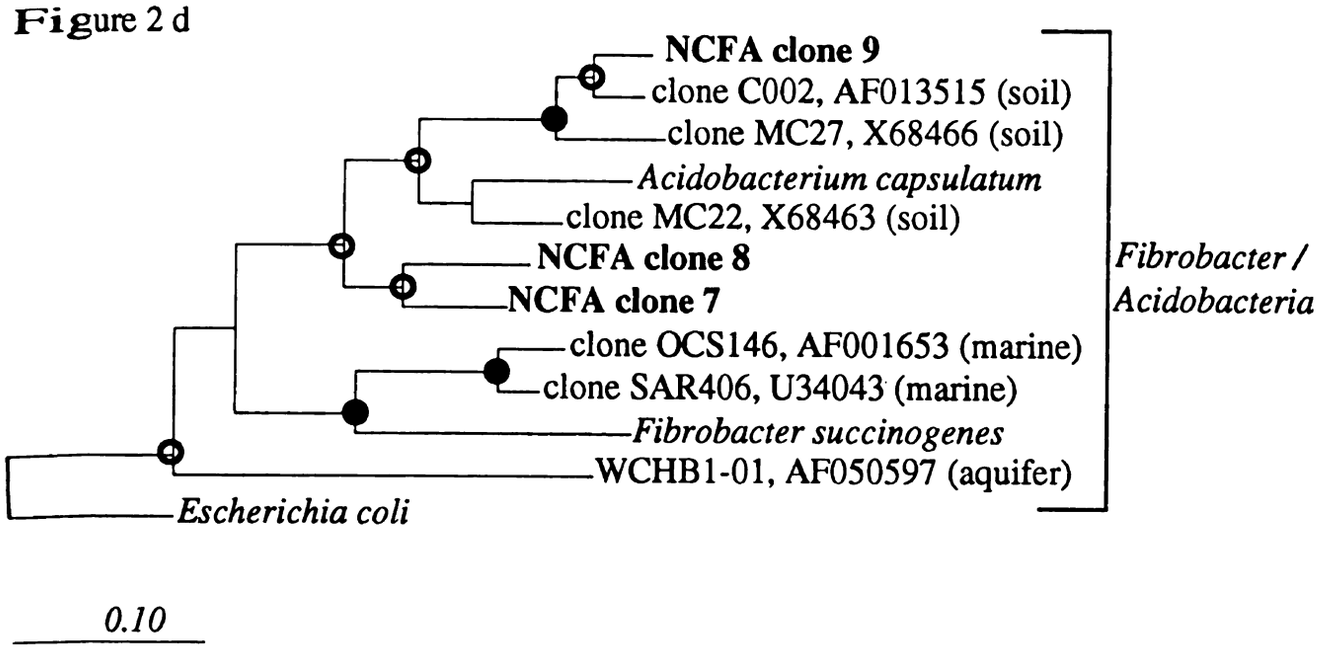
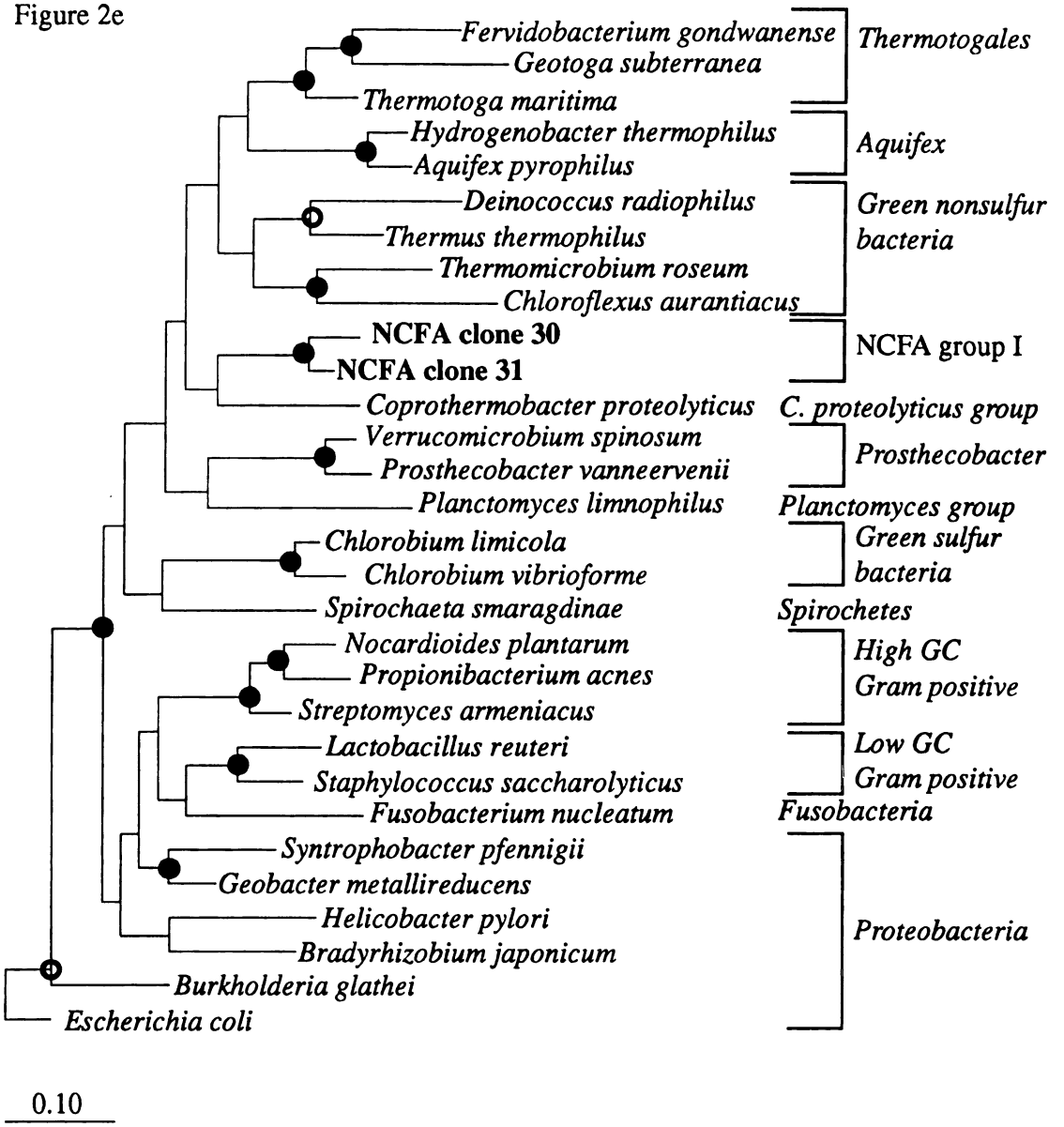

Rhodococcus rhodochrous
Corynebacterium jeikeium
Nocardia brasiliensi
Mycobacterium mageritense
Nocardioides simplex
Streptomyces coelicolor
NCFA clone 13
Arthrobacter psychrolactophilus
Arthrobacter oxydans
Propionibacterium granulosum
Geodermatophilus obscurus
Frankia (strain Dryas)
Acidothermus cellulolyticus
NCFA clone 12
NCFA clone 11
NCFA clone 10
Rubrobacter radiotolerans
Rubrobacter xylanophilus
Escherichia coli
Gram Positive bacteria
0.10

Figure 2 d



0.10

Figure 2e


Fervidobacterium gondwanense
Geotoga subterranea
Thermotoga maritima
Hydrogenobacter thermophilus
Aquifex pyrophilus
Deinococcus radiophilus
Thermus thermophilus
Thermomicrobium roseum
Chloroflexus aurantiacus
NCFA clone 30
NCFA clone 31
Coprothermobacter proteolyticus
Verrucomicrobium spinosum
Prosthecobacter vanneervenii
Planctomyces limnophilus
Chlorobium limicola
Chlorobium vibrioforme
Spirochaeta smaragdinae
Nocardioides plantarum
Propionibacterium acnes
Streptomyces armeniacus
Lactobacillus reuteri
Staphylococcus saccharolyticus
Fusobacterium nucleatum
Syntrophobacter pfennigii
Geobacter metallireducens
Helicobacter pylori
Bradyrhizobium japonicum
Burkholderia glathei
Escherichia coli
Thermotogales
Aquifex
Green nonsulfur bacteria
NCFA group I
C. proteolyticus group
Prosthecobacter
Planctomyces group
Green sulfur bacteria
Spirochetes
High GC Gram positive
Low GC Gram positive
Fusobacteria
Proteobacteria
0.10

higher in low-carbon environments because of the unsuitability of traditional media for oligotrophic species. Furthermore, while certain isolates can be derived from a given environmental sample, they are not necessarily representative of the phylogenetic, metabolic, or physiological types that dominate that environment. Using cultivation-independent techniques avoids this 'bottleneck' in detecting the dominant members of a microbial community.

In this study, we examined 12 sediments from a pristine aquifer using the culture-independent methods T-RFLP and 16S rDNA cloning and sequencing. One strength of T-RFLP analysis lies in its use in quantifying the microbial diversity in a given sample. Although it is impossible to extrapolate an exact measure of community diversity from T-RFLP profiles (42), it is possible to use T-RFLP to compare diversity between two or more samples. T-RFLP employing the bacterial-specific primer 27F and the restriction enzyme *Hha* I has been performed on samples from a number of environments. Soil typically presents 60-80 unique phylotypes (29). Clement et al (13) found between 30 and 39 fragments in clean and petroleum-contaminated sand from a coastal oil field. Liu et al. (25) detected 21 fragments in activated sludge, 33 fragments in a glucose-fed bioreactor, 36 fragments in the termite gut, and 33 fragments in trichloroethylene and jet-fuel-contaminated aquifer sediment taken from the unsaturated capillary fringe. T-RFLP profiles of the pristine aquifer sand from Oyster reveal fewer phylotypes than most of these previously studied environments. In the narrow channel focus area we detected between 13 and 39 phylotypes in the *Hha* I digestion, and profiles from the south Oyster focus area reveal between 8 and 23 phylotypes.

In comparison to soil (29), contaminated aquifer sediment (25), and the contaminated sand tested by Clement et al. (13), the microbial communities from the pristine aquifer at the Oyster study sites are relatively low in diversity. Lower diversity is consistent with the relatively lower nutrient and organic carbon concentrations in Oyster aquifer sediment. The greater the range of potential carbon sources, the greater the number of specialist populations aimed at exploiting them (12). Water-saturated aquifer sediment is also a less complex microbial environment than soil, sand, or unsaturated sediment. Unsaturated particles bear innumerable different microniches where moisture, nutrients, and dissolved gas concentrations can vary widely across small distances. Water saturation creates a bridge between sediment particles, diminishing gradients of nutrients and gases and allowing microbes to move more freely. The result is a more consistent microbial environment in the interstitial space, fewer microniches for specialized organisms, and possibly a lower microbial diversity. It can be argued that the water-saturated environment in Oyster aquifer sediment is less complex than the environments tested previously with T-RFLP, and that this is one reason for the lower diversity we detected there.

It is apparent from an inspection of the data that there was a degree of similarity between narrow channel and south Oyster T-RFLP profiles, indicating that although the geochemistry was different at the two sites, the microbial communities may have had some populations in common. T-RFLP profiles from the SOFA and the NCFA were generated using the same amount of DNA and had comparable amounts of total fluorescence per profile, allowing comparisons to be drawn between the two data sets. The two sites shared as many as 50% of the same fragments in the *Hha* I digestion and

had a comparable number of fragments in each profile, suggesting that many of the same populations may be found at both sites. This was surprising in light of the fact that the two sites had very different concentrations of oxygen and organic carbon. The two sites are within the Wachapreague formation, however, and share the same overlying soil type (14). It is reasonable to expect that sediment origin and soil nutrients have a determining effect on aquifer microbial community structure, and we believe that the similarity we observe in the profiles may be due to one or both of these factors.

It appears that the primer set used to amplify the 16S genes for T-RFLP may be an important determinant of the resulting community profile. Two different reverse primers, 1392R and 1525R, were used on identical DNA templates from the NCFA in order to study the effect of the reverse primer on T-RFLP results. For comparing profiles generated by the two primer sets we considered only those fragments that exceeded 150 fluorescence units in height as peaks below this threshold may not be consistently detectable in replicate profiles. Figure 3 is a comparison of T-RFLP profiles from NC B2 (6 m) and NC M3 (6 m) sediment generated using these primers and the restriction enzyme *Hha* I. We observe a significant effect in the NC B2 profiles: both the presence and intensity of certain terminal fragments are affected. The NC B2 (6 m) profile generated using 1392R is missing two fragments that are present in the 1525R profile: 95 and 384 bp and the 1525 profile is apparently missing fragment 477 bp. The NC M3 (6 m) profiles, however, are more comparable and we detected all fragments of over 150 fluorescence units in both profiles. It is difficult to assess the relative specificity of the two primers since most sequences in the RPDII database lack sequence information beyond base 1400, but it was noted that of the 947 matches to primer 1525R, 910 of these

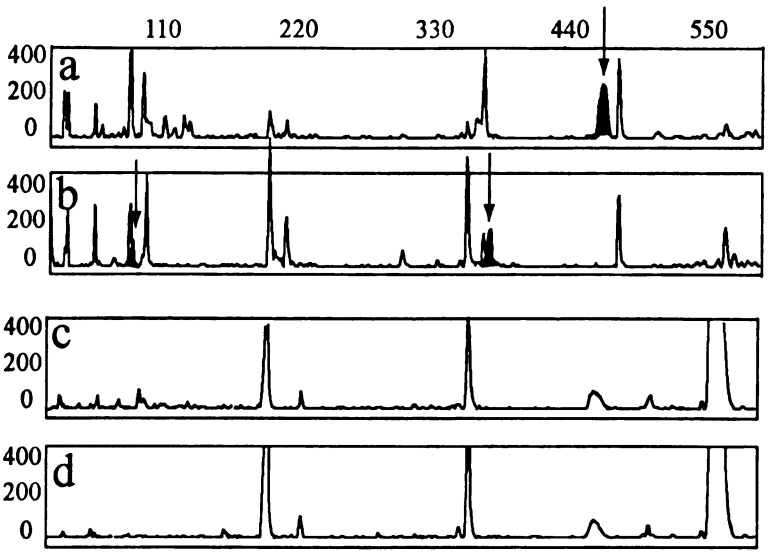


Figure 3 Differences between T-RFLP profiles generated using two different reverse primers. In comparing the profiles of template NC B2 (6 m) created with primer set (a) 27F/1392R and (b) 27F/1525R, we see the appearance and dissappearance of three significant peaks. The profiles of NC M3 (6 m) created using the same two primer sets (c) 27F/1392R and (d) 27F/1525R are more comparable and all significant peaks are found inboth profiles.

also matched the primer 1392R, suggesting that the two primers have similar specificities. In light of the differences between 1392R and 1525R profiles, it is clear that careful consideration should be given to the selection of a reverse primer for T-RFLP analysis. In the interest of making meaningful comparisons between profiles, the same reverse primer should be used in all reactions.

Cloned 16S sequences from the Oyster narrow channel site represent six major bacterial divisions (Figure 2a-e), and 28 out of 31 sequence types are sufficiently divergent (<97% similar to known species) to constitute new taxa. A number of sequences fall within the β, γ, and δ Proteobacteria (Figure 2a). Other clones were affiliated with the division *Leptospirillum / Nitrospira* (Figure 2b), the Gram positive bacteria (Figure 2c), and the division *Fibrobacter / Acidobacteria* (Figure 2d). In addition, we have detected ten sequence types (Table 2) which exhibited relatedness values of less than 0.573 when compared to sequences in the RDP II version 8.0 database and relatedness scores less than 557 when compared to sequences in GenBank using the BLAST tool at the NCBI website (1). These sequences may represent novel divisions as they fail to show a distinct relationship to any of the known divisions.

One group of unidentified clones, NCFA group I, was of particular interest as analysis of the 16S sequence places them among the groups previously identified as deeply rooted in the bacterial phylogenetic tree. The ARDRA pattern displayed by these clones was found in all libraries (Table 3). Among the clones analyzed using ARDRA, we uncovered four with this fragment pattern in the B2 (8 meters) library, three in the NC S18 (6 meters) library, and five in the NC S18 (8 meters library). We have explored the

Table 3. Presence/absence of deeply branching clone terminal restriction fragments in TRFLP profiles from the narrow channel study site

Restriction enzyme	Fragment (bp)	B2 (8 m)	S18 (6 m)	S18 (8 m)	M3 (6 m)
Hha I	61 (Type II)	+	+	+	-
Msp I	515 (Type I)	+	+	+	-
Msp I	150 (Type II)	+	+	+	-
Rsa I	498 (Type I, II)	+	+	+	-

diversity of this group in a further investigation using a 16S primer set to clone NCFA group I sequences from narrow channel sediment DNA (see chapter 3).

There was a degree of redundancy in the narrow channel clone libraries. Among the 120 clones examined with ARDRA, we observed 31 restriction fragment patterns that appear in the libraries more than once. For example, the restriction pattern for the undefined clone NCFA 21 was found in all three libraries, the pattern belonging to the "T78" clone type was found in both the NC S18 (6 meters) and the NC S18 (8 meters) libraries, and the pattern belonging to NCFA group I was found in all three libraries.

Redundancy in the clone libraries was not unexpected. The geochemistry of the narrow channel study site was more or less consistent across all sampled boreholes (21). Furthermore, similarity analysis of T-RFLP profiles from the site indicates that the sampled microbial communities are markedly similar. There are also several ARDRA patterns and clones that appear to be limited to a single clone library. It is possible that these clones are, in fact, present in each of the other libraries, but were not among the 120 clones screened or the 52 clones sequenced.

Phylogenetic data is not always a reliable indicator of the metabolism or physiology of a given organism (2), but some bacterial divisions and genera possess a consistent metabolic profile that can be extrapolated to clones found in a given environment. In the narrow channel clone libraries, three out of the 31 clone types have relationships with divisions and genera of this kind, and therefore we may speculate about their role in the environment. Clone 13 is closely related to the Hi G+C gram positive genera *Arthrobacter* (Figure 2c) and is 98% similar to *Arthrobacter oxidans*. *Arthrobacter* is a common soil bacterium which has proven to be resistant to desiccation

and starvation. Many *Arthrobacter* species have been found previous studies of the saturated subsurface (8, 15) and it is thought to be an important group in this environment because of the broad substrate range observed in many *Arthrobacter* isolates and the high numbers of these bacteria observed in the studies conducted by Balkwill et al. (8), and Crocker et al. (15). Clones 18 and 19 from the S18 (8 meters) library are phylogenetically placed within the *Pseudomonas* group of the γ Proteobacteria, and are most closely related to *P. stutzeri* and *P. azotoformans*. *Pseudomonas* strains have been isolated from subsurface environments previously (8) and are best known for their strictly respiratory metabolism and their broad range of carbon substrates.

Clones 7, 8, and 9 from the B2 (8 m) library group phylogenetically with the *Acidobacteria* of the *Fibrobacter / Acidobacter* division. To date, there exists only a single cultivated representative of the *Acidobacteria*, but the phylogenetic diversity of this group is on a par with that of other bacterial divisions (22). Acidobacterial sequences have previously been found in environments ranging from soil to hot spring mats to lake water (9, 43) and so little is currently known about this group that speculation about their role in the aquifer community is impossible.

NCFA clones 15 and 16 fall within the division *Leptospirillum / Nitrospira* which has a few cultivated species but mostly consists of rRNA sequences derived from a range of different environments. Known members of this group include *Leptospirillum ferrooxidans*, a lithotrophic, acidophilic species implicated in the evolution of acid mine drainage, and *Nitrospira moscoviensis* and *Nitrospira marina*, both nitrite-oxidizers. The relatively high nitrate concentration in this formation (3.8 – 7.1 mg N/l) (21) may be linked to the presence of these *Nitrospira*-like bacteria.

Previous cultivation-independent studies of microbial communities from uncontaminated subsurface formations provide a counterpoint to this work. Chandler et al. (1997, 1998) developed clone libraries from sediment extracted from a deep formation at the Hanford site in Washington state. As in the current study, they found clone types related to *Pseudomonas and Arthrobacter* but they also uncovered sequences related to *Bacillus, Micrococcus, Nocardioides, Clavibacter, Comamonas, Erythromicrobium*, and *Burkholderia*. In another investigation of a deep formation, Fry et al. (18) used an rRNA hybridization approach to characterize the bacterial community. They found the sediment communities to be largely bacterial (64.4-92%) with a portion of that group comprised of gram positive species (7.6 – 11.7%). Studies of shallow subsurface formations are perhaps more relevant to the current work. Shi et al. (37) used rRNA hybridization techniques to study the community from a shallow, sandy aquifer in Wisconsin. Like Fry et al. (18) they found the community from pristine sediment to be largely bacterial (Archaeal and Eukaryal signals were not detectable). As in our study, Shi et al. found the β and γ *Proteobacteria* to form a substantial part of the community (43%), that sulfate-reducing genera comprise 15-18%, and that high G+C gram positive bacteria comprise 5-10% of the community. Unlike our study, in which we failed to uncover α *Proteobacterial* clones, Shi et al. found the α *Proteobacteria* to comprise 35% of the community. It is unknown why our clone library (and those of Chandler et al. (10, 11)) failed to contain a single α proteobacterial sequence, while it seemed to be such a significant group in another shallow, sandy, aerobic aquifer. One explanation may lie in a key difference between the two environments: organic matter concentrations. The

organic carbon concentration at the narrow channel site was 1 ppm while organic matter concentrations in the Wisconsin sediment ranged from 2,000 to 32,000 ppm.

The same forward primer, 27F, was used to amplify bacterial 16S genes for T-RFLP and for cloning. Hence, one should be able to predict the terminal restriction fragment length of a given clone from the sequence of the cloned 16S genes. The terminal restriction fragments for NCFA group I was determined *in silico* and we were able to discern the terminal fragment that correlated with this group in each of the profiles from the narrow channel study site except NC M3 (Table 3). A clone library was generated from NC M3 DNA, and 40 clones were screened by ARDRA in the same manner described for the other sediments. None of the NC M3 clones possessed the NCFA group I signature ARDRA profiles (data not shown). However, we were unable to detect the terminal fragments of other NCFA clones in the T-RFLP profiles. This may be due to a number of factors. Firstly, many of the cloned sequences lacked a restriction site in the 400 bases of sequence available for analysis, so we were unable to match these with terminal fragments in the T-RFLP profiles. If the original, pre-digestion sequence held a restriction site between bases 400 and 600 it would be detected with T-RFLP. Secondly, it is possible that the labeled forward primer, 27F*, has an effect on the PCR, causing a biased reaction that, while providing a reproducible profile of the community, does not provide the same product that amplification with the unlabeled primer does.

The microbial communities of the saturated subsurface play an integral role in the functioning of aquatic ecosystems, but to date the composition of these communities has been studied in only a limited number of investigations. We have found that the diversity if the microbial communities in a shallow aquifer was significantly lower than that

observed in soil, a fact that may be attributable to the comparative dearth of organic carbon in aquifer sediment or to a lower habitat complexity. Furthermore, differences in the geochemistry between our two sampling sites do not entirely eliminate the community-level similarities that may have arisen from the common origin of the sediments. So, while flow in shallow aquifers is affected by precipitation events, the microbial communities may remain stable. The presence of *Arthrobacter* and *Pseudomonas* strains in the NCFA sediments may be a determinant of this stability: both groups are known for their broad substrate ranges, enabling them to endure the wide nutrient fluctuations that are likely in such a shallow formation. Further work in other pristine formations should be conducted in order to determine whether these groups are ubiquitous and whether they remain numerically stable over the course of short-term moisture and nutrient fluctuations.

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CHAPTER 3

DETECTION OF A NOVEL, DEEPLY-BRANCHING GROUP OF BACTERIA IN PRISTINE AQUIFER SEDIMENT

INTRODUCTION

Efforts to reveal microbial diversity have been greatly aided by the advent of cultivation-independent methods of community analysis. By circumventing the cultivation step, which can prevent the detection of >99% of microbes (1), molecular methods have afforded insight into the distribution of the recognized species of bacteria and archaea and have revealed previously undetected microbial diversity in multiple environments (5). The 16S gene, in particular, has been a useful tool in identifying the distribution of organisms which resist cultivation.

There are currently 36 accepted bacterial phylogenetic divisions, wherein a division is defined as a related group of 16S sequences which show no specific relation to any other branch in the bacterial phylogenetic tree (5). Of these, 13 are represented solely by 16S rRNA sequences and lack cultivated representatives (5). Most divisions, including the *Acidobacteria*, *Nitrospira*, *Verrucomicrobia*, and the *Planctomycetes*, contain one or more cultivated strains but are largely composed of cloned sequences from environmental DNA. The 16S sequence diversity within these divisions is comparable to that observed in the more established divisions in which many strains have been cultivated and characterized (5). The discovery of new sequences and new divisions by cultivation-independent methods continues to illustrate that the breadth of the bacterial tree is not limited to the cultivated species on hand, and that much of the phylogenetic

(and therefore metabolic and physiological) diversity of the microbial world has yet to be illuminated.

We have identified a novel group of 16S sequences in aquifer sediment from a study site in Oyster, Virginia. Using bacterial-specific primers, we amplified bacterial 16S sequences from whole community DNA and cloned the resulting sequences (see Chapter 2). This group is composed of two distinct types of clones which together comprise roughly 13% of all clones in three bacterial clone libraries. These sequences, referred to as NCFA group I, fail to show a specific relationship to any of the established bacterial divisions. Furthermore, they appear to be deeply-rooted in the bacterial phylogenetic tree, suggesting that NCFA group I may have diverged from rest of the divisions at an early point in the evolution of the kingdom. The divisions at the root of the bacterial tree, including *Aquifex*, *Thermotogales*, and *Fervidobacterium*, consist of thermophilic species (3). It has been found that bacteria closely related to thermophiles may abound in mesophilic environments (10), but mesophilic relations to thermophiles are not well represented in the literature. The detection of deeply-branching phylogenetic diversity in a mesophilic environment such as the Oyster aquifer can serve to expand our understanding of the root of the bacterial tree.

In order to elucidate the diversity of NCFA group I, we designed a primer set that specifically targets these 16S sequences and used it to construct clone libraries from several sediment samples taken from the Oyster site. The clone libraries revealed a tight cluster of deeply-branching phylotypes from points broadly dispersed across the study site.

MATERIALS AND METHODS

Sample collection Samples were aseptically collected from the narrow channel focus area (NCFA) in Oyster, Virginia in October 1998 and August 2000. Cores were placed in plastic liners, divided, placed in gamma-irradiated polypropylene tubes, and held at 4° C for seven days, after which they were stored at -80°C. Sediment samples were collected from 6.5 – 8.0 meters below the ground surface. Figure 1 is a diagram of the site and sampling points.

DNA extraction DNA was extracted from sediment samples with a Soil DNA Kit Mega Prep (MoBio) according to the manufacturer's instructions, using 10 g of sediment for each extraction. To concentrate the final solution, 0.04 volumes of 5 M NaCl and 2 volumes of ethanol was added and the mixture was centrifuged at 9,000 x g for 30 minutes. The pellet was dried and resuspended in 50 ul of water and stored at -20°C until needed.

Primer design A 16S rRNA primer was designed to specifically target the group of interest using the probe design function in the Arb genetic data environment software (12). The primer was compared against the sequences in the RDP database version 8 using the probe match function through the RDP website (http://www.cme.msu.edu/RDP/cgis/probe_match.cgi?su=SSU) to ensure specificity. The target-specific primer was designated 984R (5' - ATC CAG CAT GTC AAA CCC TG - 3').

Cloning of 16S rRNA genes Ten sediments were used for cloning experiments: S18 (6 m below the surface), S9 (6 m), M3 (6 m), and B2 (8 m) sediments were collected in October 1998. ODU2 (6 m), ODU2 (7 m), ODU3 (5 m), ODU3 (6 m), ODU4 (6 m), and

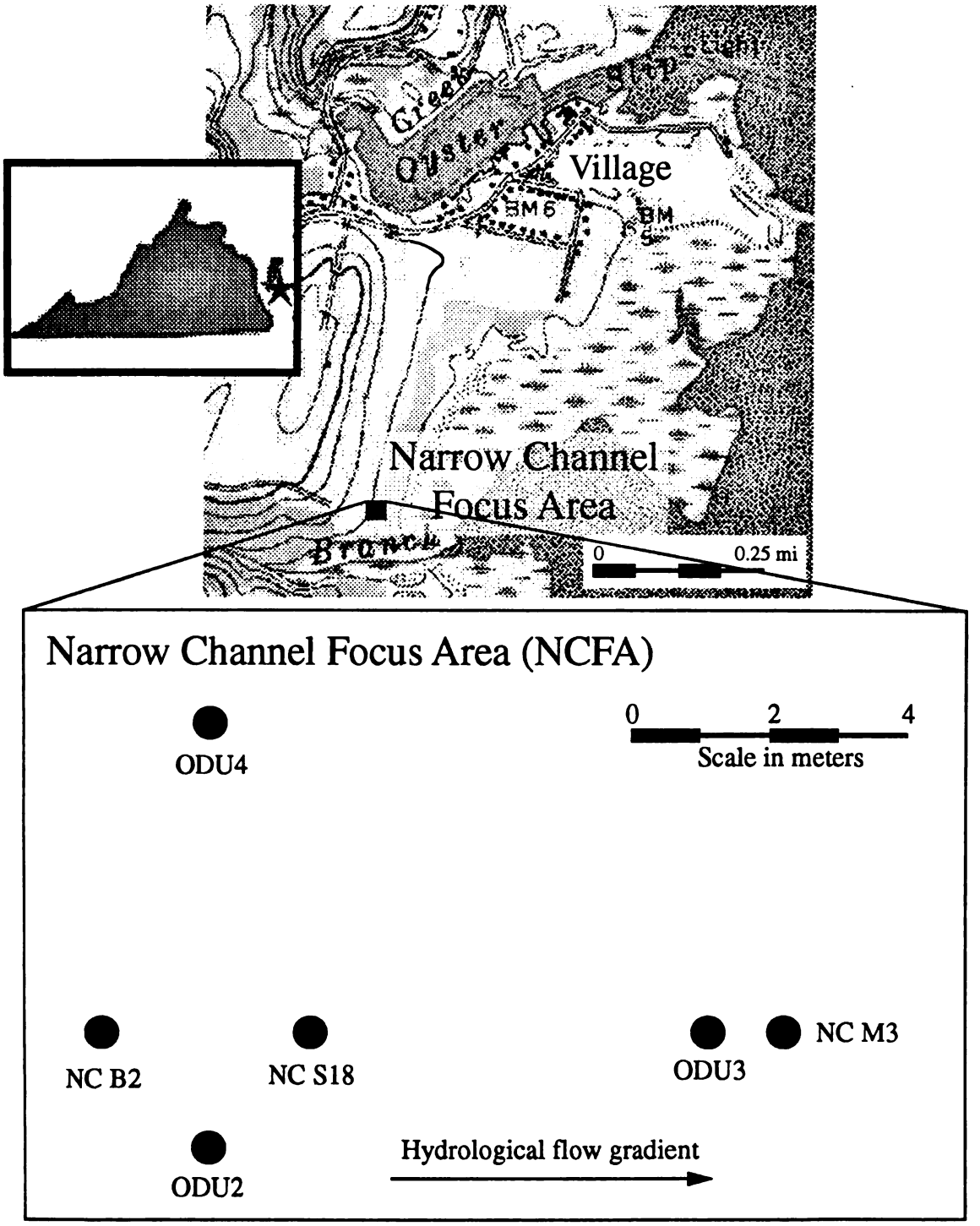

Figure 1 Map of Oyster, Virginia, indicating the location of the narrow channel focus area and the relative positions of sampling points within the site.

ODU4 (7 m) were collected in August 2000. Bacterial 16S ribosomal genes were amplified from bulk DNA in triplicate 100 ul reactions that contained 1X PCR buffer (Perkin Elmer), 5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mM of each primer, 8 ng bovine serum albumin per ul, 30 ng template DNA, and 0.02 U of AmpliTaq (Perkin Elmer) per ul. To target bacterial 16S, the forward primer 27F, which is specific for bacteria (5'-AGA GTT TGA TCC TGG CTC AG-3') (6) and the universal primer 1392R (5'-ACG GGC GGT GTG TRC-3') (8) were used. The triplicate reactions were combined and purified using a Wizard® PCR Prep (Promega) according to the manufacturer's instructions and DNA was eluted in a final volume of 50 ul. The purified PCR products were used as the template for nested PCR reactions using the NCFA group I specific primer set.

NCFA group I sequences in the bacterial 16S rDNA were amplified under the same conditions as those described for amplifying the bacterial 16S rDNA except that 50 ng template DNA was used per reaction. Triplicate reactions were combined for cloning into the vector.

The PCR products were cloned using a TOPO TA Cloning Kit (Invitrogen Corp.) in accordance with the manufacturers instructions. Plasmid DNA was extracted and purified with a Qiagen Mini Plasmid-prep kit (Qiagen).

Screening of 16S clones by ARDRA The plasmid inserts of all clones were amplified using the PCR conditions described for cloning, with 30 ng of purified plasmid DNA template per 25 ul reaction. Five ul of rDNA PCR products were digested with 10 U of the 4-base specific restriction enzyme *Cfo* I in 1X NEB buffer (New England Biolabs) in a final volume of 15 ul for 3 hrs and 30 min at 37°C. Digested DNA fragments were

separated by agarose (3.5% agarose) electrophoresis and visualized by staining with ethidium bromide and illumination on a UV transilluminator. Restriction fragment lengths were estimated by comparison with molecular weight standards which ranged from eight to 587 bp (DNA molecular weight marker V2, Roche Molecular Biochemicals), and clones with unique patterns were selected for partial sequencing.

Sequencing of rDNA clones Plasmid inserts from selected rDNA clones were amplified by the PCR using the same conditions described above for cloning. Amplified rDNA inserts were purified by using Wizard® PCR purification columns (Promega) according to the manufacturer's instructions. Sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Reaction Kit and an ABI Prism 377 DNA sequencer (PE Applied Biosystems) according to the manufacturer's directions. The primer 27F was used for sequencing six clones and four of these were selected for sequencing with the primer 984R.

Phylogenetic analyses All sequenced clones were checked for chimeric sequences using the Ribosomal Database Project II CHECK_CHIMERA version 2.7 (7). Two chimeras were detected among the clones and were eliminated from analysis. Sequences were aligned against close relatives in the Ribosomal Database Project release 8.0, and percent similarity to previously cloned NCFA I sequences was determined. For analysis of sequenced clones, a mask was generated which excluded all ambiguous positions. Dendrograms were generated using the maximum likelihood algorithm (FastDNAML) in the Arb genetic data environment package (12). The robustness of dendrogram topologies was tested by bootstrap resampling of trees using evolutionary distance (beta

version 4.0b6 of PAUP*, written by David Swofford; distance algorithm) with settings selected by execution of the dataset with ModelTest (9).

RESULTS

Description of the Study Site The site is located in the village of Oyster, Virginia, on the Delmarva Peninsula. The aquifer is currently the focus of a study on bacterial transport by the U.S. Department of Energy's Natural and Accelerated Bioremediation Research Program. The aquifer is a shallow, unconfined, sandy formation (4). The narrow channel focus area (NCFA) lies to the south-southwest of the village of Oyster (Fig. 1). The water in this part of the formation is oxic (5.5 mg O_2/l) and low in organic carbon (1000 ppb) (4). The water table lies 1.5 to 2.0 meters below the ground surface (4).

Phylogenetic analyses The NCFA group I-specific primer 984R did not match any other bacterial or archaeal 16S sequences in the RDP II database (version 8) (7). Using the 27F/984R primer pair we were able to amplify NCFA group I 16S sequences from eight of the 10 16S DNA samples tested. A separate clone library was developed from each of these PCR products. We isolated 18 clones from each library and analyzed each of these clones with ARDRA, wherein we detected six different ARDRA patterns. A representative of each type was sequenced with a single pass using the primer 27F. Two of the ARDRA types (for which we had only one representative) proved to be chimeric and were eliminated from further analysis. One representative of each of the other four ARDRA types was sequenced using both the 27F primer and 984R, providing over 970 bases of sequence for each of the clones. From prior work with a bacterial primer set (see

Chapter 2), we have 400-500 bases of nucleotide sequence of eight NCFA group I clones and 1400 bases of sequence from five NCFA group I clones.

In the former study (see Chapter 2), we detected two distinct types of NCFA group I sequences using the bacterial primer set. Types 1 and 2 are reproducibly monophyletic and are 91% identical in 16S sequence. In the bacterial clone libraries, type 1 sequences were found only in the B2 (6 m) library and type 2 sequences were found in both the S18 (6 m) library and in the S18 (8 m) library. In the present study, we were able to recover type 1 sequences from all of our clone libraries that were created using the NCFA group I-specific primer set (27F/984R). We did not detect type 2 sequences in these clone libraries.

NCFA group I sequences group deeply within the bacterial phylogenetic tree, showing a degree of relatedness to *Coprothermobacter*. Figure 2 is a maximum likelihood dendrogram of type 1 and type 2 sequences in the context of selected bacterial *16S* sequences. Bootstrap analysis with the methods of neighbor joining (100 replicates), maximum parsimony (100 replicates), and maximum likelihood (25 replicates) was used to determine the reproducibility of the branchings. Bifurcations that were supported by two or three of these methods (>75% of bootstrap resampling) are indicated with a closed circle. Open circles indicate branchings which were supported by one method.

DISCUSSION

Diversity of a deeply-branching group of bacteria We have identified two closely related 16S rDNA sequences in aquifer sediment which branch deeply within the

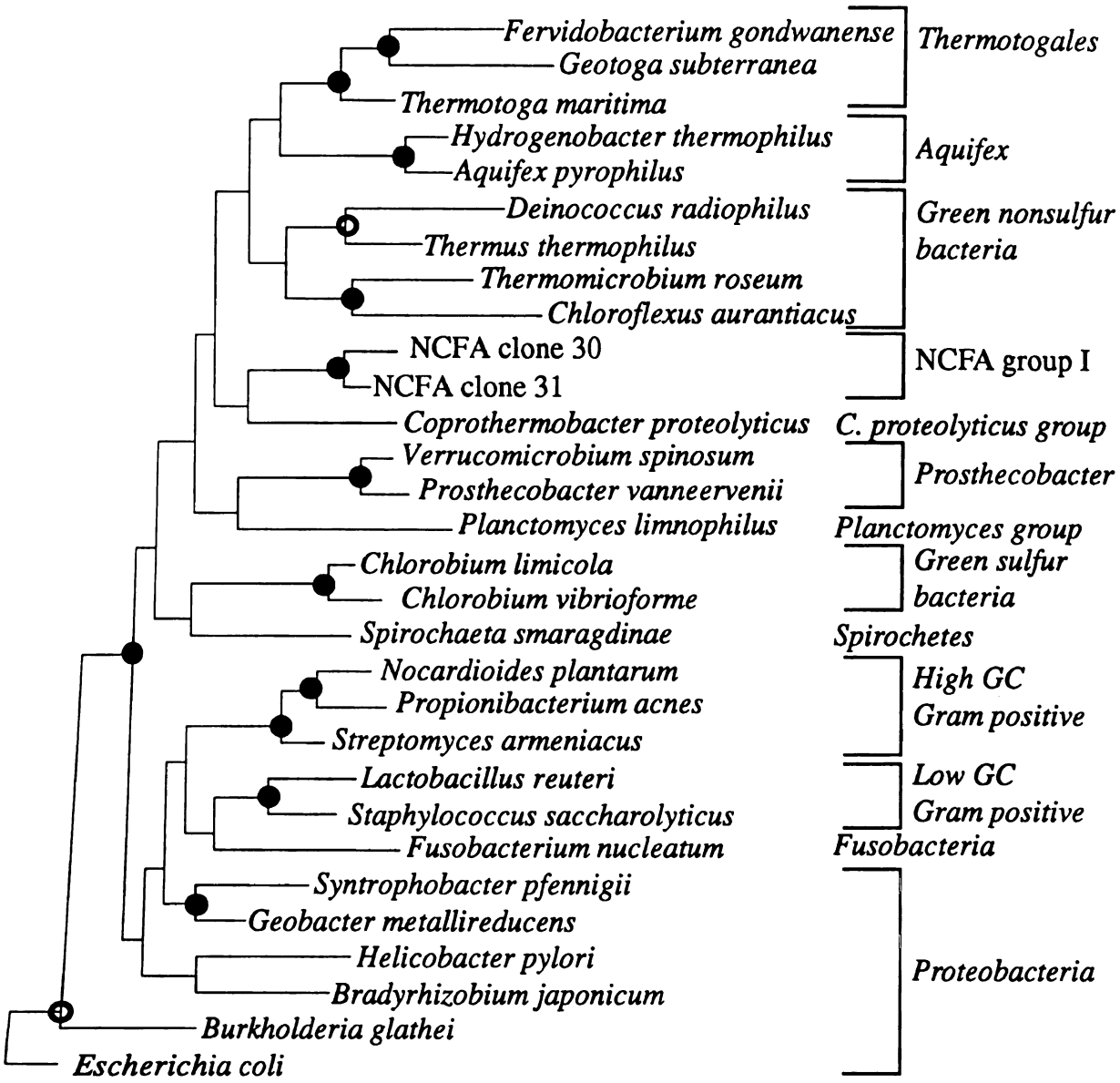

Figure 2 Maximum likelihood dendrogram of NCFA group I 16S sequences obtained from the narrow channel focus area in Oyster, Virginia. Divisions are listed outside the brackets. Optimality criteria used in bootstrap analysis of the sequences were: maximum likelihood, maximum parsimony, and neighbor joining. Bifurcations supported (bootstrap values >75%) by one optimality criteria but are only marginally supported (50-75%) or not supported (<50%) by the other criteria are indicated with open circles. Bifurcations supported by two or three of the criteria are indicated with closed circles. There were 1165 characters included in the analysis and the range of the mask (*E. coli* numbering) covered bases 92-1390.

bacterial phylogenetic tree. They are closely related (91% identical) and reproducibly monophyletic. We first discovered this group among the sequences in bacterial clone libraries (see Chapter 2), where we found type 1 clones in the B2 (6 m) library and type 2 sequences in the S18 (6 m) and the S18 (8 m) libraries. We were unable to recover either type of clone from a library that we created using DNA from sample borehole M3 (6 m). Here, we report the development and application of a 16S rDNA primer set which was specifically designed to ampify these sequences in DNA extracted from sediment at the site. We were unable to amplify these sequences in bulk DNA directly extracted from Oyster sediment, but through a nested PCR approach we were able to amplify the target sequences in 16S rDNA from eight of 10 sediments taken from the site. These samples included S18 (6 m), S9 (6 m), M3 (6 m), B2 (8 m), ODU2 (6 m), ODU2 (7 m), ODU3 (5 m), and ODU3 (6 m). We were unable to amplify DNA from the samples taken from borehole ODU4.

According to our phylogenetic analysis, NCFA group I sequences diverged from the bacterial tree early in the history of the kingdom. The species at the base of the bacterial phylogenetic tree are of interest as their characteristics may point toward the nature of the ancestor of all bacteria and toward the nature of the universal ancestor. Unfortunately, initial attempts to acquire this organism in culture have been unsuccessful (data not shown), so the metabolism of NCFA group I bacteria remains unknown. We do have an understanding, however, of the environment where these sequences are found, which can point toward certain characteristics that the group are likely to possess. Groundwater in the aquifer at the narrow channel site is aerobic, so dominant microbial groups at the site are likely to respire aerobically or fermentatively. Furthermore, the

average temperature at the site is 17°C, so NCFA group I is most certainly a mesophile. It has been argued that because the currently known species at the root of the bacterial tree are largely thermophilic, the bacterial ancestor was likely a thermophile (2). NCFA group I may constitute the earliest mesophiles in the bacterial kingdom, as there are currently no mesophilic isolates in the divisions at the base of the phylogenetic tree: *Aquifex*, *Thermodesulfobacteria*, *Coprothermobacter*, and *Thermotogales*. If other, non-thermophilic organisms are found to root deeply in the bacterial tree, lines of reasoning on the nature of the bacterial ancestor may have to be modified.

It is interesting to note that while NCFA group I sequences were not among the clones in the bacterial clone library for sediment from borehole M3 (6 m), we were able to amplify these sequences from M3 (6 m) DNA using the specific primer set. It is possible that this group was present in low numbers in the M3 (6 m) sediment and that its 16S sequence was not amplified and / or cloned in sufficient numbers because the community DNA was dominated by other species. This scenario is plausible, as the terminal 16S restriction fragment that corresponds to this group was not detected in M3 (6 m) bacterial T-RFLP profiles while it was detected in both B2 (6 m) and S18 (6 m) and S18 (8 m) profiles (see Chapter 2).

We were unable to amplify NCFA group I sequences in DNA extracted from sediments ODU4 (6 m) and ODU4 (7 m), suggesting that this group is not present at this sampling location or that it may have been present in numbers too low to detect.

It was expected that amplification of bacterial 16S DNA with the NCFA group I specific primer set would allow us to uncover further diversity within this group. This was not the case; we were only able to find type 1 sequences among the 144 clones

analyzed. Even in sediment samples in which we previously identified only type 2 sequences (S18 (6 m) and S18 (8 m)), we detected only type 1 clones. The clone-specific primer 984R is an exact match for both type 1 and type 2 sequences and should have amplified type 2 sequences present in the reactions. In an effort to avoid the bias caused by chance amplification artifacts, we carried out three separate amplifications of each template and then combined them before use in the cloning steps, but despite this step, we only recovered type 1 sequences from each sediment.

Concerns have been raised over the validity of unique sequences detected in the cloning of environmental DNA. Speksnijder et al. (11) have shown that clusters of unique, closely related rRNA sequences may be introduced in a PCR by errors cause by the polymerase. Errors include microvariations from the original sequence and chimeric assemblies from two or more sequences. We find it unlikely that NCFA group I sequences are artifacts of the amplification process. We were able to extract NCFA group I sequences in 11 separate clone libraries (three bacterial libraries and eight NCFA-specific libraries) which were developed using 11 different polymerase chain reactions. Rather, we propose that NCFA group I sequences represent a widespread population at the narrow channel focus area and, as such, they likely play a role in biogeochemical cycling at the site. Future work to isolate these sequences from other subsurface sites in Oyster or from other environmental media may prove useful in establishing the distribution and importance of this group.

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CHAPTER 4

A MOLECULAR COMPARISON OF MICROBIAL COMMUNITIES IN PRISTINE AND TETRACHLOROETHYLENE-CONTAMINATED AQUIFER SEDIMENT

INTRODUCTION

Chemical contamination of groundwater is a serious, ongoing problem in the United States, where it is estimated that 40% of the population relies on municipally supplied groundwater and 40 million people draw their household water from private wells (3). In the last 20 years, it has been observed that the microbial communities within these formations are capable of transforming many of these substances through intrinsic bioremediation (39), that is, without the addition of exogenous nutrients or organisms. Substances including crude oil (8), polyaromatic hydrocarbons (31), landfill leachate (30), trichloroethylene (TCE) (20), dichloroethylene (DCE) (21), and vinyl chloride (VC) (9) have been shown to be degraded by aquifer microbial communities either *in situ* or in microcosms. Intrinsic bioremediation of these and other compounds is a feasible option for the treatment of many sites, and its success is well documented (10, 14, 15, 45).

Contamination by tetrachloroethylene, or PCE, because of its relatively high density and recalcitrance in aerobic surface sediments, is a particular problem in groundwater ecosystems. Sources from Air Force bases to chemical companies to dry cleaning establishments have been implicated in PCE release and, once released, surface contamination moves rapidly to the underlying groundwater. The U.S. Environmental Protection Agency has determined that of the 992 sites currently listed on the National Priority List, 344 of them have been found to have contaminated the underlying

groundwater with PCE (1). It has been shown that PCE is dechlorinated either partially or completely by isolates and consortia derived from environmental samples, and intrinsic bioremediation is a promising mode of remediation for contamination at a number of sites (10, 15).

The aquifer at the Bachman Road site, located in Oscoda, Michigan, is contaminated with PCE released from a dry cleaning establishment. Studies indicate that the indigenous microbial community at the site is responsible for a measured decrease in PCE in the groundwater observed over a 14-year period (2). Bacteria capable of coupling the dehalogenation of chlorinated hydrocarbons to respiration, or reductive dehalogenators, have been isolated from sediments at the site and are thought to carry out the intrinsic remediation observed there.

Due to the demonstrated potential of intrinsic bioremediation in treating groundwater at contaminated sites, an increasing amount of work is being done to better understand the microbial communities responsible for these chemical transformations. However, because of the difficulty in aseptic collection of subsurface sediment, the microbial communities in aquifers have been little studied until recently (12). Furthermore, the communities responsible for intrinsic bioremediation in the contaminated subsurface are better understood for their functional attributes (compounds degraded, rate of transformation, limiting nutrient, etc.) than for their species-level composition. In an effort to further our knowledge of both subsurface communities and of the communities involved in intrinsic bioremediation, we have undertaken a molecular comparison of the microbial communities in clean and contaminated aquifer sediment from the Bachman Road site. We have compared the bacterial communities in the two

types of sediments using terminal restriction fragment length polymorphism (T-RFLP) to uncover broad differences in community diversity. We also developed and compared bacterial 16S rDNA clone libraries from both pristine and contaminated sediment, and phylogenetically identified 35 of these clones. Both of these analyses have been directed toward gaining a better understanding of the key differences between communities exposed to contamination and those removed from the source and between a community engaged in active reductive dechlorination of PCE and a community unexposed to the contaminant.

MATERIALS AND METHODS

Sample collection The study site is located in Oscoda, Michigan, on the shore of Lake Huron. A single intact core was aseptically collected from each sampling point in May, 1998. Sample site 4 was located in pristine sediment upstream of the plume and sample sites 1, 2, and 3 were located within the center of the plume. Cores were extracted from 11 to 19 feet below the surface, divided into two-foot segments and homogenized. The core segments used in these experiments were: 11 - 13 ft (4A, 1At, 2At, and 3At) and 17 - 19 ft (4D, 1Bb, 2Bb, and 3Bb). Samples were then divided into acid-washed glass jars and held at 4° C for one week, then transferred to gamma-irradiated polypropylene tubes and held at -80°C until use.

DNA extraction Microbial community DNA for use in T-RFLP and cloning experiments was extracted from sediment samples by a protocol described by Zhou et al. (47) with modifications. Briefly, 15 ml extraction buffer and proteinase K (final concentration 0.04 mg/ml) was added to 15 g of sediment, and the mixture was incubated

at 37°C for 30 minutes. Sodium dodecyl sulfate (SDS) solution was added for a final concentration of 18 mg/ml, and the mixture was incubated at 65°C for 2 hours, followed by three cycles of freezing in a dry ice and ethanol bath and thawing in a 65°C water bath. The supernatant was collected and the pellet was washed twice with extraction buffer and SDS and the washes and supernatant were combined. This liquid was extracted twice with chloroform and isoamyl alcohol (24:1 vol/vol) and the aqueous phases were combined. Nucleic acid was precipitated by the addition of 0.6 volume of isopropanol, then resuspended in water and precipitated by the addition of 2 volumes of ethanol. The pellet was resuspended in modified TE buffer (10 mM Tris-HCL, 0.1 mM EDTA, pH 8) and used in polymerase chain reactions.

DNA for real-time PCR reactions was extracted from sediment samples with a Soil DNA Kit Mega Prep (MoBio) according to the manufacturer's instructions, with 10 g of sediment in each extraction. DNA in the final elution volume was precipitated by the addition of 0.04 volumes 5 M NaCl and two volumes of ethanol. The solution was stored at -20°C overnight. Community DNA was pelleted by centrifugation at 10,000xg for 30 min. The pellet was dried, resuspended in 50 ul of water, and stored at -20°C until needed.

16S rDNA T-RFLP For T-RFLP experiments, DNA was extracted from duplicate samples of sediment 1At, 2At, 2Bb, and 4A. Bacterial 16S ribosomal genes were amplified from bulk DNA in reaction mixtures that contained 1X PCR buffer (Perkin Elmer), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mM of the reverse primer, 0.5 mM of the forward primer, 8 ng bovine serum albumin per ul, approximately 0.08 ng/ul of template DNA, and 0.02 U of AmpliTaq (Perkin Elmer) per ul.

The bacterial-specific forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') (27) was used for all amplifications of bacterial 16S genes and was labeled at the 5' end with the phosphoramidite dye 5-hexachlorofluorescein ("hex-labeled") (Operon Technologies, Inc.). The reverse primer used in bacterial amplifications was the universal primer 1392R (5'-ACG GGC GGT GTG TRC-3') (35). The primers used to amplify archaeal 16S genes in DNA samples were 21F (5'-TTC CGG TTG ATC CYG CCG GA-3') (6) and 915R (5'-GTG CTC CCC CGC CAA TTC CT-3') (5), both of which are specific for archaea.

Reaction mixtures were incubated in a GeneAmp 2400 PCR System thermal cycler (Perkin Elmer) at 94°C for 3 minutes, followed by 35 cycles at 94°C for 45 seconds, 60°C for 30 seconds, and 72°C for 90 seconds and a final extension step of 72°C for 10 minutes.

Triplicate reaction mixtures for each duplicate extraction were combined and purified using Wizard® PCR purification columns (Promega) and eluted with a final volume of 50 ul of modified TE buffer (0.1 mM EDTA, 10mM Tris, pH 8.0). For restriction digests, 200 ng of purified bacterial PCR product were digested with 15 U of *Hha*I, *Msp*I, or *Rsa*I (BMB) and 200 ng of archaeal products were digested with 15 U of *Hae* III, *Hha* I, or *Rsa* I (BMB) at 37°C for 3.5 hours. The lengths of the terminal restriction fragments from the amplified rDNA products were determined using an ABI Prism 377 DNA sequencer and ABI software (PE Applied Biosystems) as described by Liu et al. (28).

TRFLP analyses T-RFLP profiles were analyzed using GeneScan 3.1 software (PE Applied Biosystems). For enumerating the restriction fragments in each profile, a

fluorescence intensity threshold was set at 50 so that only peaks above this intensity were included in further analysis. Fragments shorter than 30 base pairs (bp) or larger than 600 bp were excluded from analysis in order to eliminate primer artifacts and to avoid the problems associated with identifying the length of large fragments. Only peaks present in both replicate profiles were used for further analysis, and identical peaks were taken to be those within 0.5 bp size of one another. The *Hha* 1 profiles were compared using the T-RFLP Profile Analysis tool available through the Ribosomal Database website (http://www.cme.msu.edu/RDP/cgis/trflp.cgi?su=SSU), which determines the percent similarity between two or more profiles. The number of peaks present in both profiles was divided by the number of peaks in the profile with fewer peaks.

Cloning of bacterial 16S rRNA genes Two sediments from the Bachman road site were used for cloning experiments: 4D, which was taken up gradient of the plume, and 1Bb, which came from within the contaminant plume. Bacterial 16S ribosomal genes were amplified from bulk DNA in reactions that contained 1X PCR buffer (Perkin Elmer), 5 mM $MgCl_2$, 1 mM dNTPs, 0.2 mM of each primer, 8 ng bovine serum albumin per ul, and 0.02 U of AmpliTaq (Perkin Elmer) per ul. The forward primer was 27F and the reverse primer was 1392R. The amount of template in each amplification and the cycling conditions were the same as those used for T-RFLP reactions. The PCR products were cloned using a TOPO TA Cloning Kit (Invitrogen Corp.) in accordance with the manufacturers instructions. Plasmid DNA was extracted and purified with a Qiagen Mini Plasmid-prep kit (Qiagen).

Screening of rDNA clones by ARDRA The plasmid inserts of 40 clones from each library were amplified using the PCR conditions described for cloning, with roughly 30

ng of purified plasmid DNA template per 25 ul reaction. Five ul of rDNA PCR products were digested with 10 U of the 4-base specific restriction enzyme *Cfo* I in 1X NEB buffer (New England Biolabs) in a final volume of 15 ul for 3 hrs and 30 min at 37°C. Digested DNA fragments were separated by acrylamide gel (10% acrylamide) electrophoresis and visualized by silver staining. Restriction fragment lengths were estimated by comparison with molecular weight standards which ranged from eight to 587 bp (DNA molecular weight marker V2, Roche Molecular Biochemicals), and plasmids with unique patterns were selected for partial sequencing of the rDNA insert.

Sequencing of rDNA clones Plasmid inserts from selected rDNA clones were amplified using the same conditions described above for cloning. Amplified rDNA inserts were purified by using Ultrafree MC Millipore 30,000 NMWL filter units (Millipore) according to the manufacturer's instructions. Sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Reaction Kit and an ABI Prism 377 DNA sequencer (PE Applied Biosystems) according to the manufacturer's directions. Primers for sequencing included 27F, 355F (5'-ACT CCT ACG GGR SGC AGC-3') (5) and 536R (36). Forty-two clones were partially sequenced using 27F and five clones that were of particular phylogenetic interest were fully sequenced.

Phylogenetic analyses All sequenced clones were checked for chimeric sequences using the Ribosomal Database Project II CHECK_CHIMERA version 2.7 (32). One chimera was detected among the sequences and eliminated from further analysis. Sequences were aligned against close relatives in the Ribosomal Database Project release 8.0 using the Arb software package (41), and percent similarity to known isolates and previously cloned sequences was determined. For phylogenetic analysis of sequenced clones, a

mask was generated which excluded all positions with alignment gaps or character uncertainty.

RESULTS

Description of the study site The study site is located in the town of Oscoda, Michigan and the aquifer at the site is a sandy, unconfined formation which has been contaminated with tetrachloroethylene (PCE) released from a dry cleaning establishment periodically over a number of years. Flow within the aquifer is directed eastward toward Lake Huron, and the hydraulic conductivity of the sediments ranges from 10 to 50 ft/day (2). It has been determined in previous studies that microorganisms are responsible for a sustained degradation of PCE observed at the site (2). In the center of the plume, hydrogen and redox data indicate that a gradient of conditions exists which favors halorespiring organisms at shallow and intermediate depths (nine to 16 feet below the surface) and sulfate reducers and methanogens in deeper regions (16 to 20 feet below the surface) (2). Up gradient of the plume the aquifer is aerobic and oligotrophic.

Profiles of microbial communities in clean and contaminated sediment Profiles of the bacterial communities in clean and contaminated aquifer sediment revealed a marked difference in the number of detectable phylotypes in each location. For example, in digestions with the enzyme *Hha* I, uncontaminated sediment yielded 5 reproducible terminal fragments and contaminated sediment 2Bb yielded 8 fragments, 2At yielded 12 fragments, and 1At yielded 26 fragments. The number of ribotype bands in the uncontaminated profiles was compared to the numbers observed in the contaminated profiles using a Student's unpaired t-test. The results indicate that the two data sets are

significantly different, that is, there is a 0.026 probability that the two data sets were generated by chance from the same original data set.

The bacterial profiles were compared on the basis of common peaks using the online analysis tool provided by the Ribosomal Database Project II (RDP II) available at http://www.cme.msu.edu/RDP/cgis/trflp.cgi?su=SSU. In comparing the *Hha* I profiles from clean and contaminated sediment with the RPD II T-RFLP analysis tool, the number of terminal fragments present in both profiles was divided by the number of fragments in the profile with the fewest bands. This analysis revealed that all or most of the peaks present in the uncontaminated profile are present among the fragments in the contaminated profiles. For example, each of the five terminal fragments observed in the *Hha* I uncontaminated community profile (37, 206, 370, 567, and 569 bp) are present in the 1At and 2At profiles (Figure 1), and four of the five were observed in the 2Bb profile. Certain of these terminal fragments that were common to both pristine and contaminated sediment were detected in all of the community profiles: the 495 bp fragment in the *Msp* I profiles and the 476 bp fragment in the *Rsa* I profiles. By the RDP similarity measure, the community of sediment sample 1At was 83% similar to 2At and 75% similar to 2Bb and the profile of 2At was 75% similar to 2Bb. Comparable similarities were observed in the *Msp* I and *Rsa* I digestions (data not shown). Fragments present in each of the three contaminated profiles but absent from the uncontaminated profile were: *Hha* I fragments 95, 381, and 548 bp, *Msp* I fragments 149, 528, and 530 bp, and *Rsa* I fragments 57, 459, and 496 bp.

Using the archaeal-specific primer set, we amplified archaeal 16S genes in DNA extracted from contaminated sediment, but we were unable to amplify archaeal sequences

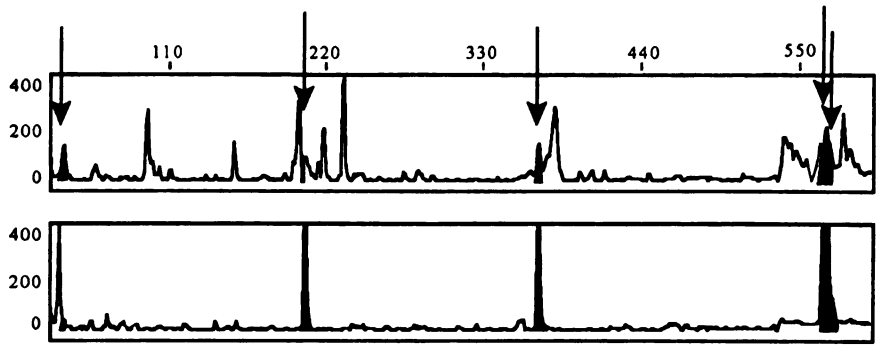


Figure 1 A comparison of bacterial community T-RFLP profiles from contaminated (top - 1At) and pristine (bottom - 4A) aquifer sediment. In this comparison, each of the five terminal fragments detected in the pristine sediment is also detected among the fragments in the contaminated sediment profile.

in DNA from uncontaminated sediment. We were able to detect between 10 and 11 terminal restriction fragments in each of the *Hae* III T-RFLP profiles, between eight and 12 in the Hha profiles, and four to seven in the Rsa I profiles. The *Hae* III archaeal profiles of sediments 1At, 2At, and 3At were 91% identical in each pair-wise comparison (which were carried out as described for the bacterial profiles). These profiles were markedly different from the 2Bb profile, which had 40%, 50%, and 50% identical fragments, respectively. The TAP-T-RFLP utility available at the RDP II website http://www.cme.msu.edu/RDP/html/analyses.html was used to identify the archaeal terminal restriction fragments in the Bachman profiles which correspond to known organisms. The profiles of 1At, 2At, and 3At displayed terminal fragments (*Hha* I 69 and 71, *Msp* I 196, *Rsa* I 608 bp) that match those of *Methanococcus vanellii* (*Hha* I 69, *Msp* I 194, *Rsa* I 606 bp) and *M. voltae* (*Hha* I 71, *Msp* I 196, *Rsa* I 608 bp). The profile of sediment 2Bb presented fragments (*Hha* I 242, *Msp* I 327, *Rsa* I 262 bp) that match those predicted for *M. thermolithotrophicus* and *M. aeolicus* (*Hha* I 242, *Msp* I 327, *Rsa* I 263 bp). However, the other terminal fragments detected in the archaeal profiles did not match the sequences in the RDP II database.

Bacterial diversity in clean and contaminated aquifer sediment The bacterial diversity in both contaminated and uncontaminated sediment at the Bachman Road site was evaluated by analyzing 16S rDNA clone libraries from sediments 1Bb and 4D, respectively. Bulk DNA was extracted from aseptically-collected sediment samples and the 16S genes therein were amplified using bacterial specific primers. These genes were then cloned and 38 clones from each library were analyzed by ARDRA. There was no overlap between the two libraries as measured by ARDRA: none of the ARDRA patterns

were detected in both libraries. Among the 38 clones from uncontaminated sediment we detected 24 distinct ARDRA patterns (63% uniqueness) and in the clone library from contaminated sediment we detected 25 distinct patterns (66% uniqueness). Fifteen clones from pristine sediment and 20 clones from the contaminated sediment were selected for single-pass sequencing. The sequences were aligned to the 16S sequences in the RDP II Version 8.0 with the Arb auto align function. Alignments were refined by visual inspection.

Figure 2 a-d depicts five maximum likelihood dendrograms in which clones from clean and PCE-contaminated sediment are depicted. Figure 2 a depicts the β and γ *Proteobacteria* clones from the site, Figure 2 b depicts the δ *Proteobacteria* clones, and Figure 2 c depicts the *Green nonsulfur* bacterial clones and the clone related to the putative division OPB80. Figure 2 d depicts the clones that are most closely related to the *Acidobacteria* and the division *Leptospirillum/Nitrospira*. Table 1 lists the Bachman site 16S clones, organized phylogenetically, their phylogenetic affiliations in the RDP II database and their nearest neighbor in the GenBank database as determined by a BLAST search. Only one clone from the Bachman site showed a high degree of similarity (>97%) to a previously discovered 16S sequence in the RDP II database: clone 1Bb1 was 97% similar to the clone WCHB1-67 (GenBank accession #: AF050536) which was first discovered at the Wurtsmith Air Force base by Dojka et al. (18).

Clone sequences were digested *in silico* to determine whether the restriction fragments of these clones could be identified among the bands in the corresponding T-RFLP profiles. Terminal restriction fragments consistent with those estimated for δ *Proteobacterial* clones 1Bb 2, 1Bb 4, 1Bb 11, and 1Bb 35 (Hha I 94 ±2, Msp I 69 ±2,

Fig. 2 a

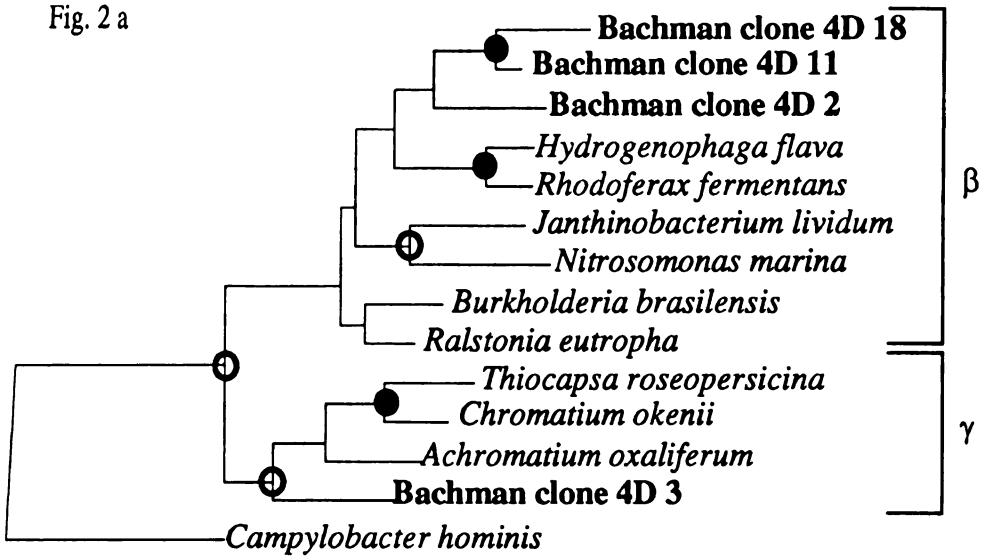
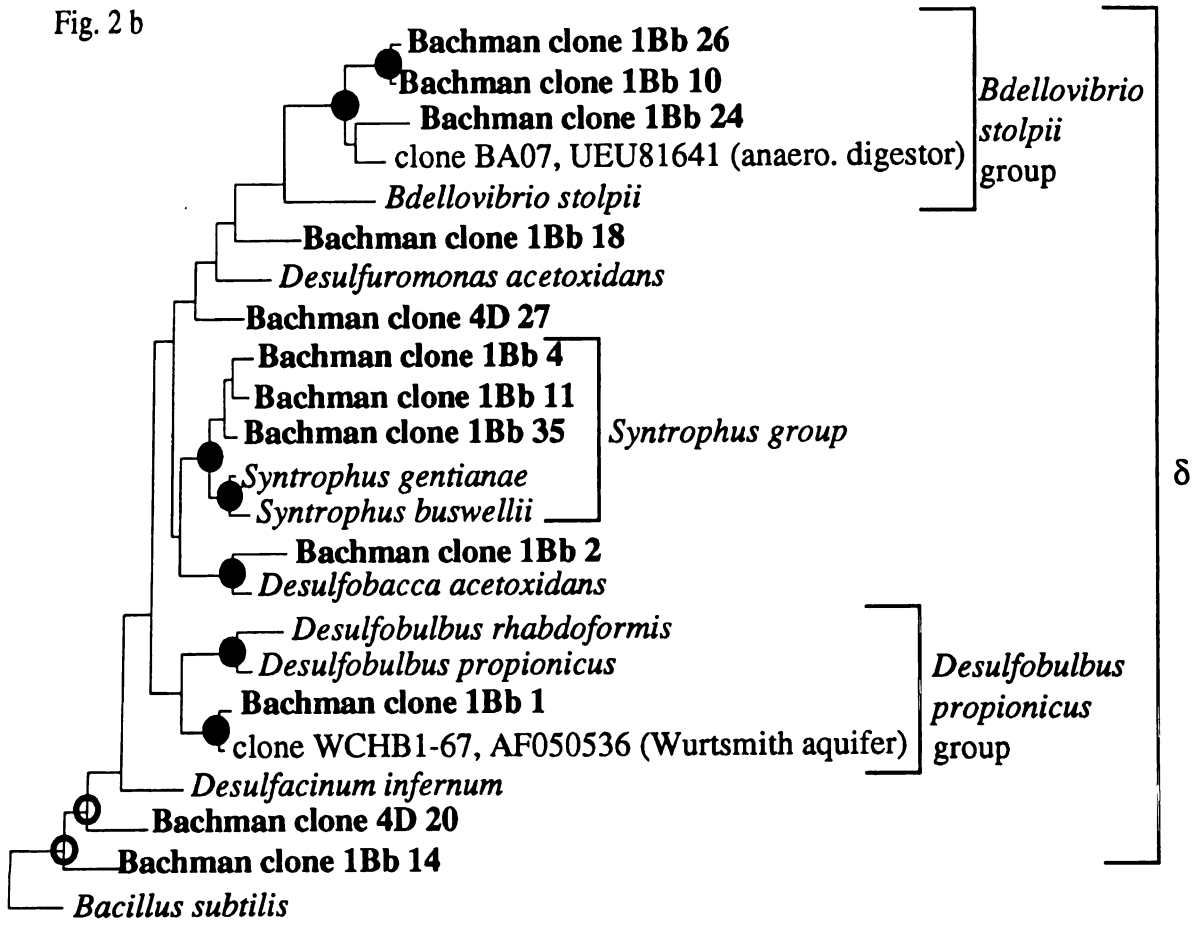


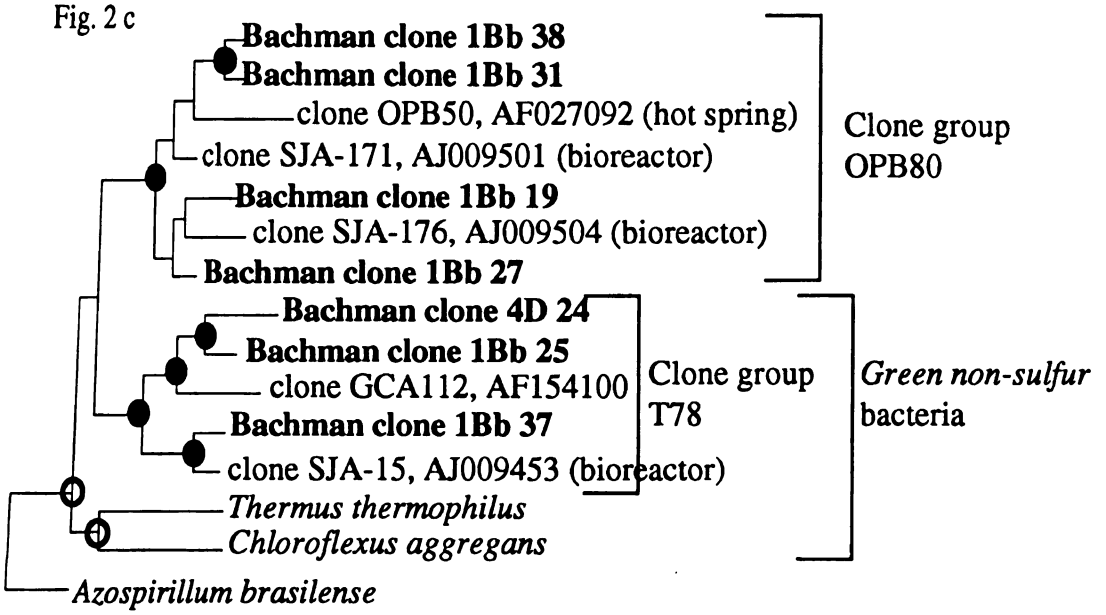
Figure 2 a-d Maximum likelihood dendrograms of bacterial 16S sequences obtained from the Bachman Road site in Oscoda, Michigan. Divisions are listed out side the brackets in panels d and c (clone group OPB80, and *Green non-sulfur*) and subdivisions are listed in panels a (*Proteobacteria*), b (*Proteobacteria*), and c (clone group T78). Optimality criteria used in bootstrap analysis of the sequences were: maximum likelihood, maximum parsimony, and neighbor joining. Bifurcations supported (bootstrap values >75%) by one optimality criteria but are only marginally supported (50-75%) or not supported (<50%) by the other criteria are indicated with open circles. Bifurcations supported by two or three of the criteria are indicated with closed circles. The number of characters in each analysis and the range of the mask (*E. coli* numbering) were as follows: a) 436 characters, bases 113-600, b) 285 characters, bases 111-422, c) 414 characters, bases 98-537, and d) 350 characters, bases 134-537.

Fig. 2 b


Bachman clone 1Bb 26
Bachman clone 1Bb 10
Bachman clone 1Bb 24
clone BA07, UEU81641 (anaero. digestor)
Bdellovibrio stolpii
Bdellovibrio stolpii group
Bachman clone 1Bb 18
Desulfuromonas acetoxidans
Bachman clone 4D 27
Bachman clone 1Bb 4
Bachman clone 1Bb 11
Bachman clone 1Bb 35
Syntrophus gentianae
Syntrophus buswellii
Syntrophus group
Bachman clone 1Bb 2
Desulfobacca acetoxidans
Desulfobulbus rhabdoformis
Desulfobulbus propionicus
Bachman clone 1Bb 1
clone WCHB1-67, AF050536 (Wurtsmith aquifer)
Desulfobulbus propionicus group
Desulfacinum infernum
Bachman clone 4D 20
Bachman clone 1Bb 14
Bacillus subtilis
δ

0.10

Fig. 2 c


Bachman clone 1Bb 38
Bachman clone 1Bb 31
clone OPB50, AF027092 (hot spring)
clone SJA-171, AJ009501 (bioreactor)
Bachman clone 1Bb 19
clone SJA-176, AJ009504 (bioreactor)
Bachman clone 1Bb 27
Bachman clone 4D 24
Bachman clone 1Bb 25
clone GCA112, AF154100
Bachman clone 1Bb 37
clone SJA-15, AJ009453 (bioreactor)
Thermus thermophilus
Chloroflexus aggregans
Azospirillum brasilense
Clone group OPB80
Clone group T78
Green non-sulfur bacteria

0.10

Fig. 2 d

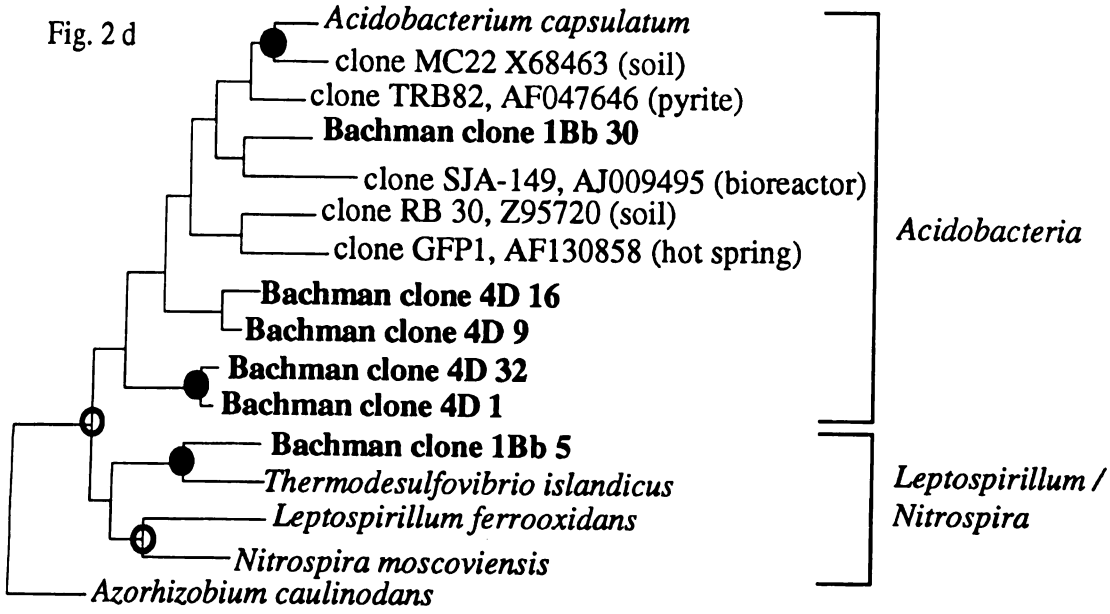

Acidobacterium capsulatum
clone MC22 X68463 (soil)
clone TRB82, AF047646 (pyrite)
Bachman clone 1Bb 30
clone SJA-149, AJ009495 (bioreactor)
clone RB 30, Z95720 (soil)
clone GFP1, AF130858 (hot spring)
Bachman clone 4D 16
Bachman clone 4D 9
Bachman clone 4D 32
Bachman clone 4D 1
Bachman clone 1Bb 5
Thermodesulfovibrio islandicus
Leptospirillum ferrooxidans
Nitrospira moscoviensis
Azorhizobium caulinodans
Acidobacteria
Leptospirillum / Nitrospira
0.10

Table 1 Phylogenetic affiliations of 16S rDNA clones from the Bachman Road site and BLAST search results

Clone	Division / subdivision	Closest GenBank relative	Score
4D 18	β *Proteobacteria*	Rhizosphere soil bacteria AJ252688	643
4D 11	β *Proteobacteria*	Rhizosphere soil bacteria AJ252688	814
4D 2	β *Proteobacteria*	Lake sediment clone AF320923	954
4D 3	γ *Proteobacteria*	Rhizosphere soil bacteria AJ232812	512
1Bb 26	δ *Proteobacteria*, *Bdellovibrio stolpii* group	Marine sediment clone AF354153	683
1Bb 10	δ *Proteobacteria*, *Bdellovibrio stolpii* group	Marine sediment clone AF354153	701
1Bb 24	δ *Proteobacteria*, *Bdellovibrio stolpii* group	Trichlorobenzene consortium AJ009448	344
1Bb 18	δ *Proteobacteria*	Methanogenic consortium AF254402	388
1Bb 4	δ *Proteobacteria, Syntrophus* group	Coal tar waste groundwater AF351220	1271
1Bb 11	δ *Proteobacteria, Syntrophus* group	Marine sediment clone AF351238	738
1Bb 35	δ *Proteobacteria, Syntrophus* group	Coal tar waste groundwater AF351238	2313
1Bb 2	δ *Proteobacteria*	Desulfobacca acetoxidans AF002671	1456
1Bb 1	δ *Proteobacteria*, *Desulfobulbus propionicus* group	WCHB1-67 Wurtsmith aquifer clone AF050536	1863
4D 27	δ *Proteobacteria*	Agricultural soil bacterium AS0252620	772
4D 20	δ *Proteobacteria*	Ferromanganous micronodule clone AF293008	486
1Bb 14	δ *Proteobacteria*	Coal tar waste groundwater AF351231	738
1Bb 38	Clone group OPB80	Trichlorobenzene consortium AJ009504	655
1Bb 31	Clone group OPB80	Trichlorobenzene consortium AJ009504	662
1Bb 19	Clone group OPB80	Trichlorobenzene consortiumAJ009501	377

Table 2 (Continued)

Clone	Division / subdivision	Closest GenBank relative	Score
1Bb 27	Clone group OPB80	Trichlorobenzene consortium AJ009490	500
4D 24	*Green non-sulfur*, Clone group T78	River sediment clone AF141416	592
1Bb 25	*Green non-sulfur*, Clone group T78	Benzene consortium clone AF029149.1	648
1Bb 37	*Green non-sulfur*, Clone group T78	Trichlorobenzene consortium AJ009453	671
1Bb 30	*Fibrobacter / Acidobacteria, Acidobacteria* group	Soil clone AF010095	370
4D 16	*Fibrobacter / Acidobacteria, Acidobacteria,* Clone group RB40	Soil clone Z95732	759
4D 9	*Fibrobacter / Acidobacter, Acidobacteria,* Env. Clone MB1228 group	Agricultural soil bacterium AJ252654	978
4D 1	*Fibrobacter / Acidobacteria, Acidobacteria* group	PCB-contaminated soil clone AJ292585	569
4D 32	*Fibrobacter / Acidobacteria, Acidobacteria* group	PCB-contaminated soil clone AJ292585	648
1Bb 5	*Leptospirillum / Nitrospira*	Lake sediment clone AF320959S1	785
4D 38	Undefined	Deep subsurface clone AF005747	442
1Bb 3	Undefined	Desulfovibrio sp. "Bendigo A" AF131324	327
1Bb 28	Undefined	Grassland soil AF078379	266
1Bb 6	Undefined	Aerothermobacter marianas AB011495	240
4D 30	Undefined	Aquatic clone AF317743	901
4D 12	Undefined	Uranium waste clone AJ296569	422
1Bb 33	Undefined	Unidentified thermophile AJ131537	336

164 ±2, 511 ±2, and Rsa I 57 ±2 bp) were detected among the bands in profiles of communities from contaminated sediment (Hha I 95 bp, Msp 67, 164, 512, and Rsa I 57). These clones were limited to the library created from contaminated sediment DNA and their signature restriction fragment sizes were not detected in the T-RFLP profile from pristine sediment. We were unable to consistently detect (i.e. in all digestions) the restriction fragments of clones from pristine sediment in the T-RFLP profiles.

DISCUSSION

T-RFLP was used to generate replicated profiles of the bacterial communities in three contaminated sediments and one pristine sediment from the Bachman Road site. The number of ribotypes detected was consistently higher in contaminated sediment than in sediment taken upstream of the PCE plume. Ribotype diversity detected by T-RFLP may be used to derive an estimate of absolute bacterial diversity in a sample, or to determine the relative diversity among a group of samples (33). The detection of greater numbers of terminal fragments in contaminated sediment profiles is an indication that a relatively higher bacterial diversity exists in PCE-contaminated sediment at the Bachman Road site.

The effect of chemical contamination on the diversity of bacterial communities has yet to be clearly resolved (42). In most cases, the influence of contamination appears to be due either to the toxicity of the material (11, 26, 37), to the utility of the contaminant as a nutrient or carbon source (13), or to a combination of these effects (17, 25) but in any case, the detailed effects of either type of contaminant are not predictable. There are indications that PCE and its degradation products may be toxic to bacterial

populations (4), but despite its potential toxicity, certain species are capable of linking dechlorination of PCE and TCE to energy conservation (34). Active microbial dechlorination of PCE has been detected at the Bachman site, and is the dominant terminal electron-accepting process in sediment at shallow and intermediate depths in the aquifer (as determined by measuring geochemical conditions) (2). Furthermore, PCE dumped at the Bachman site likely contained the co-contaminants usually present in spent dry cleaning PCE (2) including lipids, waxes, hydrocarbon solvents, detergents, and starch which may serve as carbon sources for the microbial community. Hence, it is possible that both toxicity and nutrient effects may influence diversity patterns at the Bachman Road site. The increase in ribotype diversity could, then, be due in part to the co-contaminant carbon sources present in the waste PCE, as they may serve to boost the numbers of formerly small heterotrophic populations and have the overall effect of increasing the observed genetic diversity. PCE, too, could have affected diversity by serving as an electron acceptor for reductively dechlorinating populations which were present in only small numbers in pristine sediment. The new advantage gained by these populations would foresee ably increase their numbers and render them detectable with T-RFLP. The compounds that both heterotrophs and dechlorinators release can, in turn, serves as nutrients for other populations, creating ripples down the food chain.

Most of the ribotypes observed in the profile of the uncontaminated sediment community at the Bachman site were detected among the ribotypes found in the contaminated sediment, an observation which may be explained in two ways. Populations which were dominant in the Bachman sediment prior to exposure to PCE may have persisted in the altered environment where they either carried out their normal

metabolic functions or adjusted their functions to suit the new nutrients and carbon sources. While the number of bacteria in these populations remained steady, the numbers of bacteria belonging to other groups that are capable of exploiting the new resources may have expanded. This is consistent with the fact that the nutrients and carbon sources present in the groundwater are not displaced by infiltrating PCE and co-contaminants but, rather, are supplemented by these additional sources. Alternatively, some or all of the dominant groups in pristine sediment may have been sensitive to the toxic effects of PCE and either ceased metabolism or died while smaller populations and invading species increased in numbers. Unfortunately, it is impossible to distinguish between these two hypotheses, since one drawback of the direct extraction of DNA is that dead and inactive populations may be detected (22), as their DNA is extracted and amplified along with that of active, viable cells.

Whereas the T-RFLP profiles from clean and contaminated sediment convey a degree of overlap between the two communities, the clone libraries are clearly very distinct. Among the 49 ARDRA patterns identified, no patterns common to both libraries were detected, suggesting a significant shift in the community structure. Considering the differences in geochemical conditions between the sediments 4D and 1Bb, namely differences in oxygen saturation, available carbon, and PCE concentrations, this is not an unexpected result. This contrast was reflected in the phylogenetic groups identified among the clones from the site. For example, we were able to detect one γ and three β *Proteobacterial* clones in the uncontaminated sediment, but these groups were not represented among the clones from contaminated sediment. Furthermore, we detected a number of sequences which are closely related to δ Proteobacteria in both of the clone

libraries, but only sequences from the contaminated sediment library grouped with known sulfate reducers and anaerobic syntrophs. Clone 1Bb 1 was identical to the 16S sequence of a sulfate reducing isolate, STP23 (AJ006620), discovered in the sediment of an oligotrophic lake (38). The isolate is capable of using hydrogen, acetate, formate, propionate, pyruvate, lactate, succinate, and ethanol as electron donors and sulfate, sulfite, and thiosulfate as electron donors. This species may be of particular significance in environments undergoing intrinsic bioremediation, as a nearly identical (97%) 16S sequence was also derived from the methanogenic zone of a contaminated aquifer less than five miles away at the Wurtsmith Air Force Base (18). (We have explored a method to evaluate the numbers of this species in sediment from the site; see Chapter 5.) Sequences related to the δ Proteobacteria genus *Syntrophus* have also been identified in the clone library from contaminated sediment. Below, we will discuss the role that syntrophs may play in the cycling of carbon at the Bachman site. While the differences between the clone libraries from clean and contaminated aquifer sediment may reflect a real divergence between the two sites, it is also possible that our sampling of these diverse communities was too modest to detect the presence of a significant overlap. The similarity of the T-RFLP profiles from the two sites is consistent with this possibility.

Five of the Bachman clones, one from contaminated sediment and four from pristine sediment, were shown to have a reproducible phylogenetic affiliation with the proposed division *Acidobacteria* and were 80-85% identical to *Acidobacterium capsulatum*. Many representative sequences of this division have been identified in various environments from marine sediment (46) to volcanic cinders and soil (7), and it is thought to play a role in many communities. Unfortunately, there is only one cultivated

representative to represent this group so the unifying points of metabolism among these organisms are unknown and we cannot extrapolate the characteristics of the *Acidobacteria*-related populations at the Bachman site.

Clone sequences related to both Clone Group T78 (within the division *Green-non sulfur bacteria*) and Clone Group OPB80 sequences were likewise found in both libraries. Originally isolated from activated sludge (40), Clone Group T78 has representatives from a hot spring (24), an anaerobic bioreactor (44), and lake ice (23). Clone Group OPB80 sequences come from equally diverse environments (24, 44). As in the case of *Acidobacterium*-related clones, no unifying metabolism has been identified for this group, so it is impossible to make estimates of the processes that these species carry out in the Bachman aquifer.

Using archaeal-specific primers, we were able to amplify archaeal 16S genes in bulk DNA extracted from sediment within the plume, but we were unable to amplify archaeal sequences in DNA from uncontaminated sediment. This suggests that archaea were either present in very low abundance or absent altogether from the aerobic, oligotrophic sediment upstream of the plume. Contaminated sediments, on the other hand, are anoxic and have elevated organic carbon concentrations. Indeed, archaea are expected to dominate the microbial community in deeper sediments, as geochemical data indicates that methanogenesis and sulfate reduction are the dominant terminal electron accepting processes in this zone of the aquifer (2). Analysis of archaeal T-RFLP profiles suggests that *Methanococcus* species or a closely related but hitherto unidentified genera may be an important group at the site. *Methanococcus* isolates identified to date have

been found to utilize hydrogen and formate as electron donors and to generate methane strictly by autotrophic means, that is, through uptake of CO_2 and not acetate.

It is interesting to note the rift between archaeal T-RFLP profiles from shallower sediments 1At, 2At, and 3At (11 – 13 ft) and the profile from the deeper sediment 2Bb (17 - 19 ft). Geochemical measurements at the site indicated that conditions favoring reductive dehalogenators predominate in the zone where 1At, 2At, and 3At were taken while sulfate-reducing to methanogenic conditions predominate where 2Bb was drawn (2). We were able to detect archaea, presumably methanogens, in the shallow sediments dominated by reductive dehalogenators, however, T-RFLP profiles of archaea show that these archaeal populations are different from those seen in the deeper sediments where methanogenesis has a stronger influence.

Knowing the phylogenetic affiliations of certain Bachman clones allows us to speculate about the cycling of nutrients within the deeper regions of the plume. The presence of sequences closely related to the genera *Syntrophus* is a particularly intriguing point, indicating that interspecies hydrogen or organic acid transfer may be an important process. *Syntrophus*-related species in the plume likely metabolize organic acids, producing acetate and CO_2 as well as H_2 which can be taken up and used as a valuable electron donor by other species. Both geochemical indicators and the presence of δ Proteobacterial clones which are closely related to known sulfate-reducers indicate that sulfate reducers are present in this sediment as well. The sulfate-reducing isolates most closely related to our clones, *Desulfobacterium* (1Bb 2) and STP23 (1Bb 1), are known to use hydrogen and simple acids as electron donors and they may be coupled via transfer of these compounds to the *Syntrophus*-related species at the site. Furthermore,

T-RFLP profiles of the archaeal community indicate that *Methanococcus* or a related species may be present in the contaminated sediments. The geochemical indicators at the site favor the presence of methanogens as well. *Methanococcus* and methanogenic mixed cultures isolated from other contaminated aquifers (8) are known to carry out autotrophic methanogenesis from CO_2 and H_2 or other electron donors. Thus, if autotrophic methanogenesis is the dominant type of methanogenesis at the site, then H_2 and CO_2 produced by *Syntrophus*-related species can be taken up by these archaea. Alternatively, if the methanogens at the site are acetoclastic they may use the acetate provided by the *Syntrophus*-related species to drive methane production, leaving H_2 to the sulfate-reducers. Hence, depending on which type of methanogen is dominant at the site, methanogens may have been competing with sulfate reducers for H_2 (autotrophs) or acetate (acetoclasts).

In their investigation of the bacterial and archaeal communities at the nearby Wurtsmith AFB site, Dojka et al. (18) likewise discovered *Syntrophus*-related clones and uncovered archaeal clones closely related to known acetoclastic isolates. The authors proposed that the two groups interacted via transfer of acetate and rejected the hypothesis that autotrophic methanogens were involved in the process. The authors cite previous work in mesophilic and psychrophilic environments indicating that acetoclastic methanogenesis dominates at temperatures below 20°C and in these cases available hydrogen is taken up by homoacetogens. We do find it unlikely, however, that CO_2 and H_2 are funneled to homoacetogenic populations in the Bachman aquifer, as we were unable to identify sequences related to known homoacetogens in our clone library. Further, and it has been shown that homoacetogens are at a thermodynamic disadvantage

and are hence incapable of out competing methanogens and sulfate reducers for H_2 except in a limited number of cases (16).

Furthermore, it should be noted that whatever organisms were the recipients of interspecies transfer of organic acids and hydrogen, they may have been involved in the dechlorination of PCE to TCE, cDCE, VC, and ethene which has been detected in this sediment. Reductive dehalogenators have been isolated from the Bachman Road site, where they are thought to be the dominant organisms carrying out these transformations (2). In microcosm studies using sediment from the site, it has been found that the activity of these processes is limited by electron donors, namely lactate for the conversion of PCE to cDCE and H_2 for the conversion of cDCE to ethene (2). It is possible that *Syntrophus*-related species provide the necessary hydrogen for reductive dehalogenators to carry out the transformation of cDCE to ethene. While reductive dehalogenators are at a thermodynamic advantage over sulfate reducers and methanogens in scavenging H_2 and should out compete these organisms, reductive dechlorination of chlorinated ethenes has been observed in methanogenic mixed cultures (4, 19, 43) and this activity may not be due to the activity of methanogenic populations (29). So, if reductive dehalogenators were involved in syntrophy in the deeper sediments of the Bachman site, they likely scavenged H_2 from *Syntrophus*-related species while sulfate reducers and acetoclastic methanogens competed for syntrophically-produced acetate.

In summary, T-RFLP analyses of the bacterial communities in sediment from the Bachman Road site revealed a distinct increase in phylogenetic diversity with the onset of contamination by PCE and co-contaminants, an effect which was likely brought about by higher concentrations of organic carbon available in the contaminated sediments.

Furthermore, we detected all or most of the terminal restriction fragments from the uncontaminated sediment profiles among the terminal fragments in the contaminated profiles, indicating that either the dominant populations in clean sediment persisted with the onset of contamination or that the limitations of the method permitted the extraction and detection of dead and inactive populations. In contrast, the clone libraries did not reveal an overlap between the two communities, but instead suggested that a significant shift in the microbial community structure took place with the onset of contamination. Overall, both results point to a dramatic change in the composition of the microbial community and it is possible that any overlap that may exist between the two sites was not detected in our relatively small clone libraries. Finally, the discovery of 16S rDNA genes within the plume that are closely related to the genus *Syntrophus* indicated that other species in the sediments may have been involved in the syntrophic exchange of hydrogen, CO_2, and acetate. It is possible, in such a scenario, that reductive dehalogenators were the beneficiaries of interspecies hydrogen transfer, allowing the dechlorination of cDCE to ethene. If this is the case, then efforts to ameliorate contamination at the Bachman site and others may be served by providing *Syntrophus* species with small organic acids required for the production of hydrogen for reductive dehalogenators. Further work to better define the relationship between these populations should be carried out to clarify the best mode of action to treat contamination by chlorinated hydrocarbons.

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CHAPTER 5

QUANTIFICATION OF BACTERIAL, ARCHAEAL, AND SPECIES-SPECIFIC 16S GENES IN TETRACHLOROETHYLENE-CONTAMINATED AQUIFER SEDIMENT USING REAL-TIME PCR

INTRODUCTION

Detecting the presence and abundance of particular microbial populations in environmental media can aid in evaluating the relative importance of that group in an ecosystem. Where chemical contamination has affected the microbial community, detecting the presence of certain groups can serve to achieve a better understanding of the processes that the community performs (15) or to measure ecological risk (37). Intrinsic bioremediation, the remediation of chemical contamination in the environment by the indigenous microflora, is becoming a widely-used alternative for the treatment of such sites (10-12, 46, 48). Gaining a better understanding of the abundance and distribution of microbial species that are active in these remediation processes affords researchers with another tool to evaluate the potential effectiveness of the indigenous community. Methods currently used for the purpose of detecting microbial populations of interest, including rRNA hybridization analysis, microscopy, cultivation, and quantitative PCR, each have their own individual set of advantages and limitations.

The emerging method of real-time PCR offers another approach to detecting and quantifying microbial populations. First developed by Higuchi et al (25), the method entails tracking the amplification of a target sequence in a polymerase chain reaction. The increasing number of target sequences may be signaled through the release of an intragenic fluorescent probe or through the use of a non-specific fluorescent nucleic acid

dye. The initial number of targets in the reaction can be extrapolated through comparing the evolution of the fluorescent signal with known standards. Real-time PCR has been used to quantify a range of DNA sequences, including nitrite reductase (22), glycoprotein D of human immunodefficiency virus (25), meningococcal-specific genes IS1106 , ctrA, and siaD (23), and large (28) and small subunit rRNA (13, 28, 38, 43, 47).

A prior study of the microbial communities in sediment from the Bachman Road site, a tetrachlorothylene (PCE) -contaminated aquifer in Oscoda, Michigan which is undergoing intrinsic bioremediation, has uncovered the 16S sequence of a bacterium closely related to sulfate-reducing species of the δ *Proteobacteria* (see Chapter 4). Indeed, an identical 16S sequence has since been found in a sulfate-reducing isolate from lake sediment (41), confirming the presumed metabolism of this organism. In our examination of the community in contaminated aquifer sediment, we found this sequence to comprise 26% of the 16S sequences in our bacterial clone library. In a separate study, a nearly identical 16S sequence was discovered in an aquifer at the former Wurtsmith Air Force base, approximately five miles away from our study site (15). There is evidence that intrinsic bioremediation is active in the Wurtsmith aquifer, which is contaminated with hydrocarbons and chlorinated solvents, including PCE. The discovery of this clone at two sites undergoing intrinsic bioremediation suggested that this organism might play a role, either directly or indirectly, in the treatment of these sites.

As the process of cloning can introduce biases into the analysis of microbial communities (27, 44), we were incapable of judging the true representation of the organism 1Bb1 in sediment at the Bachman and Wurtsmith sites from the composition of the clone libraries. In this study, we designed a species-specific 16S rDNA primer set to

detect and quantify 1Bb1 in sediment from the Bachman and Wurtsmith sites through real-time PCR. Furthermore, using kingdom-specific primers, we were able to measure the numbers of bacterial, archaeal, and eukaryal SSU rRNA genes in these samples and in sediment from a pristine aquifer, activated sludge, and bioreactor fluid.

MATERIALS AND METHODS

Sample collection The Bachman Road site is located in Oscoda, Michigan, on the shore of Lake Huron. A single intact core was aseptically collected from each point where injection and extraction wells were installed in August, 2000. Two injection wells (north and south) and one extraction well were installed and the cores from these points were named accordingly: IW-N, IW-S, and EW. Sampling points were arrange in a triangle: IW-N and IW-S were six feet apart, arranged perpendicularly to the hydrologic flow. Sample point EW was located 10 feet down gradient from the midpoint between the injection wells. Cores were extracted from 11 to 19 ft below the surface, divided into two-foot segments and homogenized. Samples were then divided into acid-washed glass jars and held at 4° C for one week, then transferred to gamma-irradiated polypropylene tubes and held at -80°C until use. The core segments used in these experiments were: IW-N 11 - 13 ft, EW 11 - 13 ft, IW-N 17 – 19 ft, IW-S 17 – 19 ft, and EW 17 - 19 ft.

Samples ML3 (15.5-17.5 ft) and ML3 (23.5-27.5 ft) from the Wurtsmith site were aseptically collected in November, 1999 in the manner described by Dojka et al. (15) from a point less than 10 ft downgradient of the extraction point used in that study. Sediment from Oyster, Virginia, ODU4 8.8-8.9 m was extracted in August 2000 as described in Chapter 2. Bioreactor sample LS1 was extracted from the bioreactor

described by Fernandez et al. (19) in September, 1999 and stored at -80° C until use. The activated sludge sample was extracted from the benchtop reactor described by Marsh et al. (32) and stored at -80° C until use.

DNA extraction DNA was extracted from sediment samples with a Soil DNA Kit Mega Prep (MoBio) according to the manufacturer's instructions. We used 10 g of sediment in each extraction except in the case of the two Oyster sediment samples where we used 15 g. DNA in the final elution volume was precipitated by the addition of 0.04 volumes 5 M NaCl and two volumes of ethanol. The solution was stored at -20°C overnight. Community DNA was pelleted by centrifugation at 10,000xg for 30 min. The pellet was dried, resuspended in 50 ul of water, and reprecipitated using 0.04 volumes of 5M NaCl and two volumes of ethanol. Again, the pellet was dried, resuspended in 50 ul of water, and stored at 4°C. In order to examine the reproducibility of the method two sediment samples were selected for replication: two 10 g samples were extracted for sediments IW-N (11 – 13 ft) and IW-S (17 – 19 ft).

DNA was extracted from bioreactor and activated sludge samples with a Soil DNA Isolation Kit (MoBio) according to the manufacturer's instructions. Bioreactor and activated sludge DNA was stored at 4° C until use. In order to examine reproducibility in the non-sediment samples, a single aliquot of bioreactor material was divided into two separate samples prior to extraction and analysis: 1.5 ml of bioreactor fluid was pelleted, resuspended in 200 ul of water and divided. The same division was carried out with the activated sludge sample, wherein a single pellet of 0.25 g was resuspended in 200 ul and divided.

Primer design The primer pairs for real-time PCR are listed in Table 1. Primers were selected to optimize base pairing with target sequences, achieve a T_m close to 60° C, and minimize amplicon length. The 1Bb1-specific primer 712R was designed using the probe design function in the Arb software package (42).

Real-time PCR The amplification of target sequences during the PCR was detected using the 7700 Sequence Detector (PE Applied Biosystems) to monitor the increase in fluorescence caused by the binding of SYBR Green dye (PE Applied Biosystems) to strands of double-stranded nucleic acid. The fluorescence of SYBR Green was normalized by comparison with an internal fluorescent reference dye, 6-carboxy-X-rhodamine. The threshold value, determined by the operator, is the fluorescent intensity at which the increase in fluorescence in the reactions is approximately log-linear. The C_T, or threshold cycle, is the cycle at which the normalized fluorescence in the reaction tube exceeds the threshold value. The C_T was measured and compared to the C_T values in the standard curve to determine the number of targets in a given reaction.

All optimization steps were carried out in a final volume of 25 ul and all sample and standard reactions were 50 ul. Each PCR reaction contained 1× SYBR Green PCR Master Mix (SYBR Green I Dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, passive reference dye 6-carboxy-X-rhodamine, magnesium, and PCR buffer) (PE Applied Biosystems). The concentration of each primer was optimized separately and the following concentrations were used: 1108F 300 nM; 1132Rb 300 nM; 967F 50 nM; 1132Ra 50 nM; 348F 300 nM; 552R 50 nM; 569F 300 nM; 694R 50nM. Standard DNA and sample DNA was diluted in water containing 1 ng/ul lambda phage DNA (New England BioLabs) as a carrier to prevent DNA loss to surfaces. MicroAmp optical tubes

Table 1 Primer pairs for real-time PCR

	Primer name	Sequence (5'-3') of primer	Target position	amplicon length (bp)	reference
Bacterial	1108F	ATGGYTGTCGTCAGCTCGTG	1088-1108	59	(4)
	1132R-b	GGGTTGCGCTCGTTGC	1132-1147		(49)
Archaeal	967F	AATTGGCGGGGGAGCAC	951-967	197	(3)
	1132R-a	TGGGTCTCGCTCGTTGC	1132-1148		this study
Eukaryal	348F	AGGGYTCGAYYCCGGAGA	331-348	238	(24)
	552R	GWATTACCGCGGCKGCTG	552-569		(33)
Bachman clone 1Bb1-specific	569F	GCCAGCAGCCGCCCTA	550-569	162	(4)
	694R	GAATTCCACCACCCCCTCC	694-712		this study

and caps were used (PE Applied Biosystems). The PCR cycling conditions were as follows: 10 min at 95°C followed by 40 cycles of 15s at 95°C and 1 min at 60°C. A set of standard reactions with known numbers of target SSU rRNA genes was run simultaneously with every set of sample reactions to generate a standard curve.

Samples were diluted to a final concentration of 1:100, 1:200, 1:400, and 1:800 in reactions with bacterial, eukaryal, and 1Bb1-specific primers and 1:200, 1:400, 1:800, and 1:1600 in reactions with archaeal primers. The only exception was the activated sludge DNA which was diluted to a final concentration of 1:1000, 1:2000, and 1:4000 for reactions with eukaryal primers. Each template dilution was run in triplicate. By comparison to the standard curve, the number of targets per reaction was determined and linear regression analysis (Excel, Microsoft) was used to extrapolate these 12 values to determine the target number in the undiluted sample.

Controls The DNA of the following species was used as positive control template and in the generation of standard curves: *Escherichia coli* (bacterial primers), *Methanococcus jannaschii* (archaeal primers), and *Saccharomyces cerevisiae* (eukaryal primers). For the primer set specific for the cloned 16S sequence 1Bb1, the standard template was plasmid pCR®2.1-TOPO (Invitrogen) with the cloned 1Bb1 insert.

To test for specificity, bacterial, archaeal, and clone 1Bb1-specific primer sets were compared to the species available in the RDP II database (version 8) (30). Primers used to amplify eukaryotic sequences were compared to the sequences in the Arb database (release December 1998).

In order to test whether the presence of high concentrations of non-target template affected specific amplification, a series of control reactions were carried out. For each

primer pair, a set of reactions containing a range of concentrations of standard template was spiked with 0.004 ng/ul of DNA extracted from non-target species. The following non-target templates were used: *M. jannaschii* (bacterial primers) and *E. coli* (archaeal, eukaryal, and 1Bb1-specific primers). Standard templates are listed above, and the concentrations of standard templates in these reactions were as follows: 2.3×10^{-5} to 3.0×10^{-3} ng·ul^{-1} (bacterial primers), 3.1×10^{-5} to 4.0×10^{-3} ng·ul^{-1} (archae primers), 1.6×10^{-5} to 2.1×10^{-3} ng·ul^{-1} (eukaryal primers), and 1.6×10^{-9} to 2.7×10^{-5} ng·ul^{-1} (1Bb1-specific primers). To further test the sensitivity of the primers to non-target template, these templates were tested, in the absence of target template, in a series of control reactions. The concentrations of non-target templates were as follows: 4.0×10^{-4} to 4.0 ng·ul^{-1} (bacterial primers) and 3.0×10^{-4} to 3.0 ng·ul^{-1} (archae, eukaryal, and 1Bb1-specific primers).

RESULTS

Description of the study site The study site is located in the town of Oscoda, Michigan. The aquifer at the site is a sandy, unconfined formation contaminated with tetrachloroethylene (PCE) released from a dry cleaning establishment. Flow within the aquifer is eastward toward Lake Huron. It has been determined in previous studies that microorganisms were responsible for a sustained degradation of PCE observed at the site. In the center of the plume, hydrogen and redox data indicate that a gradient of conditions exists which favors halorespiring organisms at shallow and intermediate depths (eight to 12 feet below the surface) and sulfate reducers and methanogens in deeper regions (16 to 19 feet below the surface) (2).

Several samples were used as controls for this study, including sediment from the Wurtsmith Air Force base, sediment from Oyster, Virginia, activated sludge, and methanogenic bioreactor fluid. A bacterial 16S clone which was 97% identical to 1Bb1 was identified at the Wurtsmith Air Force base site in a prior study (15). Samples from this site were selected in order to test for the relative abundance of clone 1Bb1 in this sediment. Sediment from Oyster, Virginia was selected as representative of a pristine aquifer community. Activated sludge was selected to provide a sample known to contain high numbers of microeukaryotes. Bioreactor fluid was selected to provide a well-characterized microbial community of archaea and bacteria.

Primer specificity All primer sets were found to be highly specific for their target groups. Out of 15208 bacterial sequences in the database, the bacterial primer 1108F matched 10,658 and primer 1132R-b matched 9,731. Neither bacterial primer matched any archaeal sequences in the database. Out of 1,173 archaeal sequences in the database, the archaeal primer 967F matched 703 and primer 1132R-a matched 197. Neither archaeal primer matched any of the bacterial sequences in the database. The clone specific reverse primer matched 23 bacterial sequences in the database, namely *Thermus*, *Desulfocapsa*, *Desulfobacterium*, and *Desulfofustis* species and several unidentified clones. The forward primer used in conjunction with the clone-specific primer is universal and matched 10,895 of the bacterial and archaeal sequences in the database. As the RDP database did not contain eukaryal sequences, the eukaryal primers were compared to sequences in the Arb database (release December, 1998). The forward primer 348F matched 2,144 out of 32,270 eukaryal sequences in the database. The universal reverse primer matched 13,905 sequences in the database, 2,527 of which were

eukaryal. PE Biosystems (35) recommends that for real-time PCR applications the last five bases at the 3' end of the primers should have no more than two G and/or C bases. Further, the protocols recommend avoiding runs of identical nucleotides. In designing primers for the SSU rRNA gene it was necessary to violate these recommendations.

In control reactions to test for the effect of non-target DNA template on the amplification of target template, a high concentration of non-target DNA (0.004 ng/ul) was added to reactions with variable concentrations of target template. The archaeal and eukaryal-specific primer pairs were found to be insensitive to the presence of 0.004 ng/ul of *E. coli* DNA in all reactions. The 1Bb1-specific primer set was sensitive to the presence 0.004 ng/ul of *E. coli* DNA when the number of 1Bb1 16S genes in the reaction was less than 60. This effect was deemed unimportant in the application at hand, as 60 targets per reaction was outside our reliable detection limits. The bacterial primer set was sensitive to the presence of 0.004 ng/ul *M. jannaschii* DNA only when bacterial template was less than 0.0004 ng/ul (30,000 bacterial 16S genes). Again, this effect was considered to be insignificant in the current investigation: working backwards from the numbers of targets detected in our sample reactions, most reactions held significantly more than 0.0004 ng/ul bacterial DNA, and at lower bacterial DNA concentrations archaeal DNA was equally dilute. Furthermore, for sample reactions in which the bacterial template concentration was below this threshold value, reactions with 2× and 4× as much sample template showed a linear relationship ($R^2 > 0.9$) in regression analysis to the dilute sample. Hence, as sample DNA was diluted, archaeal DNA did not compete with bacterial DNA as the amplification template.

In testing our primer pairs against variable concentrations of non-target template DNA in the absence of target template, archaeal, eukaryal, and 1Bb1-specific primer sets were found to be insensitive to the presence of non-target template at concentrations at or below 0.3 ng/ul. The bacterial primer set amplified non-target M jannaschii DNA at all concentrations tested (4.0×10^{-4} to 4.0 $ng \cdot ul^{-1}$). However, in light of the fact that the primer set was insensitive to non-target template when bacterial DNA was present in concentrations greater than 0.0004 ng/ul, this effect was deemed irrelevant.

Detection Limits In the absence of template, reactions of each primer pair (except the archaeal primers) were found to result in non-specific amplification, most likely a primer-dimer phenomenon. The C_T of these reactions was substantially greater than that of the lowest set of standards. In order to score a given group as "detected" in a sample, the C_T of all of the 12 data points for that sample must have exceeded this background level. In addition, the response in the calculated concentration of targets in these reactions must have displayed a linear response to template dilution in order for the tested group to be determined as "detected". A linear response was defined as an R^2 value greater than 0.9 in a linear regression plot of the calculated concentrations of each of the 12 data points.

Quantification Limits In order to accurately quantify the number of 16S targets in a given sample, at least half of the data points must lie within the quantifiable range of the standard curve. The quantifiable range was between 2,000 and 250,000 targets per reaction for the bacterial primer set, between 2,000 and 250,000 for the archaeal primer set, between 15,000 and 1,000,000 for the eukaryal primer set, and between 250 and 250,000 for the 1Bb1-specific primer set. The number of 18S genes in the genome of *S. cerevisiae*, which was used as a template for the eukaryote standard curve, varies

between 100 to 200 copies per genome (1). Hence, by comparison to the standard curves we were able to estimate the number of 18S targets in a given sample to within a factor of two.

For each set of unknown reactions, a set of known standard reactions was assembled and used to define a standard curve. Figure 1 illustrates four of these standard curves.

The fluorescence thresholds used to analyze the data were set to 0.02 for the amplifications with bacterial primers, 0.015 for the archaeal primer set, 0.01 for the eukaryal primer set, and 0.011 for the 1Bb1-specific primer set.

Quantification of bacterial, archaeal, eukaryal, and 1Bb1 SSU genes The numbers of bacterial, archaeal, eukaryal, and 1Bb1 SSU genes detected in each sample and the standard errors of each calculation are listed in Table 2. The percent of the total calculated number of targets of SSU genes from archaeal, bacterial, and eukaryal sources is also reported. We were able to identify clone 1Bb1 in five out of seven sediment samples from the Bachman site and in both of the Wurtsmith sediment samples. Furthermore, bacterial 16S genes were detected in all samples, archaeal 16S was detected in all sediment samples and in both replicates of bioreactor fluid, and eukaryal 18S genes were detected in saturated Wurtsmith sediment and in the activated sludge samples.

In aquifer sediment, bacterial 16S genes ranged from 2.02×10^6 to 1.72×10^7 16S genes per gram of sediment, archaeal measurements ranged from 1.20×10^5 to 4.94×10^7 genes per gram of sediment, and we measured 1.51×10^8 eukaryal 18S genes per gram of sediment in Wurtsmith sediment ML3 (23.5 – 27.5 ft). In Bachman samples, the

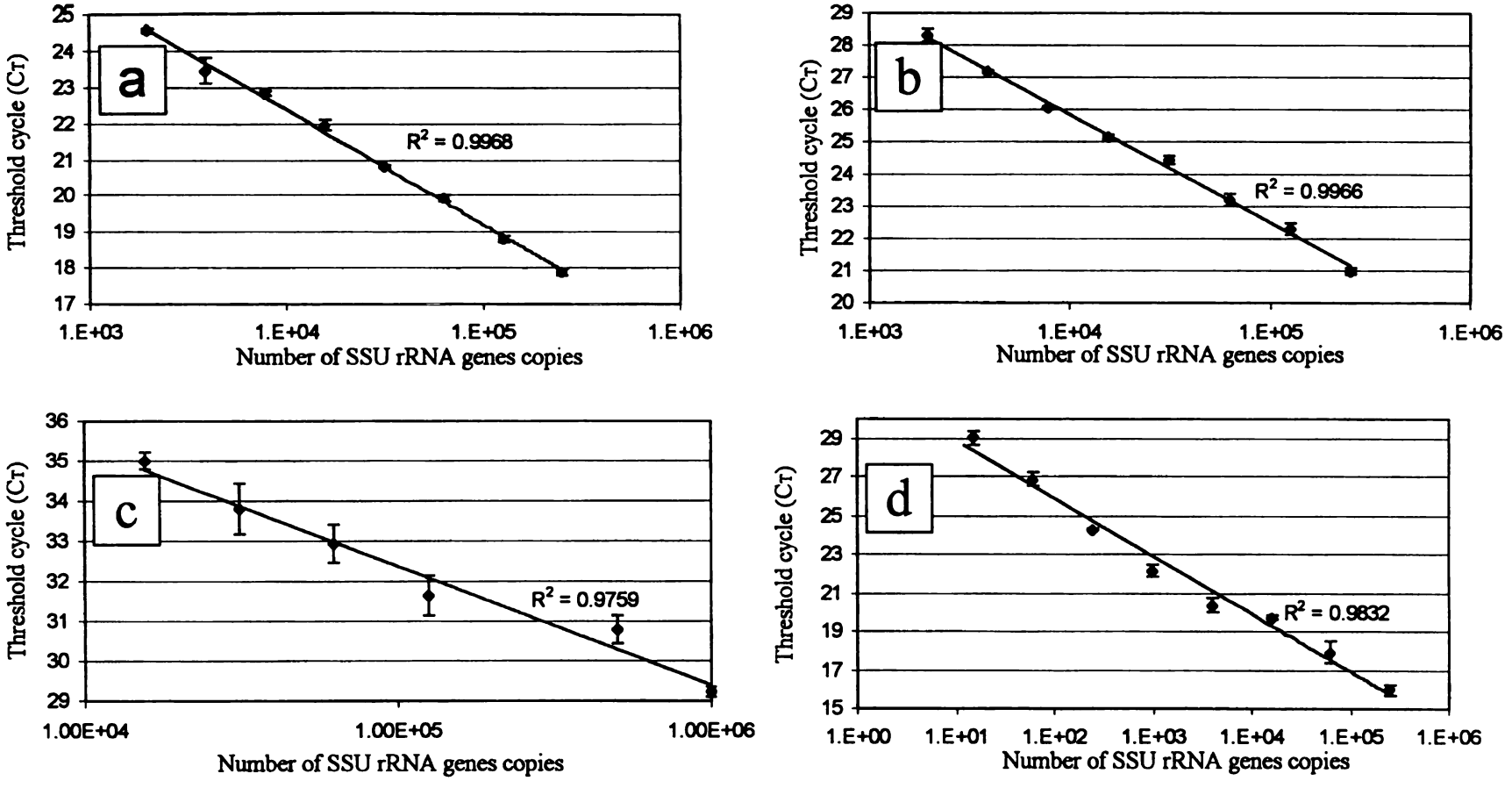

Figure 1 a-d Standard curves used to interpret real-time PCR data. Panel a depicts standard reactions with bacterial-specific primers, panel b depicts the archaeal standard reactions, panel c depicts the eukaryal reactions, and panel d depicts the standard reactions with clone 1Bb1-specific primers. Error bars indicate standard deviation among triplicate standards.

Table 2 The numbers of bacterial, archaeal, eukaryal and clone 1Bb1 SSU targets per gram of aquifer sediment (Wurtsmith, Bachman, and Oyster samples) or per ul of directly extracted DNA (bioreactor and activated sludge samples), and the standard error of each measurement. "% of total" is the percent of SSU genes that each kingdom contributed to the total SSU genes detected for a given sample. The percent contribution to the total number of bacterial 16S genes detected is reported for 1Bb1 16S sequences. The "+" symbol indicates that 1Bb1 sequences were detected in a sample but not quantitated and a "-" indicates that the group was not detected.

		Bacterial			Archaeal		
Sample origin	**Sample**	**16S genes / g sediment**	**Standard Error**	**% of total**	**16S genes / g sediment**	**Standard Error**	**% of total**
Bachman	IW-N (11 - 13 ft) Rep. I	2.96×10^6	6.88×10^3	70.4%	1.25×10^6	5.64×10^3	29.6%
Bachman	IW-N (11 - 13 ft) Rep. II	2.02×10^6	3.41×10^4	62.6%	1.20×10^6	1.62×10^4	37.4%
Bachman	IW-N (17 - 19 ft)	2.70×10^6	3.58×10^4	49.3%	2.78×10^6	3.35×10^4	50.7%
Bachman	IW-S (17 - 19 ft) Rep. I	2.14×10^6	1.59×10^4	64.4%	1.18×10^6	1.67×10^4	35.6%
Bachman	IW-S (17 - 19 ft) Rep. II	2.05×10^6	1.61×10^4	68.6%	9.37×10^5	5.45×10^3	31.4%
Bachman	EW (11 - 13 ft)	3.70×10^6	3.44×10^4	50.3%	3.66×10^6	2.25×10^4	49.7%
Bachman	EW (17 - 19 ft)	2.02×10^6	1.71×10^4	60.3%	1.33×10^6	2.05×10^4	39.7%
Wurtsmith	ML3 (15.5 -17.5 ft)	1.72×10^7	2.53×10^5	25.8%	4.94×10^7	6.17×10^5	74.2%
Wurtsmith	ML3 (23.5 - 27.5 ft)	2.22×10^6	2.14×10^4	1.4%	5.38×10^6	3.22×10^4	3.4%
Oyster	ODU4 (8.8 - 8.9 m)	6.81×10^6	2.17×10^5	98.3%	1.20×10^5	4.93×10^3	1.7%

Sample origin	**Sample**	**Targets / ul DNA extract**	**Standard Error**	**% of total**	**Targets / ul DNA extract**	**Standard Error**	**% of total**
Bioreactor	LSI Rep. I	2.06×10^6	2.92×10^4	14.4%	1.22×10^7	1.07×10^4	85.6%
Bioreactor	LSI Rep. II	1.20×10^6	9.48×10^3	15.5%	6.56×10^6	7.89×10^4	84.5%
Act. Sludge	AS Rep. I	1.36×10^6	1.05×10^4	0.3%	-	-	-
Act. Sludge	AS Rep. II	1.50×10^6	2.37×10^4	0.1%	-	-	-

Table 2 (Continued)

		Eukaryal			clone 1Bb1		
Sample origin	**Sample**	**18S genes / g sediment**	**Standard Error**	**% of total**	**16S genes / g sediment**	**Standard Error**	**% of bact. 16S genes**
Bachman	IW-N (11 - 13 ft) Rep. I	-	-	-	+	+	+
Bachman	IW-N (11 - 13 ft) Rep. II	-	-	-	-	-	-
Bachman	IW-N (17 - 19 ft)	-	-	-	1.66×10^4	6.05×10^2	0.6%
Bachman	IW-S (17 - 19 ft) Rep. I	-	-	-	+	+	+
Bachman	IW-S (17 - 19 ft) Rep. II	-	-	-	-	-	-
Bachman	EW (11 - 13 ft)	-	-	-	1.39×10^4	6.20×10^2	0.4%
Bachman	EW (17 - 19 ft)	-	-	-	9.64×10^3	3.75×10^2	0.5%
Wurtsmith	ML3 (15.5 -17.5 ft)	-	-	-	+	+	+
Wurtsmith	ML3 (23.5 - 27.5 ft)	1.51×10^8	5.33×10^5	95.2%	+	+	+
Oyster	ODU4 (8.8 - 8.9 m)	-	-	-	-	-	-

Sample origin	**Sample**	**Targets / ul DNA extract**	**Standard Error**	**% of total**
Bioreactor	LSI Rep. I	-	-	-
Bioreactor	LSI Rep. II	-	-	-
Act. Sludge	AS Rep. I	4.48×10^8	1.83×10^5	99.7%
Act. Sludge	AS Rep. II	1.61×10^9	2.50×10^5	99.9%

16S gene of our target organism, 1Bb1, ranged from 9.64×10^3 to 1.66×10^4 genes per gram of sediment.

S. cerevisiae DNA was used for building the standard curve with the eukaryotic primer set, and the number of 18S genes in *S. cerevisiae* is thought to vary between 200 and 400 copies per genome (1). The numbers of eukaryal 18S genes listed in Table 2 are conservative estimates based on the assumption that *S. cerevisiae* carried 200 18S genes per genome. Less conservative estimates may be calculated by increasing these values by a factor of two.

DISCUSSION

Real-time PCR has been shown to be a sensitive, precise method for quantifying target nucleotide sequences in both clinical and environmental samples (9, 22, 25, 26, 28, 38, 47). Through the use of four kingdom- and species-specific primer sets, we have used real-time PCR to detect and quantify the 16S rRNA genes in DNA extracted from environmental microbial communities. Each of the primer sets was found to be highly specific for the target group and we were able to detect and quantify as few as 2,000 bacterial, 2,000 archaeal, 8,000 eukaryal, and 250 SSU rRNA genes from the clone 1Bb1 per reaction.

In order to employ real-time PCR accurately, we validated three aspects of the method: specificity, sensitivity, and reproducibility. The primer pairs used in this study were found to be highly specific for their target groups. Furthermore, real-time PCR exhibited sensitivity to even very low concentrations of target sequences: reactions were sensitive to the presence of as few as 250 rRNA genes per tube. Finally, we evaluated the reproducibility of the method from one DNA extraction replicate to another. We

extracted and analyzed replicate sediment samples 1W-N (11-13 ft) I and II and 1W-S (17-19 ft) I and II, replicate bioreactor samples I and II, and replicate activated sludge samples I and II. In general, a more reproducible detection of absolute numbers of bacteria and archaea was observed in replicate sediment samples (replicate samples were within 4-46% different) than in replicate activated sludge and bioreactor samples (replicate samples within 11 and 86% different) which were derived from a single cell pellet. However, in terms of the representation of bacterial and archaeal 16S calculated for a given sample, the bioreactor replicates were more consistent (1-8% difference) than the replicate sediment samples (7-26% difference). Replicate sediment samples did not agree in the detection of clone sequence 1Bb1, suggesting that sediment heterogeneity, even within replicate samples from the same core, can affect the detection of small populations.

The primary focus of this study was the detection and quantitation of our target of interest, cloned 16S sequence 1Bb1, in sediment from the contaminated aquifer at the Bachman site. We were able to detect this sequence in five of the seven Bachman sediments tested and in both Wurtsmith sediments (Table 2). Clone 1Bb1 was not detected in any of the samples from the aquifer in Oyster, Virginia, the bioreactor, or activated sludge. Although this clone comprised 26% of a bacterial clone library derived from contaminated sediment at the Bachman site (see Chapter 4), we determined that it only comprised up to 0.6% of the total bacterial 16S in Bachman sediment. This discrepancy may have been caused by a bias in the PCR reaction used to amplify 16S for cloning (for a review of these considerations, see reference (44)) or to bias in the cloning

process, including toxicity of vector inserts to the transformed host, or the choice of cloning kit (27).

The discovery that the organism of interest, 1Bb1, constitutes less than 1% of the bacterial community at the Bachman site does not preclude its importance in the natural attenuation detected at the site. It is possible that the relative contribution of 1Bb1 to the activity of the microbial community is disproportional to the number of 16S genes detected in the sediments. In one scenario, a high number of dead or inactive cells that contribute their 16S genes in the extraction of DNA from a sample could reduce the comparative importance of this species. It is possible that 1Bb1 is directly involved in reductive dechlorination at the site, as sulfate-reducing isolates and mixed cultures have been found to reductively dechlorinate chlorinated ethenes (5, 20, 34). Further work with the isolate most closely related to this strain, STP23 (41), may help to define the processes that it undertakes at the Bachman site.

Bacterial 16S genes in Bachman sediment ranged from 2.02×10^6 to 3.70×10^6 per gram of sediment, archaeal 16S genes ranged from 9.37×10^5 to 3.66×10^6 per gram, and eukaryal 18S genes were not detected. Bacterial 16S genes outnumbered archaeal genes in these samples: the relative proportion of bacterial 16S genes to the total number of sequences detected ranged from 50.3% to 70.4% (Figure 2). In Wurtsmith sediment samples, bacterial 16S genes ranged from 2.22×10^6 to 1.72×10^7 per gram of sediment, archaeal 16S ranged from 5.38×10^6 to 4.94×10^7 genes per gram, and eukaryal 18S was determined to be 1.14×10^8 per gram in the deeper, saturated sediment. Eukaryal 18S genes dominated the numbers of rDNA detected in the saturated Wurtsmith sediment: they comprised 93.7% of the total SSU genes detected (Figure 2).

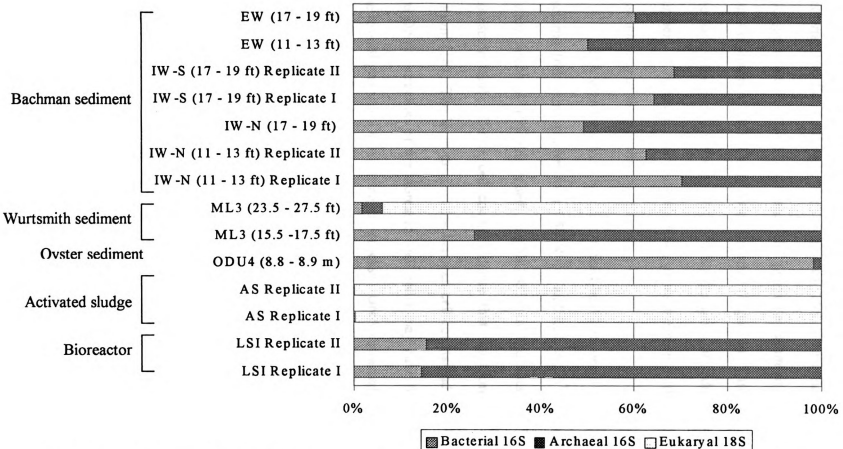

Figure 2 The relative contributions of bacterial, archaeal, and eukaryal SSU rRNA genes to total numbers of rRNA genes detected in DNA from activated sludge, bioreactor fluid, PCE-contaminated aquifer sediment from the Bachman site, pristine aquifer sediment from the Oyster site, and a contaminated aquifer at the former Wurtsmith Air Force Base. Archaeal 16S was not detected in either of the activated sludge replicates or in sample ODU4 6.3-6.4 m. Eukaryal 18S was detected only in Wurtsmith sediment ML3 (23.5 – 27.5 ft) and in the activated sludge samples.

In pristine, aerobic aquifer sediment from Oyster, Virginia, the number of bacterial 16S far outstripped that of archaeal 16S. We measured 3.81×10^6 bacterial 16S genes and 1.2×10^5 archaeal 16S genes per gram of sediment, so bacterial 16S genes represented 98.3% of all those detected (Figure 2). Direct cell counts at the Oyster site have measured approximately 10^7 cells per gram of sediment (21), and the relative numbers of bacterial and archaeal rRNA genes measure in this study suggests that the vast majority of theses cells are bacterial.

DNA extracted from bioreactor fluid held between 1.20×10^6 and 2.06×10^6 bacterial 16S genes and between 6.56×10^6 and 1.22×10^7 archaeal 16S genes per ul. Hence, archaeal 16S comprised roughly 85% of the total SSU genes detected in bioreactor fluid. Direct cell counts indicate that the bioreactor holds roughly 10^{10} cells/ml (16). The large discrepancy between cell counts and rRNA genes indicates that the DNA extraction of the bioreactor cell pellet may have been inefficient, resulting in the loss of genomic DNA during the process. DNA extracted from activated sludge held between 1.5×10^5 and 1.36×10^6 copies of bacterial 16S and 1.21×10^9 to 3.37×10^8 eukaryal 18S genes per ul, so eukaryotes represented over 99% of the total SSU genes detected (Figure 2).

In our measurements of SSU rRNA genes, replicate bioreactor and activated sludge samples varied by as much as 86% and replicate sediment samples varied by as much as 46%. In light of this, it is advisable when using real-time PCR to detect and quantify kingdom-level groups or small populations to thoroughly homogenize environmental samples and to use replicate samples for extraction and analysis.

The relative numbers of prokaryotic rRNA genes detected in DNA from aquifer sediment samples are consistent with the relative textures of these samples. Each of the sediment samples was examined and their relative textures were visually compared to the others in terms of relative texture (data not shown). The finest, sandiest textured sediments, ODU4 (8.8 – 8.9 m) and ML3 (15.5 – 17.5 ft), yielded the highest numbers of bacterial 16S, and the sediment samples with the coarsest texture, the Bachman sediments, yielded the lowest numbers. Although the number of bacterial 16S targets in the Oyster sediment sample ODU4 (8.8 – 8.9 m) was high, the organic carbon concentration in Oyster sediment, 1,000 ppb (21), was lower than that measured in sediment from either Bachman (as high as 2,400 ppb TCE alone (2)) or Wurtsmith (as high as 13,650 ppm (15)). These facts suggest that for our samples, the texture of a given sediment was a greater determinant of bacterial density than organic carbon concentration.

The ratios of archaeal 16S genes to bacterial 16S genes measured in sediment from the Bachman site and the Wurtsmith site and in biomass from the methanogenic bioreactor were higher than ratios of rRNA derived from hybridization data. At the Wurtsmith site, rRNA hybridization analysis has shown that archaeal rRNA comprises between 1.2 and 1.6% of the total rRNA in unsaturated sediment and between 0.9 and 9.5% of the total in saturated sediment (48). Hybridization analysis of the community in the methanogenic bioreactor has revealed that archaeal rRNA comprises up to 25% of the total (16), and work on other methanogenic bioreactors has revealed similar ratios of bacterial to archaeal rRNA (39). Of the combined numbers of archaeal and bacterial genes detected, we observed percentages of archaeal 16S DNA to be 70.8 to 74.2% in

Wurtsmith aquifer sediment and 84.5 to 85.6% in the bioreactor. We have confirmed these data by re-examining the concentration of DNA in reactions used to generate the standard curve, verifying that the standard curve was calibrated correctly (data not shown). We also ruled out the possibility that bacterial DNA affected amplification with archaeal primers by assembling artificial community DNA samples, in which *E. coli* and *M. jannaschii* DNA were combined in three different ratios. We were able to measure the relative contribution of bacterial and archaeal 16S targets to within 8% of the correct value (data not shown). Taking this line of investigation a step further, we developed control reactions with archaeal primers, varying concentrations of archaeal template, and 4.0×10^{-3} ng/ul bacterial DNA. Amplification of archaeal 16S was not affected by the presence of bacterial template. Furthermore, we verified that, in the absence of archaeal template, the presence of bacterial template in reactions with archaeal primers did not result in a false-positive amplification: we detected amplification greater than that seen in no-template controls only when the amount of bacterial DNA in the reaction exceeded 0.3 ng/ul. We estimated, from the number of bacterial and archaeal targets measured in our samples, that there was a maximum of 0.1 ng/ul of total template DNA in our reactions, which is well below this limit. Having validated the method of quantifying archaeal 16S using real-time PCR, we consider our data on archaeal 16S targets in aquifer sediment and bioreactor DNA to be accurate.

There are a number of possible explanations for the high archaeal:bacterial rDNA ratios in Wurtsmith sediment and bioreactor samples measured in this study. Firstly, differential cell retention times for bacteria and methanogenic archaea in the LS1 bioreactor may have affected these analyses. In the bioreactor, F_{420} fluorescent cells,

presumably methanogens, frequently formed aggregates. The cell retention time of inactive methanogens (with low rRNA content and ambient DNA content) in aggregates may have been affected by the differences in settling properties, leading to overrepresentation of archaeal cells in samples taken from the reactor. This would explain why high amounts of bacterial rRNA, which indicates metabolic activity, were detected in hybridization analysis, while the scales tipped in favor of archaea in the analysis of rDNA. The most likely explanation for the disparity between rRNA content and rDNA content, however, lies in the possibility that archaea maintain a higher rDNA:rRNA ratio than bacteria. Studies of *Sulfolobus acidocaldarius* and *M. jannaschii* have shown that, because of a characteristically eukaryal cell cycle, these species carry between 2 and 5 genome equivalents during stationary phase (6, 8, 31, 36). *E. coli* carries, on average, one genome equivalent during stationary phase (17). If relatively small populations of archaea in the Wurtsmith sediments and in the methanogenic bioreactor maintained multiple genome equivalents while bacterial cells maintained a single equivalent, comparisons of rDNA from these environments would be skewed in favor of the archaea. More study of the archaeal cell cycle may confirm that the maintenance of multiple chromosomes during stationary phase is ubiquitous among the archaea, as the machinery of cell division observed in *S. acidocaldarius* and *M. jannaschii* are thought to be shared by all members of the kingdom Archaea (7). If this is the case, then researchers using DNA-based comparisons, like real-time PCR, of archaeal and bacterial populations will have to address the relationship between activity and genome equivalents in members of these two kingdoms.

The detection of eukaryal 16S sequences in activated sludge DNA was expected, as municipal wastewater treatment sludge is known to harbor high numbers of diverse microeukaryotes (32). Eukaryal 16S genes were also detected in saturated sediment from the Wurtsmith site. Again, this is not an unexpected result, as previous work using rRNA hybridization has shown that eukaryal sequences comprise from 1.1 to 4.4% of the total detected SSU rRNA in sediment from 7.5 m below the surface (48). In the same study, rRNA from shallower, unsaturated sediment, was found to be 5% eukaryal. However, we did not detect eukaryal sequences in the unsaturated sediment from the Wurtsmith site, possibly due to temporal heterogeneity at the site.

The detection of eukaryotes in saturated sediment and their absence in unsaturated sediment is contrary to the trend noted by Madsen et al. (29), who measured higher numbers of culturable protists in unsaturated sediment than in saturated sediment from the same contaminated aquifer. They conclude from prior work (18) that the higher protozoan numbers in the unsaturated sediment indicate faster bacterial growth rates. If higher protozoan counts do signify faster bacterial growth, then growth rates may be higher in the saturated sediment at Wurtsmith than in the unsaturated sediment.

The numbers of eukaryotic 18S genes listed in Table 2 are conservative estimates based on the assumption that the DNA standard template, *S. cerevisiae*, yields 200 18S genes per genome (15 Mb). However, the number of 18S genes in the yeast genome may be as high as 400 (1). If this is the case, the number of 18S genes detected in our samples may be twice that reported in Table 2. In future work with real-time PCR, the eukaryal-specific primer set could be used to more precisely quantify these targets in a given sample through the development of a standard template for which the number of

SSU targets per ng of DNA is known. Since the number of 18S operons per genome can vary in eukaryotes, eukaryal 18S target sequence may have to be inserted into a cloning vector in order to develop a reliable standard template.

Where detected, the number of eukaryal 18S genes far exceeds that of bacterial and archaeal 16S genes (Table 2). In the activated sludge samples, eukaryal 18S comprises 99.7 to 99.9% of all SSU genes detected. In activated sludge, protozoa number about 5,000 per ml or 5% of the total dry weight (40). Further, seventy percent of protozoa in activated sludge are ciliates (14), a group which has been found to yield as many as 9,000 SSU genes per cell (45). Bacteria have been found to number as high as 10^{10} per ml in activated sludge. Assuming that eukaryotes maintain 9,000 copies of the rRNA operon and bacteria maintain approximately 5 copies, and using the observation that 99.8 of the detected SSU genes are eukaryal, these data translate into approximately four bacteria for every eukaryote. This is a distinct underestimate of measured ratios of bacterial to eukaryal organisms in activated sludge (10^{10}:5000), a discrepancy which may be due to errors in the aforementioned assumptions or differential DNA extraction efficiency from bacterial and eukaryal cells. In any event, eukarya are important members of the microbial community in activated sludge but the differences in rRNA copy number between bacteria and eukarya make population comparisons problematic.

In Wurtsmith sample ML3 (23.5 – 27.5 ft) eukaryal 18S genes comprise 95.2% of all SSU genes detected. In the aforementioned study of saturated Wurtsmith sediment communities, eukaryal rRNA was determined to represent from 1.1 to 4.4% of the total detected SSU rRNA (48). The differences in rDNA and rRNA representation in the

larger community suggests that the relative contributions of eukaryotic 18S rDNA and rRNA to total community pools are significantly different.

The use of a species-specific primer set in real-time PCR appears to hold a great deal of promise for the detection and quantitation of microbial species of interest. Even in conjunction with a universally-specific SSU primer, using the 1Bb1 primer set we were able to quantify as few as 250 16S targets per reaction. Future work with species and group-specific primer sets in real-time PCR could avoid some of the limitations of rRNA hybridization analysis while achieving quantitation of a species or genus of interest. For example, real-time PCR does not require handling of RNA, which can be unstable and is easily degraded, or radioactively-labeled probes, which can be of concern to individual or environmental health. However the potential for a disparity between the number rRNA genes per cell and the activity of that organism may complicate conclusions about the contribution of a species to the functioning of the whole community drawn from real-time PCR data. Further work in comparing real-time PCR with SSU rRNA and real-time PCR with a single-copy gene may help to correct for some of this potential for error. Another area for exploration lies in combining reverse-transcription PCR and real-time PCR, wherein SSU rRNA would be extracted from an environmental sample and amplified by reverse transcriptase in a real-time PCR reaction. This approach would avoid potential problems associated with tying rRNA gene abundance to activity, while sensitively detecting the presence and abundance of groups of interest in environmental samples. In any case, real-time PCR promises to be a valuable method in the detection and quantification of SSU rRNA from environmental communities.

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CHAPTER 6

CONCLUSIONS

The terrestrial subsurface is a relatively new frontier for microbial ecology. Prior to the advent of aseptic sampling of the subsurface in the 1980s little work was, in fact, possible. It is becoming clear, however, considering the sheer size of the terrestrial subsurface, subsurface microbes likely play a substantial role in global cycling of bioactive elements. Furthermore, the ecological connectivity of groundwater with surface systems means that the subsurface is not an isolated area, but instead has significant impacts on macro-ecological systems. And the human demands on subsurface water are substantial: we pump 77 billion gallons of groundwater per day in the US alone. So, the subsurface and groundwater in particular are key underpinnings of global nutrient cycles, ecosystem health, and human water requirements, and microbes are largely responsible for the chemical properties of these resources. Understanding the communities and the roles of individual populations within them will help us to manage groundwater resources and to better understand the role of the terrestrial subsurface in global nutrient cycling.

In an effort to further our understanding of subsurface microbial communities, the microbial diversity in aquifer sediment from two different sites was described using molecular, 16S rRNA-based approaches. The first site was a pristine, shallow, aerobic formation in Oyster, Virginia. The narrow channel focus area, or NCFA, is currently the focus of a Department of Energy – Natural and Accelerated Bioremediation study in bacterial transport, and has been extensively characterized for this effort. The aquifer is

an unconfined formation consisting of unconsolidated to weakly cemented, medium- to fine-grained sand and pebbly sand. These sediments are, in decreasing order of abundance: quartz, feldspar, clay, and iron and aluminum hydroxides, with the smaller sized quarts particles encrusted with clay and hydroxide coatings (3). Dissolved oxygen was measured at 5.5 mg/L and determined to be consistent across the study site, organic carbon was determined to be 1000 ppb, and the groundwater pH was 6.1(1).

The second study site was a tetrachloroethylene (PCE)-contaminated aquifer in Oscoda, Michigan referred to as the Bachman Road site. The Bachman aquifer is a similar formation to that in Oyster, Virginia: it is a shallow, sandy formation, composed of unconsolidated, medium-grained glacial sand. Within the plume, dissolved oxygen concentrations range from 1.8 mg/L 8 ft below ground level to 0.2 mg/L at 19 feet, and redox potentials range from –151 to –247 respectively. The concentration of organic carbon has not been measured in these sediments, but PCE alone has been found to reach as high as 10,000 ppb and within our sampling area the concentration of cis-dichloroethylene (cis-DCE) was determined to reach as high as 3,600 ppb (2). Measurements of groundwater pH within the plume range between 5.8 to 7.6 (2).

The bacterial communities at both sites were assessed using terminal restriction fragment length polymorphism analysis (T-RFLP). While analysis did reveal diverse communities at both sites, with Oyster communities presenting as many as 39 terminal restriction fragments in the *Hha* I digestion and Bachman contaminated sediment presenting as many as 26 and uncontaminated sediment presenting 5 terminal fragments, these communities appear to be less diverse than soil bacterial communities. Soil bacterial communities present between 60 and 80 terminal fragments when analyzed

using T-RFLP with the same primers and restriction enzyme. This suggests that the properties that the Oyster and Bachman sediments share that surface soils do not, most significantly water saturation and low concentrations of recalcitrant organic carbon, are strong determinants of bacterial diversity. Further work to relate indices of bacterial diversity to environmental heterogeneity and to concentration and complexity of organic carbon would prove valuable in better understanding these observations.

Furthermore, the phylogenetic identities of members of the bacterial communities at both sites were determined using 16S cloning and sequencing. Both sites exhibited previously unknown bacterial diversity: ten clones from Oyster and seven clones from Bachman failed to show a specific relationship to any of the known bacterial divisions. The subsurface is a relatively new environment for study, so it is not surprising that molecular microbiological techniques should reveal novel groups. While there was no overlap between the 16S sequences detected at Bachman and Oyster, three divisions were detected in clone libraries from both sites: *Acidobacteria*, *Leptospirillum/Nitrospira*, and the *Proteobacteria*. It could be that the characteristics the two sites share, including relatively low organic carbon concentrations, absence of light energy, low nutrient concentrations, water saturation, and low seasonal temperature fluctuations are driving these similarities.

The only isolated representative of the division *Acidobacteria*, *Acidobacterium capsulatum*, is an acidophilic chemoorganotroph. *Acidobacteria*-related sequences have been identified in many environments, including soil, subsurface sediment, and marine sediment. The diversity of these sequences suggests that the division *Acidobacteria* is likely quite a metabolically diverse group, but the lack of isolates from more

representative environments is limiting. Representatives of the division *Leptospirillum/Nitrospira* have not previously been identified in the subsurface. Isolates from three genera compose this division: *Leptospirillum, Nitrospira,* and *Thermodesulfovibrio. Leptospirillum* isolates are chemolithotrophic sulfur or iron oxidizers from acid mine drainage. *Nitrospira* are chemolithoautotrophic, oxidizing nitrite for energy, and *Thermodesulfovibrio* is a thermophilic sulfate-reducer. It is not known what roles the members of the divisions *Acidobacteria* and *Leptospirillum/Nitrospira* play in the subsurface, but further work to isolate members of these groups from subsurface sediment may help to better understand their functions. Isolates and 16S sequences of the division *Proteobacteria,* on the other hand, have been identified in most studies of subsurface bacterial communities and appear to be ubiquitous in these environments. There are innumerable isolates of this metabolically diverse division, but the sequences from Bachman and Oyster suggest that our knowledge about the diversity of the *Proteobacteria* is not complete, as most of these sequences failed to show relationships to any of the known genera. Again, more work to isolate novel *Proteobacteria* from subsurface sediment could help in our understanding of the activity of these species at Bachman and Oyster.

Sampling at the Oyster and Bachman sites may be seen as inadequate in light of the potential for sediment variation across very small distances to affect microbial abundance (4) and presumably community structure. Ideally, numerous cores from a particular sampling point (in a meter square area, for example) would be gathered and analyzed equally, but this is simply not feasible in the case of most subsurface investigations. Regardless, I find it unlikely that selective sampling of any environment

as complex as an aquifer will reveal the entirety of microbial diversity of that site. With the tools currently available to researchers, we have no choice but to extract and analyze a limited number of samples from and extrapolate those results appropriately.

The discovery of previously unidentified 16S sequences and sequences belonging to divisions about which we still know very little is an indication that a great deal of research remains to be carried out to characterize the microbial communities of the saturated subsurface. Further work should be focused on isolating and characterizing these organisms and defining their distribution in other subsurface environments using molecular techniques.

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APPENDIX A

CULTIVATION TECHNIQUES USED TO ISOLATE NCFA GROUP I BACTERIA

INTRODUCTION

A unique group of 16S sequences was identified at the Narrow Channel focus area at a study site in Oyster, Virginia, and was designated NCFA group I (see Chapters 2 and 3). Cultivation experiments were designed and implemented in an attempt to obtain these organisms in culture, but were unsuccessful. These experiments are described here.

Briefly, aliquots of sediment from two depths within the same borehole were serially diluted in minimal media with added sulfate, thiosulfate, nitrate, peptone, and yeast extract. Replicate dilutions were held at 10° C under three different gaseous conditions: anaerobic, microaerophilic with hydrogen, and microaerophilic without hydrogen. Cultures were periodically checked for the development of NCFA group I by whole cell PCR of an aliquot of culture using primers specific for the target organisms.

Media was created by adding ingredients to water in order listed in Table 1 then making up to the final volume. Table 2 lists the ingredients of the trace metals solution used in the media. The mixture was boiled on a hot plate and cooled in an ice tub under a flow of nitrogen gas. Under nitrogen, 18 ml of media was transferred to individual Balsch tubes and sealed with rubber stoppers and aluminum crimp tops. The tubes were cooled and 90 ul of 10% dithiothreitol (DTT) solution was added to the tubes to be used for anaerobic cultures and microaerophilic cultures under hydrogen. The tubes were autoclaved for 45 minutes.

Table 1 Media used for cultivation of NCFA group I. (Quantities are in units of g per L). Store metals solution at 4 degrees C.

NaCl	0.05
MgCl2·6H2O	0.5
KH2PO4	0.2
K2HPO4	0.25
NH4Cl	0.3
KCl	0.3
CaCl2·2H2O	0.015
Yeast extract	0.1
Peptone	0.1
Agar	1
Na2SO4	0.142
KNO3	0.05
Na2S2O3·5H2O	0.25
Resazurine	0.001
Trace metals solution	1 ml

Table 2 Trace metals solution for growth media. (Quantities are in units of g per L)

Nitrotriacetic acid	12.8
PH 6.5 then add in order, dissolving each one:	
FeCl3·3H2O	1.35
MnCl2·4H2O	0.1
CoCl2·6H2O	0.024
ZnCl2	0.1
CuCl2·2H2O	0.025
H3BO4	0.01
NaCl	1
Na2SeO3·5H2O	0.026
NiCl2·6H2O	0.12
Na2Wo4·2H2O	0.033

Two sediment samples were used for inoculation, both of which were recovered from the site in August, 2000: ODU2 7.7-7.8 m and ODU2 6-7 m. Media was inoculated in the anaerobic hood (90% nitrogen and 10% hydrogen atmosphere), keeping the media and sediment on ice as temperatures exceeded 37° C inside. Approximately 2 grams of sediment was added to the first tube in the series (1:10 dilution w:v), and the tube was capped and mixed by shaking. The mixture was allowed to settle for 10 seconds, then 2 ml was transferred to the next tube in the series. The dilution series was carried out eight times for a total of nine tubes. Each sediment was used as inoculum for three replicate dilution series. The replicate series destined for anaerobic conditions was recapped in the hood and handled anaerobically for all subsequent sampling. The replicate series destined for microaerophilic conditions with hydrogen were loosely capped with culture tube caps (not rubber stoppers) and transferred to gas pack jars with Campy Pak Plus microaerophilic envelopes (Becton Dickinson Microbiology Systems) which provided a microaerophilic environment with hydrogen and carbon dioxide. The microaerophilic replicate series was loosely capped with culture tube caps. All replicate series were placed at 10° C in the dark.

Cultures were sampled in the anaerobic hood every other day as they grew turbid, up to 18 days after inoculation. Two depths of each culture were sampled (1 cm from the meniscus and 1 cm from the bottom) using sterile Pasteur pipettes and 1.25 ul was used for each of two PCR screening reactions using (1) the NCFA group 1-specific primer set (see Chapter 3) and (2) bacterial primers 27F and 1392R. The amplification conditions,

including cycle parameters and reagent concentrations are identical to those outlined in Chapter 2 for bacterial 16S cloning.

APPENDIX B

T-RFLP DATA FROM CHAPTERS 2 AND 4

Table 1 Chapter 3 data.

SOUTH OYSTER FOCUS AREA SEDIMENT

SEDIMENT B2 (5 m)

Hha I		Msp I		Rsa I	
Terminal Fragment	Peak area	Terminal Fragment	Peak area	Terminal Fragment	Peak area
32.73	536	32.75	333	32.97	377
33.9	1260	33.91	951	34.12	945
35.64	361	38.98	1369	39.15	1406
38.99	1858	39.96	1496	40.12	1367
39.98	1595	41.54	2098	41.68	1917
41.57	3140	48.81	151	79.99	281
48.88	2037	125.9	7732	82.08	3849
60.43	876	141.98	1556	109.63	404
66.07	7207	143.8	3163	110.71	399
181.14	935	172.97	3879	116.81	5058
182.11	302	177.18	1478	119.55	30299
182.92	467	197.19	346	475.34	38551
183.57	1297	234.71	636	482.51	2094
187.37	580	431.87	10567	489.17	492
187.97	690	433.76	37121	492.06	1207
189.34	822	440.56	1733	492.7	1146
206.2	31812	477.37	8082		
370.75	1797	492.09	3043		
567.63	3358	494.02	31662		
568.83	3923				
574.52	1007				

SEDIMENT M3 (6 m)

Hha I		Msp I		Rsa I	
Terminal Fragment	Peak area	Terminal Fragment	Peak area	Terminal Fragment	Peak area
33.92	2493	34.11	1881	33.92	1338

Table 1 (cont'd)

39.16	1741	39.95	2610	38.97	1022
39.95	1825	41.52	2050	39.95	1290
41.52	2978	48.94	215	41.52	1540
48.94	324	140.02	375	117.61	381
65.61	618	433.14	600	119.7	5274
206.38	7413	435.02	4649	430.7	1043
569.07	9922	493.99	5126	431.48	1479
		495.58	14597	472.02	523
				476.4	2632
				477.75	2219

SEDIMENT S10 (5 m)

Hha I		Msp I		Rsa I	
Terminal Fragment	Peak area	Terminal Fragment	Peak area	Terminal Fragment	Peak area
34.11	3780	32.97	443	32.97	343
35.65	787	34.12	1956	33.93	1463
37.98	1926	37.2	352	39.15	1621
39.16	2454	39.15	1505	39.93	1283
39.95	2961	40.12	1732	41.49	2087
41.52	5007	41.49	2522	117.78	439
48.94	591	48.88	187	464.27	4241
50.7	1513	125.92	158	467.61	319
65.99	2064	133.43	1459	476.18	26912
67.72	1637	152.36	609		
196.93	27974	154.35	15683		
225.15	3378	174.43	1512		
227.3	6353	186.3	584		
234.61	9779	293.87	4411		
372.07	2000	492.56	2521		
569.67	1231	495.75	1991		
		500.38	4023		
		517.27	1147		

Table 1 (cont'd)

SEDIMENT S14 (5 m)

Hha I		Msp I		Rsa I	
Terminal Fragment	Peak area	Terminal Fragment	Peak area	Terminal Fragment	Peak area
32.94	393	32.96	422	32.96	287
33.91	1377	34.11	1331	34.11	1236
38.98	1667	39.16	1870	39.16	1704
39.96	1512	40.14	1756	39.95	1493
41.54	2494	41.52	1992	41.52	1695
48.81	984	126.07	3408	100.83	571
65.74	3966	138.35	846	117.61	3153
77.28	980	140.16	2200	119.54	9311
202.37	781	142.13	531	430.55	4964
203.46	983	432.34	2391	471.85	4996
206.58	29426	434.23	7721	475.89	46424
210.19	678	459.1	758		
368.76	834	460.86	2392		
370.59	2187	477.64	1006		
566.59	3991	492.08	19866		
569.01	14108	494	29789		
		494.95	36754		
		600.2	2536		

SEDIMENT S18 (6 m)

Hha I		Msp I		Rsa I	
Terminal Fragment	Peak area	Terminal Fragment	Peak area	Terminal Fragment	Peak area
32.56	507	33.92	205	31.99	518
33.91	751	39.16	583	33.92	435
37	384	39.95	756	38.97	918
37.99	541	41.52	1107	39.95	1086
39.17	1202	84.73	960	41.52	1310
39.96	1251	86.25	1968	67.92	745
41.54	3056	88.9	1690	89.28	717
49.01	332	126	1616	116.8	3815
89.84	1753	140.35	678	119.54	10041

Table 1 (cont'd)

121.06	446	142.16	780	126.81	900
122.54	721	156.39	327	127.79	538
123.52	1364	158.56	489	292.17	951
150.74	2227	443.03	350	307.31	2629
164.43	371	446.19	1202	476.63	6897
165.44	964	449.21	1855	482.22	3710
206.06	19491	450.64	1363		
206.85	25477	451.59	778		
370.85	1059	452.23	811		
374.2	520	453.02	672		
543.3	1112	454.45	1560		
566.14	4448	455.73	1489		
569.07	20229	456.85	889		
574.43	3728	457.48	867		
		458.28	3179		
		484.38	1069		
		493.36	9910		
		494.47	16520		
		495.59	48757		
		515.74	625		
		517.31	1226		
		600.12	4484		

SEDIMENT T2 (4 m)

Hha I		Msp I		Rsa I	
Terminal Fragment	Peak area	Terminal Fragment	Peak area	Terminal Fragment	Peak area
32.75	396	33.91	838	31.82	359
33.91	655	38.98	1235	32.78	372
37.2	350	39.96	1170	33.93	407
38.96	1559	41.54	1580	39.13	1322
39.94	1817	67.67	1784	39.9	1500
41.5	2969	88.7	435	41.45	2010
48.72	5370	98.15	1991	81.8	1943
65.6	2831	100.07	1096	89.24	1211
120.26	3483	100.83	1090	101.04	1061
175.56	817	103.62	1340	109.72	2288

Table 1 (cont'd)

206.32	33716	104.72	699	110.71	2416
369.66	12317	105.26	786	111.68	2834
374.08	2944	106.7	646	116	12216
375.77	878	107.77	1319	118.82	32803
397	1108	110.87	1531	170.56	3452
567.43	4881	112.1	750	171.64	3155
569.15	18831	113.16	872	427.14	4511
577.49	522	114.21	1078	475.05	31411
		120.7	566		
		124.92	212		
		126.06	6920		
		127.37	3867		
		172.81	1262		
		432.22	23210		
		433.79	28376		
		443.91	1464		
		477.37	5117		
		490.48	677		
		492.4	5008		
		493.53	5929		
		495.45	28178		
		600.25	1764		

SEDIMENT T2 (6 m)

Hha I		Msp I		Rsa I	
Terminal Fragment	Peak area	Terminal Fragment	Peak area	Terminal Fragment	Peak area
33.91	2763	33.92	2570	33.92	1779
39.17	3036	39.16	2051	38.97	1644
40.16	2360	39.95	2078	39.95	1671
41.54	5005	41.52	3165	41.32	2137
48.81	243	67.72	401	117.46	3176
65.74	4150	138.42	360	119.57	6737
67.86	388	140.23	1638	430.55	5499
77.28	849	142.37	663	472.02	1971
202.56	346	434.23	2246	473.37	2798
203.65	1558	459.1	544	476.57	4117

Table 1 (cont'd)

206.32	8304	460.86	1725
368.75	542	492.4	10229
369.82	984	493.36	9765
566.56	2603	495.59	23181
568.98	14926	600.36	606

NARROW CHANNEL FOCUS AREA SEDIMENT

SEDIMENT B2 (8 m)

Hha I

Primer set 8F-1392R		Primer set 8F-1525R	
Terminal fragment	Peak area	Terminal fragment	Peak area
39.27	1311	30.33	9983
40.22	305	40.22	475
41.98	1127	41.82	1417
64.16	928	64.16	1747
69.59	441	92.72	2233
87.14	355	95.21	1032
92.72	3510	102.94	484
94.51	482	105.72	5621
103.2	3225	205.39	6079
105.53	994	206.91	3347
107.36	596	210.1	848
108.7	773	211.91	687
120.38	961	219.18	2084
121.55	521	220.59	1301
128.56	358	313.51	1378
136.16	1261	365.77	7154
138.51	485	379.34	2673
141.14	1024	382.62	756
205.36	1292	384.76	2317
207.03	763	487.75	3966
219.18	750	573.72	3075
365.78	819	581.14	819
373.14	1162		

Table 1 (cont'd)

375.27	1088
378.83	8222
474.5	3220
476.76	5737
487.57	4538
574.17	1231

Msp I

Primer set 8F-1392R

Terminal fragment	Peak area
39.27	1191
67.99	1047
70.38	1606
86.98	563
103.41	2800
105.41	372
106.33	473
107.38	597
108.71	761
120.35	871
121.51	464
129.93	2178
136.05	1207
138.38	378
139.84	2425
141.01	1040
161.09	4021
163.45	2927
175.02	426
180.31	478
206.69	427
225.63	734
282.78	576
289.66	456
478.2	927
488.54	975

Primer set 8F-1525R

Terminal fragment	Peak area
31.16	11259
69.68	504
86.89	3625
129.99	1389
139.9	1792
143.4	596
144.13	1147
148.22	1522
161.05	699
162.66	679
163.97	486
171.85	475
180.17	1647
186.13	1023
205.52	480
225.46	5176
282.66	5351
289.65	956
292.6	2541
297.22	2941
474.29	1423
479.08	674
488.84	392
495.48	7409
499.08	754
505.04	1253

Table 1 (cont'd)

495.48	2639	515.11	2292
513.82	3872	521.42	650
519.56	4851	534.09	395
574.16	1785		

Rsa I

Primer set 8F-1392R		Primer set 8F-1525R	
Terminal fragment	Peak area	Terminal fragment	Peak area
34.16	2203	40.29	1097
39.27	878	85.94	2887
40.7	1047	243.38	1843
56.66	429	418.06	946
63.2	501	450.82	1714
68.79	633	455.2	9893
69.43	655	468.23	2060
86.02	458	477.85	10305
103.41	3263	484.11	3621
105.41	276	485.67	2392
106.33	550	489.65	5685
107.38	680	490.47	16611
109	1414	498.18	5531
120.19	731		
136	1344		
141.09	840		
243.28	2877		
455.59	1502		
458.08	3961		
460.1	8185		
478.74	4668		
484.29	1492		
488.32	307		
489.5	1407		
497.08	13878		
502.9	6893		

Table 1 (cont'd)

SEDIMENT S18 (6 m)

Hha I

Primer set 8F-1392R		Primer set 8F-1525R	
Terminal fragment	Peak area	Terminal fragment	Peak area
37.91	345	68.47	657
38.73	494	69.27	940
40.52	1257	96.04	1204
61.39	450	107.36	830
68.73	411	114.02	606
86.66	387	124.6	661
92.53	494	129.99	359
103.33	1566	132.18	483
211.9	1975	137.13	493
370.15	691	139.76	1854
375.68	739	142.38	4767
379.1	2046	143.99	782
389.12	959	145.59	3385
402.45	2709	148.22	827
487.73	2133	149.67	416
550.11	575	151.42	880
570.14	579	162.66	813
573.74	2183	163.68	760
		177.74	421
		186	1619
		282.63	3688
		285.71	1120
		293.46	1136
		294.72	3804
		300.2	690
		305.25	4696
		469.29	807
		471.13	681
		471.89	361
		472.39	377
		473.02	919
		477.62	883

Table 1 (cont'd)

485.2	482
491.58	3673
495.22	4159
507.41	1448
509.11	386
511.7	902
527.56	1243
589.32	1258

Msp I

Primer set 8F-1392R		Primer set 8F-1525R	
Terminal fragment	Peak area	Terminal fragment	Peak area
39.27	350	40.22	773
69.43	392	41.82	1979
70.22	715	62.24	262
87.14	808	79	1191
88.89	431	79.96	458
98.58	356	92.72	730
103.41	1281	95.38	1182
139.7	1561	105.72	407
142.33	981	154.14	578
145.4	979	184.14	512
147.74	407	211.8	10271
149.35	1456	223.6	4543
163.74	681	227.46	1379
305.51	591	230.27	2562
428.21	804	302.95	829
489.03	797	311.26	538
495.92	1687	313.52	2523
499.51	1116	363.79	675
514.3	978	365.89	4330
520.65	1511	370.09	462
528.62	1107	371.64	589
		376.01	1816
		378.13	781
		382.67	1439

Table 1 (cont'd)

384.95	597
402.19	11289
410.04	643
487.73	3401
534.55	1739
573.88	3937

Rsa I

Primer set 8F-1392R		Primer set 8F-1525R	
Terminal fragment	Peak area	Terminal fragment	Peak area
34.32	523	41.02	1230
39.43	233	84.59	1231
40.86	528	86.02	2660
69.43	863	180.74	2211
86.02	1102	297.8	634
103.41	1754	418.38	467
105.41	540	437.48	2407
437.13	408	446.52	1228
449.98	1093	457.39	11823
458.09	3006	461.62	7231
461.94	1266	466.67	524
469.69	889	470.68	3984
471.31	528	473.39	1164
479.65	1980	474.41	625
481.62	2676	475.77	682
485.56	1683	479.33	3074
491.89	763	482.89	2502
493.15	880	484.75	1352
497.26	4305	485.6	1140
502.81	5433	486.78	2397
		488.48	934
		491.73	5665
		492.99	9888
		498.18	1304
		499.91	2670

Table 1 (cont'd)

502.74	609
517.18	661

SEDIMENT S18 (8 m)

Hha I

Primer set 8F-1392R		Primer set 8F-1525R	
Terminal fragment	Peak area	Terminal fragment	Peak area
39.11	486	69.74	406
40.06	439	96.24	326
41.82	1059	124.44	497
62.24	707	139.75	707
69.43	265	142.38	2026
86.98	920	145.6	1103
92.72	702	185.86	662
103.2	2242	282.52	537
114.87	478	294.71	822
117.9	405	305.38	816
123.18	400	491.28	8601
124.06	681	495.4	1740
137.39	648	527.51	622
151.9	433		
169.36	885		
206.86	556		
212.01	1583		
225.77	511		
370.13	797		
379.06	1522		
380.77	476		
389.21	847		
402.52	2089		
488.06	1854		
573.96	2869		

Table 1 (cont'd)

Msp I

Primer set 8F-1392R		Primer set 8F-1525R	
Terminal fragment	Peak area	Terminal fragment	Peak area
39.27	392	40.22	459
64.64	305	41.82	1056
69.43	239	79	600
70.38	553	95.38	336
87.14	786	211.87	3515
88.89	397	216.08	562
103.41	1815	221.43	444
114.76	694	223.44	1962
123.95	494	230.28	763
136.29	463	313.32	665
137.16	658	365.96	668
139.64	1357	382.67	641
142.26	677	402.54	5061
145.32	897	410.02	583
149.41	1249	487.12	1726
151.6	853	572.71	4911
163.76	460		
169.35	614		
468.61	573		
489.02	739		
495.76	4636		
499.66	913		
514.73	722		
520.73	995		
529.14	707		

Rsa I

Primer set 8F-1392R		Primer set 8F-1525R	
Terminal fragment	Peak area	Terminal fragment	Peak area
34.16	1825	40.45	753
39.27	356	84.48	445
40.7	412	85.94	855

Table 1 (cont'd)

56.82	228	180.88	1087
63.2	860	433.2	1456
64.16	281	437.76	818
67.03	350	456.37	508
69.43	504	458.09	6597
85.86	696	462.46	2016
103.36	1349	466.53	424
117.89	393	470.66	1170
190.74	386	478.91	5683
458.86	1726	483.21	1175
462.87	577	484.37	915
479.56	531	487.18	1196
480.37	826	488.34	1666
482.16	1747	493.31	8095
486.73	1298	498.05	856
497.96	2285	499.94	763
504.21	3302		

SEDIMENT M3 (6 m)

Hha I

Primer set 8F-1392R		Primer set 8F-1525R	
Terminal fragment	Peak area	Terminal fragment	Peak area
39.27	337	31.16	8097
69.59	452	204.2	2830
86.98	324	206.55	31473
103.36	1117	234.24	1450
107.51	686	362.53	611
204.18	1887	369.93	11955
206.53	16851	470.48	1614
234.12	1155	471.12	439
369.72	10465	472.08	1011
469.53	1286	473.53	1122
470.48	537	475.45	571
471.12	668	476.58	330
471.92	561	515.49	1004

Table 1 (cont'd)

472.89	893	566.94	115605
474.01	463		
475.62	1045		
515.01	515		
515.65	312		
516.78	876		
566.8	118058		

Msp I

Primer set 8F-1392R		Primer set 8F-1525R	
Terminal fragment	Peak area	Terminal fragment	Peak area
31.16	11215	158.92	604
39.84	373	206.56	877
69.84	488	419.24	2198
87.21	308	420.74	1073
103.24	1166	422.41	1453
107.36	773	430.16	630
206.42	1284	493.86	167712
369.18	858		
420.99	2389		
421.89	694		
423.41	2374		
493.84	152150		

Rsa I

Primer set 8F-1392R		Primer set 8F-1525R	
Terminal fragment	Peak area	Terminal fragment	Peak area
34.32	1630	119.81	4655
38.63	200	329.02	974
39.43	276	397.66	5742
43.26	530	399.48	1691
63.36	394	401	3466
69.59	425	433.17	47840
103.36	765	469.24	445
107.36	754	476.12	66974
119.79	2910	651.4	6278

Table 1 (cont'd)

395.19	1978
396.53	1836
397.57	854
398.32	700
398.91	1361
400.26	2177
403.24	726
433.36	22555
475.77	63608

Table 2 Chapter 4 bacterial T-RFLP data

SEDIMENT 1At

Hha I

Data from 4/10		**Data from 4/27**		**After eliminating inconsistent peaks**
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment
36.81	764	37	1386	37
38.14	290	58.18	445	58.18
57.91	300	59.85	398	59.85
59.62	275	95.14	2971	95.14
95.03	2656	97.01	473	98.48
98.62	479	98.48	636	155.84
155	1294	111.28	417	197.9
197.49	825	155.84	1711	201.42
201.33	3975	197.9	751	204.53
204.27	750	199.07	562	206.46
205.82	1574	201.42	4789	215.45
215.27	578	204.53	1232	218.94
218.94	1631	206.46	1743	232.84
233	2509	208.39	689	369.78
370.1	1592	215.45	751	374.41
373.97	599	218.94	2379	375.56
376.14	506	232.84	4412	380.78
381.12	4935	364.02	605	539.79
539.61	710	368.2	537	540.75
540.99	1013	369.78	2619	542.65
542.71	830	374.41	1134	544.57
544.27	1264	375.56	1552	548.42
546.7	304	377.59	1004	565.19
548.44	1739	379.48	2985	569.17
565.22	841	380.78	6001	581.07
567.19	756	406.8	703	585.65
569.16	1938	415.2	481	
571.5	1347	539.79	2472	
580.94	1815	540.75	2670	
584.98	793	542.65	997	
585.9	1071	544.57	3302	
		548.42	4334	

Table 2 (cont'd)

554.07	824
556.02	2011
560.26	1546
561.9	705
565.19	2587
569.17	7632
574	555
575.01	347
576.85	1346
581.07	4862
585.65	4047
590.78	1704
599.42	544

Msp I				**After eliminating**
Data from 4/10		**Data from 4/27**		**inconsistent peaks**
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment
34.33	3813	75.84	496	75.84
34.33	4219	93.1	518	93.1
75.17	518	134.16	472	149.32
77.64	393	149.32	471	156.16
93.24	635	156.16	1105	158.23
149.35	630	158.23	1489	161.9
155.66	1044	161.9	779	163.82
157.83	1231	163.82	1855	165.9
161.86	628	165.9	880	172.93
163.54	1880	170.55	388	448.48
165.73	689	172.93	649	450.29
172.8	654	206.22	520	463.91
207.25	1256	207.56	904	466.19
230.96	546	229.18	410	481.85
448.35	2232	285.53	410	482.97
450.15	835	286.46	470	491.97
463.68	1071	288.43	926	495.76
466.17	1172	376.58	429	505.44

Table 2 (cont'd)

481.62	514	437.82	577	507.26
482.87	1033	448.48	3242	512.25
490.84	655	450.29	830	513.76
492.34	1469	463.91	1361	520.87
494.17	1579	466.19	1364	522.54
496.17	3018	481.85	873	523.6
505.81	1909	482.97	1094	527.98
507.64	2762	490.15	936	529.8
512.44	2228	491.97	1885	532.22
514.09	3940	493.64	1794	534.03
519.54	1362	495.76	4103	569.24
520.86	1157	505.44	2589	
521.69	792	507.26	3204	
522.51	801	512.25	2979	
523.5	1193	513.76	4077	
524.98	1635	520.87	3131	
527.12	356	522.54	2075	
527.78	782	523.6	2780	
529.59	861	527.98	1326	
532.22	804	529.8	1358	
534.03	656	532.22	1099	
568.91	999	534.03	698	
		569.24	1802	
		570.9	858	

Rsa I

Data from 4/10		**Data from 4/27**		**After eliminating inconsistent peaks**
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment
33.38	1045	56.89	595	56.89
34.14	2653	122.1	827	122.1
34.33	4219	130.43	308	131.69
56.8	440	131.69	830	446.11
121.94	526	298.9	892	451.71
131.64	560	421.23	628	453.07

Table 2 (cont'd)

445.83	504	428.68	665	454.14
451.71	1746	446.11	941	459.46
453.01	667	451.71	2973	460.98
454.33	1837	453.07	1250	464.64
459.58	1186	454.14	2857	473.07
460.74	998	459.46	2112	476.63
464.7	1049	460.98	1593	478.73
473.37	2001	464.64	1531	489.04
476.91	1569	466.17	350	495.93
478.51	5441	466.78	765	502.3
489.12	2377	473.07	3250	
496.32	4727	476.63	2875	
502.47	553	478.73	8997	
		489.04	3468	
		495.93	7416	
		502.3	1357	

SEDIMENT 2At

Hha I

Data from 4/10		**Data from 4/27**		**After eliminating inconsistent peaks**
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment
36.62	1403	36.81	2382	36.81
59.86	1495	56.61	402	59.96
69.97	599	57.73	621	94.96
81.55	403	59.96	2806	204.08
95	2019	62.19	763	206.01
143.23	372	72.72	345	209.29
144.05	472	82.42	923	211.69
205.58	2064	94.96	4799	369.79
209	1127	97.21	396	381.23
211.19	452	98.51	410	548.71
211.98	598	101.05	474	568.95
287.63	2686	176.18	776	585.43
370.09	4679	204.08	586	
379.7	4066	206.01	3626	

Table 2 (cont'd)

381.72	3744	209.29	958
548.85	4139	211.69	1079
567.32	5288	341.57	930
569.3	11368	369.79	6924
582.8	756	375.57	1154
585.2	1744	379.05	6091
		381.23	4245
		406.67	431
		407.25	490
		484.25	759
		538.84	2273
		541.69	1058
		543.91	860
		548.71	8494
		552.9	958
		555.66	648
		556.31	401
		557.28	385
		558.59	833
		560.55	872
		565.48	5393
		568.95	26683
		576.97	479
		578.48	715
		579.83	571
		585.43	5995
		589.18	715
		590.73	375
		596.25	622

Msp I

Data from 4/10

Terminal fragment	Peak area
65.34	582
127.91	689
131.15	1402

Data from 4/27

Terminal fragment	Peak area
33.11	610
42.88	313
65.16	620

After eliminating inconsistent peaks

Terminal fragment
65.16
128.28
131.59

Table 2 (cont'd)

149.18	891	128.28	491	149.61
162.53	1088	131.59	1275	162.93
207.88	938	149.61	506	207.7
209.75	803	150.88	397	288.44
288.12	858	162.93	1151	431.77
432.3	788	165.16	541	465.88
466.01	1423	207.7	323	495.62
494.01	7080	288.44	1004	502.29
496.01	10282	290.41	445	513.34
502.82	1430	431.77	968	528
513.1	5351	438.95	471	529.81
528.11	1469	465.88	1003	600.8
529.26	1413	493.65	9200	
598.26	738	495.62	12666	
600.15	1583	501.23	637	
		502.29	2003	
		511.38	952	
		513.34	3459	
		515.46	1380	
		528	1922	
		529.81	1572	
		599.06	669	
		600.8	1683	

Rsa I

Data from 4/10		**Data from 4/27**		**After eliminating inconsistent peaks**
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment
33.38	544	33.11	335	33.11
54.12	260	56.98	525	56.98
56.8	270	284.44	578	430.34
130.09	426	430.34	3098	459.46
430.54	1496	459.46	2249	460.99
459.89	1806	460.99	2410	474.53
460.89	1382	474.53	5253	476.47
474.61	3550	476.47	13815	484.21
476.91	13210	480.66	1915	496.38

Table 2 (cont'd)

484.34	719	484.21	1112
496.66	4619	494.86	2902
498.15	2039	496.38	4502
		501.24	678

SEDIMENT 2Bb

Hha I

Data from 4/10		**Data from 4/27**		**After eliminating inconsistent peaks**
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment
36.04	1725	35.16	1152	61.89
60.24	625	37	3995	91.3
62.17	299	55.77	312	94.95
91.23	573	56.7	334	206.11
95.36	1391	57.81	444	369.64
205.74	1099	59.85	1840	380.5
207.44	416	61.89	1229	548.71
287.79	1710	82.43	934	569.12
370.08	1930	86.6	613	
373.17	246	91.3	1663	
373.79	584	94.95	4230	
377.51	1375	101.02	461	
380.46	2387	143.21	331	
381.86	1690	175.37	629	
548.85	1439	176.13	1177	
550.43	752	182.67	940	
567.15	1456	206.11	3145	
569.31	6839	208.96	1179	
		357.44	1850	
		359.81	660	
		362.29	547	
		363.01	844	
		369.64	6851	
		375.14	1302	
		376.87	3732	
		379.48	3913	

Table 2 (cont'd)

380.5	5738
403.43	654
415.22	741
540.58	3869
543.91	1013
548.71	7291
554.85	311
556.14	568
559.4	942
561.04	551
563.34	836
565.31	2106
569.12	16391
574.95	655
576.63	247
577.81	697
581.69	1543
584.23	2458
587.3	1530
589.35	392
590.21	517
591.59	256
592.62	432
594.17	515

Msp I

Data from 4/10		**Data from 4/27**		**Consistent peaks**
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment
34.14	5938	64.73	300	64.73
43.12	242	65.84	661	93.1
65.07	708	93.1	297	139.68
93.05	272	139.68	818	149.32
139.3	698	149.32	656	151.23
149.18	765	151.23	1510	174.79
150.84	1005	158.87	330	207.65
159.51	799	160.3	673	217.12

Table 2 (cont'd)

161.86	1193	162.38	1447	491.82
163.54	697	164.3	742	493.8
174.56	450	174.79	585	495.62
207.97	1158	176.33	507	502.6
210.02	626	207.65	600	509.87
217.16	885	208.99	919	512.13
491.84	1021	217.12	1019	514.25
494	5568	465.87	869	516.37
496.01	11580	491.82	1289	519.69
502.33	519	493.8	7094	521.66
503.5	442	495.62	11803	523.47
509.98	681	500.47	1212	525.13
512.14	2227	502.6	1418	528.15
514.13	3252	509.87	1047	529.81
516.29	3993	512.13	2873	598.87
519.44	961	514.25	4345	600.61
521.76	967	516.37	4283	
523.25	490	519.69	1447	
525.07	375	521.66	1234	
528.06	1090	523.47	670	
529.88	1940	525.13	616	
598.58	807	528.15	1934	
600.66	1390	529.81	2377	
31		598.87	881	
		600.61	2509	

Rsa I

Data from 4/10		**Data from 4/27**		**Consistent peaks**
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment
54.12	375	56.98	651	56.98
56.8	806	166.04	850	166.04
126.25	447	424.61	572	430.43
166.04	967	430.43	2621	445.59
430.45	2161	445.59	3311	451.17
432.7	1003	448.3	420	451.77
445.5	2419	449.36	464	452.83

Table 2 (cont'd)

447.13	1938	451.17	923	455.4
450.72	782	451.77	613	456.77
451.87	748	452.83	1042	459.65
452.85	1002	455.4	1193	464.82
455.47	1431	456.77	498	467.25
456.95	502	459.65	3754	474.65
459.58	4295	463.75	348	476.57
464.37	765	464.82	468	493.8
467.35	1083	467.25	1010	495.77
470.53	793	471.28	419	501.09
474.43	2903	474.65	3158	
476.91	16521	476.57	16152	
493.98	1786	483.77	800	
496.14	7830	493.8	2428	
501.44	2565	495.77	7000	
22		499.42	1084	
		501.09	2358	

SEDIMENT 4A

Hha I

Data from 4/10		**Data from 4/27**		**Consistent peaks**
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment
37	604	37	773	37
40.08	454	205.98	7695	205.98
41.04	716	369.77	6867	369.77
56.39	339	538.98	715	567.18
69.79	524	549.15	626	568.99
205.58	3964	567.18	6140	
287.46	2430	568.99	36653	
369.89	2742	579.32	579	
567.19	4785	584.72	539	
569.36	22244	588.8	317	
		589.49	459	

Table 2 (cont'd)

Msp I

Data from 4/10		**Data from 4/27**		**Consistent peaks**
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment
40.09	729	32.92	705	207.61
41.05	951	207.61	771	209.56
207.97	499	209.56	856	491.82
209.99	684	461.9	312	493.5
491.84	455	491.82	983	495.47
493.84	7042	493.5	19572	598.62
496.01	21256	495.47	48311	600.52
598.53	796	541.04	334	
600.61	1291	542.15	668	
		545.33	879	
		547.09	484	
		549.97	519	
		551.26	493	
		552.7	520	
		576.84	512	
		584.93	686	
		589.69	417	
		591.39	445	
		593.45	569	
		594.31	239	
		595.51	562	
		598.62	2829	
		600.52	3639	

Rsa I

Data from 4/10		**Data from 4/27**		**Consistent peaks**
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment
33.38	1751	126.58	638	126.58
40.09	319	421.02	592	430.46
126.5	504	430.46	19894	474.53
430.56	4792	474.53	5718	476.63
474.43	2048	476.63	47428	

476.91 15222	488.71	543
	499.45	430
	500.97	492
	584.98	1036
	589.72	434

Table 3 Chapter 4 Archaeal T-RFLP data

SEDIMENT 1At

Hae III		**Hha I**		**Rsa I**	
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment	Peak area
69.23	1937	60.15	608	40.78	2230
70.7	702	62.19	7258	78.43	2272
175.56	675	90.03	1985	80.25	716
207.8	2298	175.56	459	241.71	2598
208.99	6359	195.23	5898	262.15	7115
214.4	19069	224.11	712	294.96	6954
240.69	19001	327.83	22176		
246.13	1864	338.14	7577		
272.52	1958	357.9	2502		
298.9	699	359.52	6201		
317.46	2716				

SEDIMENT 2At

Hae III		**Hha I**		**Rsa I**	
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment	Peak area
69.46	377	62.08	15787	40.59	3276
70.74	1093	88.77	563	242.55	7278
175.56	830	89.86	1224	262.35	1545
208.99	1918	175.56	1041	263.67	1049
214.55	18797	196.14	719	295.09	9036
240.27	30076	327.68	14024		
245.96	1923	338.74	9229		
272.36	2627	358.78	9970		
298.76	2265				
311.15	558				
317.17	1456				

Table 3 (cont'd)

SEDIMENT 2Bb

Hae III		**Hha I**		**Rsa I**	
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment	Peak area
38.12	627	38.13	1444	40.78	17001
191.34	8522	52.04	399	91.12	429
209.89	1267	60.23	557	242.4	3482
214.4	45307	62.26	4191	258.21	8992
240	6221	76.05	766	262.24	7100
242.15	2873	196.29	897	295.28	1326
246.37	670	327.31	48106	297.1	1151
311.1	827	333.13	9991		
316.96	1529	338.48	4972		
		358.64	2394		

SEDIMENT 3At

Hae III		**Hha I**		**Rsa I**	
Terminal fragment	Peak area	Terminal fragment	Peak area	Terminal fragment	Peak area
38.14	537	38.13	574	40.78	3509
69.41	1139	62.08	15397	242.55	773
70.7	778	88.77	828	262.35	2295
175.25	698	89.86	3920	295.11	15264
208.99	5855	175.56	721		
214.55	10713	195.95	471		
240.41	36801	224.29	2502		
245.97	1996	327.69	17300		
272.49	2211	333.35	588		
298.87	1302	339	1287		
317.71	1087	359.09	15655		
		362.72	665		

APPENDIX C

Merry S. Riley, Vaughn S. Cooper, Richard E. Lenski, Larry J. Forney, and Terence L. Marsh Rapid phenotypic change and diversification of a soil bacterium during 1000 generations of experimental evolution. *Microbiology* 2001 147: 995-1006.

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Rapid phenotypic change and diversification of a soil bacterium during 1000 generations of experimental evolution

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Evolutionary pathways open to even relatively simple organisms, such as bacteria, may lead to complex and unpredictable phenotypic changes, both adaptive and non-adaptive. The evolutionary pathways taken by 18 populations of *Ralstonia* strain TFD41 while they evolved in defined environments for 1000 generations were examined. Twelve populations evolved in liquid media, while six others evolved on agar surfaces. Phenotypic analyses of these derived populations identified some changes that were consistent across all populations and others that differed among them. The evolved populations all exhibited morphological changes in their cell envelopes, including reductions of the capsule in each population and reduced prostheca-like surface structures in most populations. Mean cell length increased in most populations (in one case by more than fourfold), although a few populations evolved shorter cells. Carbon utilization profiles were variable among the evolved populations, but two distinct patterns were correlated with genetic markers introduced at the outset of the experiment. Fatty acid methyl ester composition was less variable across populations, but distinct patterns were correlated with the two physical environments. All 18 populations evolved greatly increased sensitivity to bile salts, and all but one had increased adhesion to sand; both patterns consistent with changes in the outer envelope. This phenotypic diversity contrasts with the fairly uniform increases in competitive fitness observed in all populations. This diversity may represent a set of equally probable adaptive solutions to the selective environment; it may also arise from the chance fixation of non-adaptive mutations that hitchhiked with a more limited set of beneficial mutations.

Keywords: *Ralstonia*, phenotypic radiation, diversity, mutation

INTRODUCTION

Micro-organisms have proven useful for studying the mechanisms and consequences of evolution. By virtue of their small size, fast growth rates and comparatively simple genetic systems, questions that could not be addressed with macro-fauna can be answered in a robust and statistical manner with micro-organisms (Helling *et al.*, 1987; Dykhuizen, 1990, 1993; Lenski & Travisano, 1994; Lenski *et al.*, 1998; Rainey & Travisano, 1998). Moreover, because of the resilience of some micro-organisms to cryogenic preservation, a detailed historical record of an evolving population can be preserved and used comparatively in the dissection of evolutionary processes (Lenski & Travisano, 1994).

Our present work extends previous investigations of the experimental evolution of a soil bacterium (Korona *et al.*, 1994; Korona, 1996; Nakatsu *et al.*, 1998). The original intent of this work was to extend investigations performed on domesticated *Escherichia coli* to a recently isolated undomesticated soil bacterium. In addition, two different environmental conditions were used as selective regimes during the propagation of lines derived from this isolate for 1000 generations. Twelve replicate populations were maintained in a liquid shake

Abbreviations: FAME, fatty acid methyl ester; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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ask and six populations were cultivated on agar. The former provided a mass-action environment that is highly homogeneous throughout, while the latter provided a structured environment with complex gradients of nutrients as well as metabolites produced by the bacteria themselves. The soil bacterium used as the ancestral founder of all these populations is strain TFD41, which was identi ed as a *Ralstonia* species based on its 16S rRNA sequence (Nakatsu *et al.*, 1998). Previous work described a substantial increase in competitive tness of all 18 evolved populations, measured relative to the common ancestor in the experimental environments (Korona *et al.*, 1994). Variations in colony morphology were noted (Korona *et al.*, 1994), as were certain genome changes, both chromosomal and plasmid encoded (Nakatsu *et al.*, 1998).

In this paper, we extend these observations with a systematic examination of cell morphology, substrate utilization, bile salts sensitivity, adhesion properties and fatty acid methyl ester (FAME) analysis of the evolved populations and their ancestor. Our objectives were to identify common phenotypic motifs that may indicate parallel genetic adaptations, as well as differences among populations within and between the two selective regimes. The notion of an adaptive landscape (Wright, 1932, 1988) suggests that a population of organisms may have many potential evolutionary solutions to a selective challenge. It seems reasonable, too, that the diversity of potential solutions would be correlated with the complexity of the selective environment. Consistent with this postulate is the observation that variation in colony morphology was greater amongst the populations evolved on solid media than those evolved in liquid (Korona *et al.*, 1994; Korona, 1996).

METHODS

Strains and media. Strain TFD41 was originally isolated from soil based on its ability to grow on 2,4-dichlorophenoxyacetate (Tonso *et al.*, 1995). The isolation of streptomycin and nalidixic acid resistant mutants of TFD41 and the maintenance of 18 replicate populations for 1000 generations have been described previously (Korona *et al.*, 1994). The ancestor, antibiotic resistant mutants and isolates from generation 1000 of the evolved populations were maintained at −80 °C in glycerol stocks. Cultures derived from frozen stocks were maintained on plates for no longer than 2 weeks. Cells were routinely grown on either nutrient agar or R2A agar at 30 °C unless otherwise indicated.

Microscopy. Light microscopy was performed on a Zeiss Axioskop microscope. Cells were taken from freshly streaked nutrient agar plates that had been incubated at 30 °C for 48 72 h. The presence of outer capsule material was detected with an India ink stain (Doetsch, 1981) and representative micrographs were made at 1000× magni cation with an oil immersion lens (Zeiss). Cells were evaluated for the presence of capsule as well as cell shape and mean length. Images were captured Son Kodak TMAX lm.

Scanning electron microscopy (SEM) was performed on cells grown on nutrient agar plates as described for light microscopy. The cells were xed in 4% glutaraldehyde, mounted on polylysine-coated coverslips, dehydrated in an ethanol series (25%, 50%, 75% and 95%), critical-point dried and sputter coated with gold. At each step the cells were treated as gently as possible so as to preserve the integrity of the outer cellular architecture. The mounted cells were viewed on a JEOL scanning electron microscope at several different magni cations. Images were captured and stored electronically. At least ten elds, at approximately 2500× magni cation, were viewed for each lineage to obtain a representative sample of morphology. Cell length of well-isolated cells was measured from end to end, excluding surface appendages. The mean cell length was calculated from a minimum of 25 cells in a minimum of two elds. The mean cell length of an evolved population was deemed to depart signi cantly from that of the ancestor if there was no overlap between the respective means ± twice the standard error.

Transmission electron microscopy was performed on the ancestor and three evolved populations. The cells were grown as described above for light microscopy, scraped from the agar plate and pelleted by centrifugation. The pellets were resuspended in a 4% agar solution and allowed to solidify. The agar was diced into 1 mm^3 squares then xed in 2% glutaraldehyde buffered with 100 mM sodium cacodylate (pH 7·2) for 2 h. The xed cubes were washed three times in 100 mM sodium cacodylate buffer (pH 7·2), post- xed in 1% osmium tetroxide for 1·5 h and then washed four times in deionized water. The specimens were dehydrated in a graded acetone series and in ltrated in a graded Quetol resin series and embedded. Preparations were stained with uranyl acetate/lead citrate. All preparation steps were conducted at room temperature. Scanning and transmission electron microscopies were performed at the Michigan State University Center for Electron Optics.

BIOLOG assays. Carbon utilization pro les of the ancestor and evolved populations were determined using BIOLOG GN plates and the protocol provided by the vendor (modi ed to accommodate a *Ralstonia* sp.). Brie y, an aliquot of an overnight culture grown on 1/3× Trypticase soy broth was transferred to fresh media and cells were grown at 30 °C in a reciprocating shaker to mid-exponential phase. Cells were harvested by centrifugation and resuspended in 0·145 M NaCl to an of OD_{590} 1·0. One lineage, L16, had anomalously low cell density at an OD_{590} of 1·0 and therefore was adjusted to OD_{590} 2·0. Cells were then starved for 15 min at room temperature prior to the inoculation of the microtitre plates with 150 µl cell suspension per well. Each strain was tested in duplicate. The plates were incubated at 30 °C and the OD_{590} measured using a Bio-Kinetics microtitre plate reader (model EL 312E, Bio-Tek Instruments) every 2 h for a total of 8 h. Plates were vigorously shaken prior to each reading and the OD_{590} was corrected for the zero substrate control well. Some substrates had a variable response where one of the replicates was unambiguously positive (OD_{590} > 0·1 after zero substrate correction) and the other borderline. These substrates are indicated (see Fig. 3).

Analysis of BIOLOG data. BIOLOG optical density (OD) readings from the plate reader were imported into a database and fed into a Microsoft Visual Basic-Excel program that approximated the area under the growth curve for each of the 95 substrates for each genotype replicate. Following the general method of Guckert *et al.* (1996), the program calculates a trapezoidal area that approximates the result of tting and integrating each individual growth curve. This curve area approach collapses several values from a growth curve into a single value and integrates several properties (e.g. duration of lag, growth rate, yield). Individual values for each substrate,

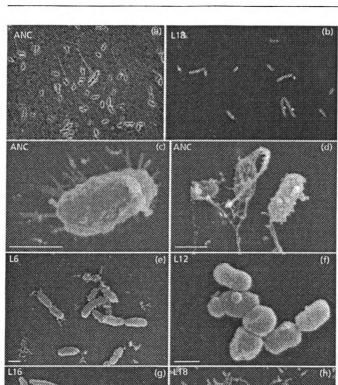

Fig. 1. Light and scanning electron microscopy of the ancestor, *Ralstonia* strain TFD41, and several evolved lines. (*a*) Light micrograph of ancestor. (*b*) Light micrograph of evolved L18. (*c*, *d*) Scanning electron micrographs of ancestor. (*e–h*) Scanning electron micrographs of evolved lines L6, L12, L16 and L18. Bars, 1 μm.

which we refer to as catabolic area , were subjected to a hierarchical cluster analysis (SYSTAT, v. 7.0, SPSS) to determine the relationships between the ancestors and the evolved lineages based on their patterns of carbon source utilization. The area data were expressed on a continuous numeric scale, and therefore normalized Euclidean distances were calculated for our clustering analysis. Ward s linkage method (Ward, 1963) was applied to adjust for covariance and to focus on mean values within clusters. Other linkage methods were also tested and none gave substantially different cluster patterns (data not shown). The source codes for the above procedures are available by request from the author (V.S.C. at cooperva@pilot.msu.edu).

Bile salts MIC. Overnight cultures grown on 1/3× TSB were diluted 1:100 into fresh broth containing a range of bile salts (Difco) concentrations (0·1 g l^{-1} to 20 g l^{-1}). The cultures were incubated at 30 °C and scored for growth at 24, 48 and 72 h. Any increase in turbidity above the initial OD was scored as a positive. All evolved popularions were tested in duplicate, and no differences were detected between these replicates.

Adhesion. The adhesion assay was based on the retention of bacterial cells in a saturated sand matrix supported in a column and has been described previously (DeFlaun *et al.*, 1990). Brie y, A + N minimal medium (Wyndham, 1986) supplemented with 30 mM L-aspartic acid (AN-asp) as the sole carbon source was inoculated with the ancestor or an evolved population from a fresh nutrient agar plate and grown overnight at 30 °C in a reciprocating shaker. Prior screening indicated that the ancestor and all 18 evolved lineages could utilize L-aspartic acid. The culture was diluted into fresh AN-asp and grown to late-exponential phase. The cells were harvested by centrifugation, resuspended in phosphate buffered saline (PBS) and adjusted by dilution with PBS to an OD_{540} of 0·5 0·6 (1 cm light path).

Bio-Rad Econocolumns (5 × 1·5 cm inner diameter) were packed with 11 g − 50 + 70 mesh sand (Sigma S-9887) and rinsed with approximately 15 ml PBS. The cell suspension (3·5 ml) was pipetted gently onto the column, an entire pore volume was allowed to pass into the matrix (3·5 ml void volume) and then ow was stopped. The loaded column was incubated undisturbed at room temperature for 1 h to allow cells to bind to the matrix. The column was then eluted with 14 ml PBS, collected in four fractions. The fraction of cells eluted was determined spectrophotometrically in a 1 cm path length quartz cuvette (Hewlett Packard 8452A Diode Array Spectrophotometer) and the fraction of cells bound to the column was calculated as fraction bund = 1 − total OD

Table 1. Morphological characteristics of the evolved populations

Minimal medium with 2,4-dichlorophenoxyacetate as the carbon source was used for selection (see Methods).

Strain*	Selection	Colony type†	Light microscopy‡	SEM		
				Cell surface	Mean cell length (nm)§	2(SE)‖
Ancestor	Soil	+	C+ P++	++Capsule	1·95	0·14
L1, Str	Liquid	−	C− P−	Smooth	1·8	0·12
L2, Nal	Liquid	−	C− P−	Smooth	2·95¶	0·59
L3, Str	Liquid	±	C− P−	Smooth	5·64¶	0·92
L4, Nal	Liquid	+	C− P+	±Capsule	1·39¶	0·09
L5, Str	Liquid	+	C− P+	Smooth	4·08¶	0·68
L6, Nal	Liquid	−	C− P−	Smooth	1·46¶	0·13
L7, Str	Liquid	−	C− P−	Smooth	3·44¶	0·52
L8, Nal	Liquid	−	C− P−	±Capsule	3·21¶	0·46
L9, Str	Liquid	−	C− P−	Smooth	5·19¶	0·81
L10, Nal	Liquid	−	C− P−	±Capsule	3·59¶	0·75
L11, Str	Liquid	−	C− P−	Smooth	3·23¶	0·47
L12, Nal	Liquid	−	C− P−	Smooth	1·88	0·16
L13, Str	Agar	+	C− P+	±Capsule	4·53¶	0·64
L14, Nal	Agar	−	C− P−	±Capsule, blebs	1·97	0·34
L15, Str	Agar	±	C− P−	±Capsule	1·51¶	0·08
L16, Nal	Agar	−	C− P−	Smooth	9·14¶	2·73
L17, Str	Agar	−	C− P−	Smooth	4·08¶	0·7
L18, Nal	Agar	±	C− P+	Smooth, blebs	1·93	2·07

* Str, streptomycin resistant; Nal, nalidixic acid resistant.

† +, Highly mucoid; ±, intermediate mucoid; −, non-mucoidal.

‡ C+, detectable capsule with India ink; C−, capsule not detectable with India ink; P++, prostheca-like appendages present in greater than 75% of cells; P+, prostheca-like appendages present in less than 25% of cells; P , prostheca-like appendages absent.

§ Mean of at least 25 cells from a minimum of two SEM elds; a total of ten elds of each population were viewed.

‖ Standard error (SE) = standard deviation/($\sqrt{n}$).

¶ Statistically different from the ancestor.

eluted/total OD loaded. All populations were tested in triplicate.

FAME analysis. This was conducted on a subset of the derived lineages (L1, L5, L6, L8, L9, L11, L12, L16, L17 and L18), the original ancestor as well as the Str^{R} and Nal^{R} derivatives of the ancestor. Strains were streaked from −80 °C stocks onto 1/3× Trypticase soy agar, incubated at 30 °C and harvested after 2 d by scraping the plates with a sterile spatula directly into dry-heat-sterilized glass culture tubes. The resulting cell pellet was stored at −80 °C until assayed. Each sample was extracted and run in triplicate as described previously by Sasser (1997). FAME analysis was performed on a Hewlett Packard 5890 series II gas chromatograph equipped with a FID detector. A Hewlett Packard Ultra2 column (cross-linked 5% phenyl methyl silicone) of 25 cm × 0·22 mm with 0·33 μm lm thickness was used with ultra-high purity hydrogen as the carrier gas at 50 ml min^{-1} using a 50:1 split ratio. The initial temperature, 170 °C, was ramped to 270 °C at a rate of 5 °C min^{-1}. The column was baked for 2 min at 300 °C after each run. The injector and detector temperatures were maintained at 250 °C and 300 °C, respectively. FAME data were analysed with the Sherlock MIDI system and the Dendrogram program (MIDI). The former identi es the closest microbial relative based on the fatty acid pro les, while the latter compares the lineages based on a clustering analysis using unweighted pair-group method with arithmetic averages (UPGMA).

RESULTS

Microscopy

One of the rst phenotypic changes noted in the evolved populations was colony morphology (Korona *et al.*, 1994). Because these differences were suggestive of changes in capsule production, the cell morphology of the ancestor and evolved populations was systematically assessed using both light and electron microscopy. Fig. 1(a) shows the cell morphology of the ancestor visualized with a capsule counter-stain (India ink). Cells of the ancestral strains were heavily encapsulated, as evidenced by the light-bright halo surrounding the cells. In addition, very long prostheca-like appendages were present on most cells. All of the evolved populations showed a substantial reduction in encapsulation as judged by light microscopy. Four lineages (L4, L5, L13 and L18) showed some indication of prostheca-like appendages but the length of the appendages and the

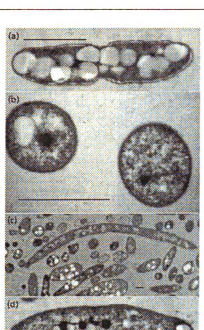

Fig. 2. Transmission electron micrographs of the ancestor and two evolved lines, L16 and L18. (a, b) Ancestor. (c) Evolved line L16. (d) Evolved line L18. Bars, 1 μm.

number of cells with appendages were considerably less than for the ancestor. Several lineages had cells that were consistently longer than the ancestor. Cells from population L18 are shown in Fig. 1(b). L18 is an example not only of the lack of encapsulation but also of the occasional presence of vestigial prosthecae. Light-bright blebs were occasionally seen associated with the cell surface or with a prostheca-like appendage, as can be seen in this photomicrograph. The compiled data are presented in Table 1.

Scanning electron micrographs of the ancestor and four evolved populations are also shown in Fig. 1. The ancestor (Fig. 1c and d) presented a complex contoured surface with clear indications of heavy encapsulation as well as prostheca-like appendages. The appendages were not as long as those observed using light microscopy, perhaps due to the more rigorous preparative steps required by SEM. SEM micrographs of evolved populations L6, L12, L16 and L18 are shown in Fig. 1(e h). These lineages do not display evidence of encapsulation. Cells from the evolved populations L12 and L18 do not vary signi cantly in length from the ancestor (Table 1). L16 was remarkable in that its mean cell length was 4·7 times the mean length of the ancestor and occasional cells of 20 25 μm length were observed. Frequently, one terminus of the rod-shaped cell had greater light and electron opacity than the other. L18 showed evidence of vestigial prostheca-like appendages, some evidence of capsule material, and the unusual light-bright blebs mentioned above. L6 is one of three lineages that was signi cantly shorter than the ancestor. Eight of the 12 lineages evolved in liquid and three of six evolved on agar had signi cant increases in mean cell length, ranging from 1·5 to 4·7 times that of the ancestor. No signi cant differences between the original ancestor and the streptomycin or nalidixic acid resistant mutants were detected with microscopy. Table 1 gives the mean cell lengths for all of the lineages based on SEM.

Transmission electron microscopy (TEM) was performed to con rm the differences in morphology between the ancestor and two lineages, and these images are shown in Fig. 2. As viewed with TEM, L16 and L18 (Fig. 2c and d, respectively) had internal cellular details similar to those seen in the ancestor (Fig. 2a and b), including structures consistent with poly-β-hydroxybutyrate storage granules. The TEM images also con rm the integrity of the cytoplasm throughout the length of the unusually long cells of L16.

BIOLOG

To monitor changes in the catabolic breadth of the replicate lines, BIOLOG GN plates testing the ability to metabolize 95 different carbon sources were used. The salient features of the BIOLOG data (Fig. 3) are as follows. First, the ancestor was capable of utilizing 43 49 of the 95 substrates in the BIOLOG GN plates in a pattern consistent with the type strain description of *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*; Krieg & Holt, 1984). Second, no two derived genotypes had the same pattern of carbon utilization. Third, the greatest range of utilization patterns appeared amongst the populations evolved on agar. Fourth, while there was tremendous phenotypic variation among the evolved populations, there was one discernible pattern detected among the lineages evolved in liquid as described below.

The results of hierarchical cluster analysis on the BIOLOG data using all 18 lineages evolved either in liquid or on solid media are presented in Fig. 4. Note that the antibiotic resistance marker determines the primary grouping for the lineages evolved on liquid. Lineages founded with the streptomycin-resistant ancestor (L1, L3, L5, L7, L9 and L11) group with the original ancestor and the two antibiotic resistant variants whereas those founded with the nalidixic acid resistant strain (L2, L4, L6, L8, L10 and L12) group separately. No such ancestor-dependent clustering is seen among the lineages evolved on agar. This marker effect was examined statistically by two *t*-tests: one which divided all evolved populations based on their antibiotic marker state, and another which only divided

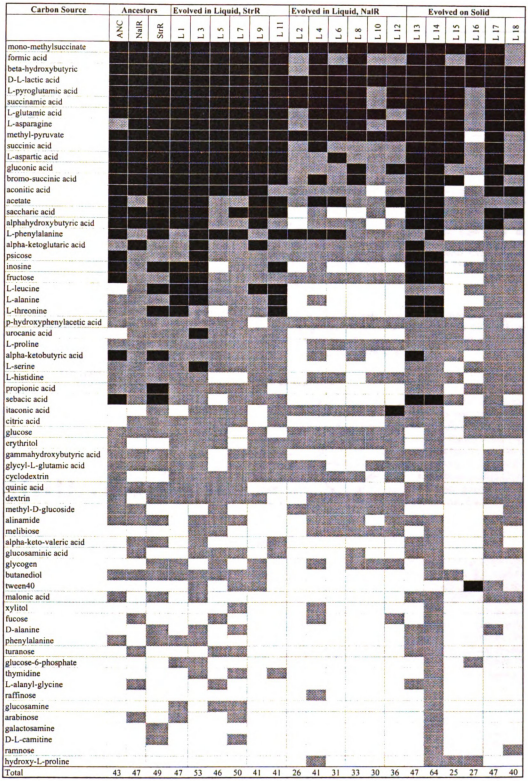

Fig. 3. For legend see facing page.

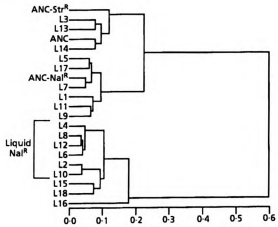

Fig. 4. Clustering based on catabolic profiles of the 18 evolved lines, their two proximate ancestors that differ in antibiotic resistance markers (ANC-Str^R and ANC-Nal^R), and the original *Ralstonia* strain TFD41 (ANC). The tree was constructed by hierarchical cluster analysis based on the method of Ward (1963), adjusted for covariance, using SYSTAT v. 7.01. See Table 1 for strain identification. ANC indicates the original *Ralstonia* strain TFD41, while ANC-Str^R and ANC-Nal^R denote antibiotic resistance marker variants thereof.

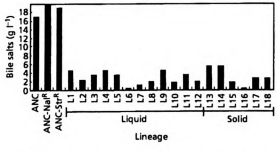

Fig. 5. MIC of bile salts. Each strain was tested in duplicate. The MIC was defined as the concentration at which no detectable increase in turbidity was observed after 5 d at 30 °C. ANC indicates the original *Ralstonia* strain TFD41, while ANC-Str^R and ANC-Nal^R denote antibiotic resistance marker variants thereof.

liquid-evolved populations. We found that Nal^R populations had diminished breadth in carbon source utilization compared to the Str^R populations, but the difference was only clearly signi cant among the liquid-evolved populations (all evolved lines, $t = 1{\cdot}91$, d.f. $= 16$, $P = 0{\cdot}074$; liquid evolved only, $t = 3{\cdot}59$, d.f. $= 10$, $P = 0{\cdot}005$; both two-tailed tests).

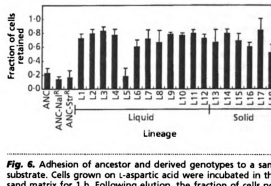

Fig. 6. Adhesion of ancestor and derived genotypes to a sand substrate. Cells grown on L-aspartic acid were incubated in the sand matrix for 1 h. Following elution, the fraction of cells not attached to the sand was determined spectrophotometrically and the fraction retained was calculated by subtraction. All assays were performed in triplicate. Error bars indicate standard deviation. ANC indicates the original *Ralstonia* strain TFD41, while ANC-Str^R and ANC-Nal^R denote antibiotic resistance marker variants thereof.

MIC of bile salts

In Gram-negative bacteria, changes to the outer envelope can alter sensitivity to antibiotics and detergents. To determine if any of the evolved populations changed in this respect, the MIC of bile salts was determined (Fig. 5). The ancestor and its antibiotic-resistant mutants were able to grow in media with bile salts concentrations above 16 g l^{-1}. In contrast, all 18 evolved populations were substantially more sensitive, with growth inhibition observed at concentrations below 6 g l^{-1} in all cases.

Adhesion

The ancestors and evolved populations displayed very different adhesion properties (Fig. 6). All of the evolved strains, with one exception (L5), had high affinity for the sand matrix, which retained more than 50% of the cells applied to the column. The ancestral strains, on the other hand, did not adhere strongly to the sand, with about 80% of the cells loaded onto the column passing through the sand matrix. L5, the only exception among the evolved populations, behaved like the ancestor in that 80% of cells applied to the column were recovered in the effluent.

Fig. 3. Carbon utilization patterns of the ancestor, Str^R and Nal^R mutants thereof, and the 18 evolved lines after 1000 generations. Black cells indicate the carbon source was consistently metabolized; grey cells indicate variability in the catabolism of the particular carbon source across replicate BIOLOG plates. Carbon sources were sorted first by overall mean (mean performance of all lineages on a particular substrate), in descending order, and then by coefficient of variation (the standard deviation divided by the overall mean), in descending order. The total number of carbon sources used by each strain is shown on the bottom row, including both consistently positive and variable carbon sources. The lines that evolved in liquid media are grouped by their ancestral antibiotic resistance markers.

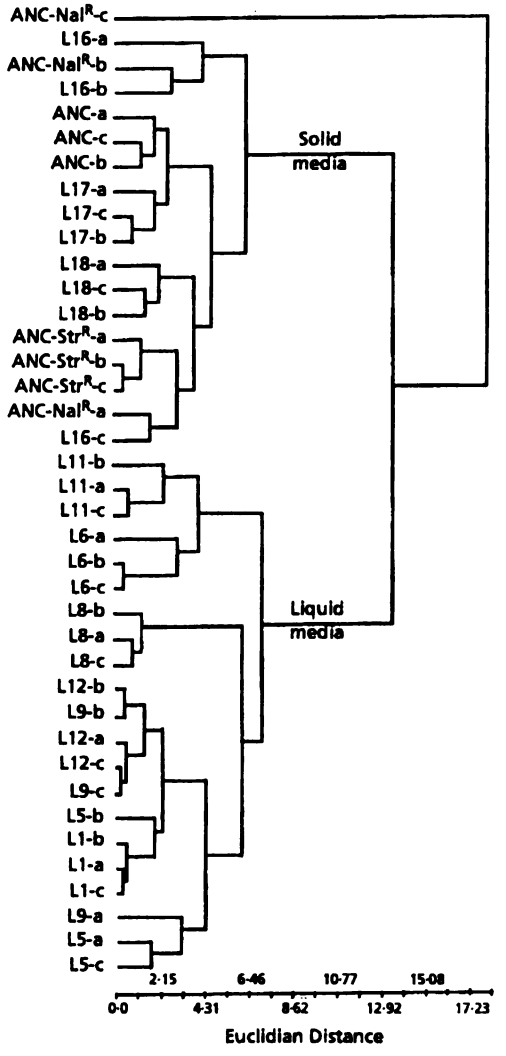

Fig. 7. Cluster analysis of FAME data from the ancestral strains and ten evolved lineages. L1, L5, L6, L8, L9, L11 and L12 evolved in liquid medium, whereas L16, L17 and L18 evolved on agar. Each strain was tested in triplicate (denoted a, b and c). ANC indicates the original *Ralstonia* strain TFD41, while ANC-Str^R and ANC-Nal^R denote antibiotic resistance marker variants thereof.

FAME analysis

FAME analysis of the ancestors and a subset of ten evolved lineages consistently revealed nine peaks corresponding to fatty acids 14:0, 14:0 3-OH/16:1 iso I, 16:1 $\omega 7c/15$ iso 2-OH, 16:0, 17:0 cyclo, 16:0 2-OH, 18:1 $\omega 9c/\omega 12t/\omega 7c$, 18:0 and 18:1 2-OH. Note that in this nomenclature a slash indicates that two or more fatty acids co-elute under these conditions and they are indistinguishable from one another. MIDI analysis indicated that all lineages resemble *Pseudomonas pickettii* or *Burkholderia cepacia* most closely among those organisms in the database. A cluster analysis of these data placed the ancestral strains and the lineages evolved on solid medium together in one group that was distinct from the lineages evolved in liquid (Fig. 7). The reproducibility of this major division is quite high, as indicated by the frequent clustering of the three replicates for each population. Only the Nal^R parental type showed variability in this regard. The distinguishing features in the FAME pro les that account for the clustering were the relative areas of three peaks that correspond to between three and six fatty acids. The 18:1$\omega 7c/\omega 9t/\omega 2t$ peak was dominant in the liquid-medium derived lineages, whereas 16:1$\omega 7c/15$ iso 2-OH and 16:0 were the dominant peaks in the ancestor and in those lineages evolved on agar.

DISCUSSION

After 1000 generations of evolution of *Ralstonia* strain TFD41 in two different selective environments, we observed several different patterns of phenotypic evolution, depending on the particular trait: (1) changes common to most or all derived lines, indicating parallelism; (2) changes that were speci c to the particular selective environment; (3) changes that depended upon which particular antibiotic-resistant variant of the ancestor was used as the founder, indicating historical contingency; and (4) changes that appeared more stochastic and led to phenotypic diversity among the evolved populations.

Morphological changes in the outer cell envelope occurred in all 18 evolved lineages, including reductions in the capsule (Table 1), increased sensitivity to bile salts (Fig. 5), and with one exception, increased cell adhesion to a sand matrix (Fig. 6). A genomic change also occurred in all of the evolved lineages, including a deletion of a genomic region detected by the loss of a REP-PCR fragment (Nakatsu *et al.*, 1998); it will be interesting to determine whether these parallel genetic and phenotypic changes are related to one another. Changes that were speci c to the particular selective environment were evident in the FAME pro les (Fig. 7), as well as the observation that competitive tness was more variable among the agar-evolved lineages than among the liquid-evolved populations (Korona *et al.*, 1994). Evidence for historical contingency was found in the carbon utilization patterns, which grouped the liquid-evolved populations as a function of the antibiotic-resistance marker borne by their immediate ancestors (Fig. 4). Finally, substantial phenotypic diversity of cell morphology and carbon utilization patterns was observed across all of the evolved populations, irrespective of differences in their selective environments or parental genotype. For example, L16 and L18 were both evolved on agar, and both were founded by the Nal^R parental strain. L18 remained similar in mean cell length (Table 1) and carbon utilization pro le (Fig. 3) to the ancestor, whereas L16 has increased almost ve-fold in mean length and has a substantially narrowed catabolic pro le.

In broad outline, but not in detail, this mixture of parallel and divergent patterns is similar to that seen in a long-term evolution experiment with 12 replicate populations of *E. coli*, each propagated in a de ned glucose medium for thousands of generations (Lenski, 1995; Lenski *et al.*, 1998). In that study, all the lines improved in tness to a similar degree when in competition against their ancestor in the glucose medium (Lenski & Travisano, 1994). However when the competitiveness of derived lines was tested against the ancestor on different substrates, their performance was much more variable, which implies a diversity of underlying physiological changes (Travisano & Lenski, 1996). All 12 lines also increased their mean cell volume, but the degree to which volume increased and the resulting cell shape were variable (Lenski & Mongold, 2000). Substantial differences among the lines in genomic mutation rates also arose, as several lines evolved defects in methyl-directed mismatch repair (Sniegowski *et al.*, 1997) while other lines exhibited bursts of activity of certain IS elements (Papadopoulos *et al.*, 1999). However, unlike the present study with *Ralstonia*, in which resistance mutations used as genetic markers in the ancestor in uenced the path of subsequent evolution, none of the differences among *E. coli* lines was strongly affected by the arabinose-utilization marker that was used to discriminate between those lines. Also, the main long-term *E. coli* experiment used only a single selective environment; however, when new lines derived from these original populations evolved in different thermal regimes, there emerged systematic differences among them (Lenski, 1995; Mongold *et al.*, 1996).

In considering the many changes to *Ralstonia* that have accrued over 1000 generations, it is worthwhile to consider in some detail the observed alterations to the outer envelope. The outer envelope is the rst part of the cell to interact with the external environment, serving both as a barrier to unwanted factors (viruses, antibiotics, etc.) and as a conduit for essential resources. Components of the outer envelope may therefore be the most sensitive and responsive cellular constituents to the selective environment. As Parke *et al.* (2000) put it: The cell surface is where the rubber hits the road in bacterial evolution. The components of the outer envelope in uence shape, chemical resistance, adhesion and metabolic properties of cells, and indeed all these properties were substantially altered during 1000 generations of evolution in *Ralstonia* strain TFD41. However, the underlying genetic and physiological bases of these changes are not yet sufficiently understood to interpret unambiguously the selective advantages conferred by these traits, either in the laboratory experiment or in nature. In the following paragraphs, we discuss some possible adaptive scenarios.

Morphological changes

Both light and electron microscopy clearly show that all the evolved populations have changed from the ancestral morphology in terms of the cell-surface architecture and, in many cases, cell length (Table 1). Most populations have lost all prostheca-like appendages and all experienced either partial or complete loss of their capsule. Variation in capsule production in bacteria is not uncommon (Roberts, 1996; see also Fletcher, 1996; Whit eld & Valvano, 1993) and has been cited as an induced response that may confer an advantage in appropriate environments. In *E. coli*, for example, the expression of capsule material is increased when cells are subjected to an arid environment, thereby providing protection against desiccation (Ophir & Glutnick, 1994). The cell surface also determines adhesive properties of the cell. A capsule can promote or prevent adhesion depending upon the relative chemistries of the capsule and substratum. Various extracellular polymers, including polysaccharides, that promote adhesion have been identi ed (Roberts, 1996). One study identi ed two types of extracellular polymers in the same organism: one that promotes attachment and the other that promotes detachment (Wrangstadh *et al.*, 1990). Non-adhesive variants of *Pseudomonas uorescens* that possess a capsule have also been described (Williams & Fletcher, 1996). Simoni *et al.* (1998) speculate that heterogeneity within bacterial populations expressed at the level of the outer envelope can in uence their transport in groundwater aquifers. Among the evolved populations of *Ralstonia* in this study all but one showed a substantial increase in adhesion to a sand matrix in comparison to the ancestor. Inasmuch as all of the evolved populations show reduced encapsulation, the capsule of the ancestral soil-dwelling *Ralstonia* strain must have served some function other than providing a matrix for adhesion to silicates. Another possible bene t for capsule-bearing strains is resistance to anti-bacterial compounds (Nikaido, 1996). Consistent with this possibility is the observation that, in a selective environment lacking such compounds, all 18 populations evolved increased sensitivity to bile salts (Fig. 5). Hence, in *Ralstonia*, the primary bene t of the capsule in the soil environment may be protection against chemicals and desiccation rather than adhesion to soil particles.

Perhaps the most striking phenotypic changes were in the mean cell length: 14 of 18 evolved lines showed signi cant changes in cell length. Of the 12 liquid-derived populations, eight are signi cantly longer than the ancestor and two are shorter. The six lines evolved on agar also include both shorter (one of six) and longer (three of six) morphologies, including lineage L16 that increased its mean length almost vefold. The unusually long cells of this population were not caused by incomplete septation, as TEM clearly showed a normal cytoplasm (Fig. 2). Changes in cell length have been correlated with survival strategies including, for example, the avoidance of predation in the case of long laments (Hahn *et al.*, 1999; Jürgens *et al.*, 1999), but it is unclear what factors promoted such conspicuous changes in this study. The fact that some lineages evolved greater cell length and others became smaller, while all showed substantial improvements in competitive tness (Korona *et al.*, 1994), suggests that cell

length was not itself a direct target of selection in these experiments.

Biochemical changes

Mechanisms for recognition and transport of nutrients also have essential components in the cell envelope. Mutations that alter either speci c transport complexes or their supporting structural matrix could change carbon utilization patterns. For example, in *E. coli* at least 90 genes are required for biosynthesis of the outer membrane and capsule which in turn provides structural scaffolding for the products of at least 37 genes encoding integral outer-membrane proteins (see for example the *E. coli* genome database at http://mbgd.genome.ad.jp). If the absence of capsule is a selected phenotype, as suggested by the loss of capsule in all lineages, then any mutation in the biosynthetic pathways of outer envelope synthesis might contribute the loss of capsule and the resulting increase in tness. Given the structural and biochemical complexity of the outer envelope, a number of possible genotypic pathways leading to loss of the capsule exist. The complex and variable carbon utilization pro les of the evolved populations are consistent with this scenario. Several populations have much narrower pro les than their ancestors, including all the lines founded from the Nal^R ancestor and evolved in liquid as well as two lines evolved on agar surfaces (L15 and L16), whereas another surface-evolved line (L14) has a much broader pro le than its ancestor (Fig. 3).

The capsule has been viewed as a molecular sieve that can in uence the accessibility of a substrate to the cell (Nikaido, 1996). Given the diverse and complex changes in morphology and surface architecture (Fig. 1 and Table 1), it is easy to imagine that carbon utilization patterns would be somehow affected, but difficult to discern any simple association between these classes of data. We did detect the acquisition of catabolic activity (Fig. 3). In particular the ability to metabolize Tween 40 was detected in several lineages. It seems likely that the loss of the copious capsule produced by the ancestor permitted increased access of some carbon sources to existing metabolic capabilities of the cell (Nikaido, 1996). Indeed, the loss of capsule may facilitate the assimilation of the substrate 2,4-dichlorophenoxy-acetate on which the replicate lines were evolved.

There is, however, one striking pattern in the carbon utilization pro les. In particular, a signi cant clustering was detected amongst the 12 liquid-evolved populations (Fig. 4), in which the two dominant clusters derive from the two different antibiotic-resistant ancestors that were each used to found half of the evolving lines (Korona *et al.*, 1994). In particular, all of the liquid-evolved populations that were founded with the Nal^R ancestor had much narrower catabolic pro les than did those founded by the Str^R ancestor (Fig. 3). It is important to emphasize that this narrower pro le was not directly attributable to the mutation that conferred Nal^R, because the Nal^R ancestor had a catabolic breadth comparable to the common ancestor. Moreover, this association with the founding ancestor was not seen with the agar-evolved lines. Interestingly, all six of the lines that were initially Nal^R and evolved in liquid reverted to sensitivity to nalidixic acid during the evolution experiment, whereas all three surface-evolved lines retained the Nal^R phenotype (Korona *et al.*, 1994). These data collectively indicate that the initial resistance marker in uenced subsequent evolution in the liquid environment, although the reasons for this remain speculative. In *E. coli*, Nal^R phenotypes can be produced by mutations in the *gyrB* locus that encodes DNA gyrase (Yamagishi *et al.*, 1986) as well as by mutations in other loci that cause reduced permeability of the outer membrane (Hrebenda *et al.*, 1985). If the nalidixic acid resistance of the ancestral strain was expressed at the level of the cell envelope, then a subsequent evolutionary change in this structure (of the sort observed in the evolved lines) could negate the phenotypic expression of the resistance mutation.

FAME analysis indicated that the liquid-evolved lineages changed from the ancestral composition, whereas the surface-evolved lines have not (Fig. 7). In particular, all six of the liquid-evolved lines that were tested had changes in the relative amounts of three elution peaks, corresponding to three to six co-eluting fatty acids, compared to the ancestors and the three tested lineages that had evolved on solid media. While these patterns are consistent, it is not obvious how these alterations relate functionally to the two different selective regimes, except perhaps to note that the greater deviation of the liquid-evolved populations from the ancestral state suggests that this regime was more different from the natural environment than was the surface regime. It is also interesting to point out that a Euclidean distance of 10 in the FAME analysis has been proposed as an indication of species-level differences (MIDI). By this standard, the liquid-evolved lineages have evolved into a new species in only 1000 generations. This interpretation is unreasonable, and so one might be tempted to suggest that the experimental populations became contaminated by some other species, but this possibility is rejected by molecular genetic analyses that show all the evolved lines, from both liquid and surface regimes, to be derived from ancestral TFD41 (Nakatsu *et al.*, 1998). In our view, the idea that such differences in FAME composition re ect species-level differences is evidently awed, at least as a general proposition.

Concluding remarks

Considering all of the phenotypic traits together, we nd remarkable both the extent of change from the ancestral condition and the diversity among the derived lines. It is tempting to suggest that all this diversity represents different adaptive solutions achieved by each line in response to the selective environment. An alternative explanation is that a substantial fraction of the phenotypic diversity was the result of non-adaptive genotypic

changes that spread by hitchhiking (linkage) with bene cial mutations. To the extent that the selective environment was stressful and thereby mutagenic to the bacteria, this explanation becomes more likely, as the opportunity for neutral and even deleterious mutations to hitchhike becomes greater the higher the genomic mutation rate. It has been shown that some *Ralstonia* strains undergo substantial stress-induced changes in their genomes, including deletions and rearrangements (Taghavi *et al.*, 1997), and similar kinds of events were seen in this evolution experiment with strain TFD41 (Nakatsu *et al.*, 1998). Indeed, recent evidence indicates that *Ralstonia* strain TFD41 undergoes deletions similar to those found in the evolved lines when subjected to certain stresses, and these deletions occur at high frequency (T. L. Marsh, unpublished data), so that they could spread by hitchhiking. We do not know whether batch culture (including lag and stationary phases, as well as growth) on 2,4-dichlorophenoxyacetate represented a comparable stress, but it is certainly possible that stress-induced genotypic changes contributed to the phenotypic diversity observed (Finkel & Kolter, 1999). We are currently assessing the extent to which stress-induced changes in the TFD41 genome may have caused changes in cell morphology and other traits during the evolution experiment as well as determining the molecular bases of the increase in relative tness exhibited by the derived lineages at 1000 generations.

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