

AN ABSTRACT OF THE DISSERTATION OF

Stephanie A. Connon for the degree of Doctor of Philosophy in Microbiology
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Abstract approved ~~_____~~
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~~_____~~ Stephen J. Giovannoni

Research on natural environments, over the last decade, is replete with microbial diversity studies that used culture-independent approaches. The cloning and sequencing of the 16S rRNA genes has been the driving force in the expansion of awareness about the great diversity of previously undiscovered microorganisms. Well-known uncultured groups of microorganisms are numerous, and half of the known phylogenetic divisions of bacteria are not represented in any culture collection. It is no longer assumed that cultures acquired from an environment represent the dominant or physiologically important organisms from that environment. A high throughput culturing (HTC) technique was developed in an attempt to bring into culture some of these widespread and uncultured microorganisms. Over the course of 3 years, 2,484 culturing attempts were screened for microbial growth from sample collections off the coast of Oregon and 576 attempts from groundwater at McClellan Air Force Base (MAFB). However, using the HTC approach up to 14% of the microorganisms counted by direct microscopy were cultured. In contrast, less than 1% of the microorganisms from natural environments that are observed under a microscope can be grown using

standard agar plating techniques. This newly developed technique was successful at bringing into culture 11 previously uncultured or undescribed Proteobacteria. Four were isolated from the marine environment including, members of the SAR11 clade (alpha subclass), OM43 (beta-subclass), SAR92 (gamma subclass), and OM60/OM241 (gamma subclass). SAR11 was transiently cultured in this study but was later successfully brought into culture using these HTC techniques by Mike Rappé. Eight were isolated from a trichloroethene (TCE) and cis-dichloroethene (*cis*-DCE) contaminated aquifer, including members of the MHP14 clade (alpha subclass), 4-*Org*1-14 clade (alpha subclass), *Herbaspirillum/Oxalobacter* clade (beta subclass), HTCC333 (beta subclass), HTCC410 (beta subclass), PM1 clade (beta subclass), Boom-7m-04 clade (beta subclass) and OM43 clade (beta subclass). Culturing microorganisms is an important step towards understanding their physiology and ecology, and in most cases is necessary for the formal systematic description of a new species. For microorganisms of global significance, such as the major uncultured bacterioplankton and soil microbiota, obtaining cultures is a prerequisite for obtaining complete genome sequences and understanding the relevance of these microorganisms to biogeochemical cycles.

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CULTURING UNCULTURED ENVIRONMENTAL MICROORGANISMS

by
Stephanie A. Connon

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APPROVED:

Redacted for privacy

~~Major Professor~~, representing Microbiology

Redacted for privacy

Chair of the Department of Microbiology

Redacted for privacy

Dean of the Graduate School

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Stephanie A. Connon, Author

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CONTRIBUTION OF AUTHORS

Lewis Semprini¹ was principal investigator for the cometabolic-sparging project at McClellan Air Force Base, Sacramento, California, detailed in Chapter 3. He provided invaluable mentoring, guidance and financial support for Chapters 3 and 4. Adisorn Tovanabootr¹ collected all non-biological data related to the cometabolic-sparging project in Chapter 3. He also collected and mailed many of the samples for DNA extraction during his stay at the study site. Mark Dolan¹ took over data processing for Adisorn Tovanabootr after he became ill. He also provided mentoring and guidance for Chapter 3. Kevin Vergin² made the clone library discussed in Chapter 3 and 4 and screened this library for the presence of the 385 and 366 base pair LH-PCR fragment, Chapter 3.

¹ Department of Civil, Construction, and Environmental Engineering, Oregon State University, Corvallis, OR 97331

² Department of Microbiology, Oregon State University, Corvallis, OR 97331

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CULTURING UNCULTURED ENVIRONMENTAL MICROORGANISMS

CHAPTER 1:

INTRODUCTION

Stephanie A. Connon

INTRODUCTION

Over the past two decades microbiologists have become increasingly aware of the great diversity of uncultured microorganisms. Carl Woese set the stage for these discoveries by pioneering a powerful molecular technique for inferring evolutionary relationships among microorganisms that led to the discovery of a whole new realm of uncultured microorganisms (Carl R. Woese and Fox 1977). His approach was to purify and sequence the 16S rRNA from pure cultures of bacteria and archaea (C. R. Woese, et al. 1980). These sequences were then aligned and the differences were measured to determine evolutionary divergence between the genes. The most difficult aspect of this method at the time was acquiring the sequences for phylogenetic analysis.

Researchers at Indiana University began to develop methods to acquire these sequences more efficiently (Lane, et al. 1985). They amplified 16S rRNA, purified from cultures, by reverse transcriptase using oligonucleotides that target universally conserved regions of the gene. The development of a culture independent approach to collecting the 16S rRNA gene sequences soon followed. The first culture-independent approach developed was the “shotgun” clone library technique, where total DNA collected from an environment was purified, directly cloned into bacteriophage lambda and later screened for 16S rRNA genes (Olsen, et al. 1986; Pace, et al. 1986). This technique was very laborious and the number of 16S rRNA genes acquired were only 0.2% of the library (Weller and Ward 1989).

Two techniques were soon developed to increase the efficiency of acquiring these culture-independently collected 16S rRNA genes. The first, was a rather time consuming technique of reverse transcribing extracted RNA using 16S specific oligonucleotides and ligating the resulting cDNA into a plasmid that is cloned into *E. coli* (David M. Ward, et al. 1990; Weller and Ward 1989). The second technique relied on the polymerase chain reaction (PCR), which had been recently invented (Saiki, et al. 1985), and the discovery of thermal stable *Taq* polymerase (Saiki, et al. 1988). It involved PCR amplification of the 16S rDNA followed by cloning into bacteriophage lambda (Giovannoni, et al. 1990).

Britschgi and Giovannoni developed the clone library approach that is still used most commonly today by combining aspects of these two approaches. The 16S rDNA is PCR amplified and the resulting fragments are ligated into a plasmid and cloned into *E. coli* (Britschgi and Giovannoni 1991). An explosive increase in the number of sequences in the Ribosomal Database Project (RDP) and GenBank databases that have no known cultured representatives soon followed (Barns, et al. 1994; Choi, et al. 1994; E. F. DeLong 1992; Fuhrman, et al. 1993; Liesack and Stackebrandt 1992; Schmidt, et al. 1991; D. M. Ward, et al. 1994). Every new environment from which the 16S rRNA genes were cloned revealed novel sequences.

As sequences of 16S rRNA genes filled the databases, other molecular approaches to study microbial diversity and ecology were soon developed as a result of this new source of information including denaturing gradient gel

electrophoresis (DGGE) (Muyzer, et al. 1993), terminal restriction length polymorphism (T-RFLP) (Liu, et al. 1997), and length heterogeneity-PCR (LH-PCR) (Suzuki and Giovannoni 1996; Suzuki, et al. 1998). These three PCR based techniques had an advantage over 16S rRNA clone libraries in that a larger number of samples could be screened quickly to assess differences in microbial diversity.

DGGE was adapted for application in microbial community analysis from protocols for detecting point mutations in the human genome. This technique is used to separate 16S rRNA PCR amplification products that are usually between 150-900bp in length by taking advantage of the melting kinetics of double-stranded DNA, which is determined by the base pair composition of the fragment. Base pair differences result in different melting temperatures, which are detected on gels containing a linear gradient of denaturants (urea and formamide). As the DNA migrates through the gel, the different fragments are separated and visualized as discrete bands. These bands can be cut out and sequenced to identify the microorganisms represented by the fragment.

T-RFLP and LH-PCR techniques both start with a 16S rRNA PCR amplification product that has been amplified using one fluor-labeled and one unlabeled primer. T-RFLP can also be prepared using a fluor label on each primer; usually each primer is labeled with a different fluor molecule. For T-RFLP, the product is then cut by a restriction enzyme, and for LH-PCR no restriction digest is performed. The products are then run on an ABI gel or capillary sequencer to separate and visualize the fragments. T-RFLP relies on the primer site and the

enzyme cut site to define the fragment size. LH-PCR relies solely on the two primers to define the fragment size. The primers are designed to amplify a fragment that includes a sequence variable region of the otherwise highly conserved 16S rRNA gene. The T-RFLP and LH-PCR fragment sizes of different species of microorganisms can be predicted based on the 16S rRNA sequence information in the RDP and GenBank databases.

In situ hybridization of whole cells with oligonucleotide probes was another powerful approach developed to use this new repository of 16S rRNA sequence data. It allows a direct look at a species or group of microbes in a particular environment with the advantage of being able to directly count the cells and view their morphology. This technique was pioneered by Giovannoni et al. using ³⁵S-labeled probes that target the 16S rRNA molecule, which is abundant in the cytoplasm of prokaryotic cells (Giovannoni, et al. 1988). The probes were applied to whole, fixed cultures of cells, which were then viewed by microautoradiography. They were able to distinguish between the three major kingdoms of life Bacteria, Eucarya and Archaea using kingdom specific probes. Since microautoradiography was a time consuming process, a variation of this technique, fluorescence *in situ* hybridization (FISH), which uses an oligonucleotide probe attached to a fluorochrome was soon developed by Edward F. DeLong, et al. (1989) and Amann, et al. (1990). They used FISH with species and phylogenetic-group specific probes that were hybridized to whole cells and then viewed microscopically within hours rather than days of applying the probe. While FISH was very successful when

relatively large cultured cells with high ribosome content were hybridized, most environmental cells are small and have low ribosome content, and the cells are not bright enough to reliably visualize (Amann 1995). However, recent research in the Giovannoni laboratory has overcome this low signal problem by using multiple probes, a high quality microscope, and a cooled CCD camera (Morris, et al. 2002). FISH is most successfully applied to the detection of environmental microorganisms that have already been detected by a clone library, DGGE, T-RFLP or LH-PCR analysis.

All these culture-independent molecular techniques have led to an understanding of the widespread importance of uncultured microorganisms, and they have been applied to almost every environment on earth. New microorganisms were discovered in the open ocean by phylogenetically analyzing 16S rRNA sequences cloned from these environments. These novel groups included SAR11, SAR86, SAR92, SAR116, SAR202, SAR324 and SAR406, which were named after one clone from each of these novel phylogenetic clusters (Britschgi and Giovannoni 1991; Giovannoni, et al. 1990; Mullins, et al. 1995; Wright, et al. 1997). The ecology of uncultured marine bacteria was studied by determining the placement of their 16S rRNA gene sequences in the water column using a clone library approach (Field, et al. 1997; Giovannoni, et al. 1996; Gordon and Giovannoni 1996; Wright, et al. 1997). This series of studies published by Giovannoni and colleagues resulted in a better understanding of the distribution of some of the major uncultured groups of bacterioplankton. Two different

phylogenetic clusters of SAR11 also had different depth distributions occupying either the surface or 250 m water in the open ocean (Field, et al. 1997). SAR324 was found to be more abundant in deep water than at the surface with depth maxima between 160 and 500 m (Wright, et al. 1997). SAR406 was found to be more abundant at 200 m than at the surface, and the surface population of SAR406 was found to be positively correlated with chlorophyll a levels (Gordon and Giovannoni 1996). SAR202 was found to have a depth maximum of 200 m in the Western Sargasso Sea (Giovannoni, et al. 1996). A clone library and oligonucleotide hybridization of 16S rRNA blots were used to study bacterial placement in the water column of the very oligotrophic Crater Lake in Oregon (Urbach, et al. 2001). The uncultivated *Actinomycetes* group ACK4 and a newly discovered and uncultured *Verrucomicrobia*, CL120-10, were found to dominate the euphotic zone. The deep water, 300 to 500 m, was dominated by a newly discovered uncultured green non-sulfur group, CL500-11, and group I crenarchaeota. The concentrations and placement of microorganisms in the ocean have been determined using FISH for the uncultured marine archaea. Archaea were found to sometimes dominate the microbial community in deep ocean waters (Fuhrman and Ouverney 1998). Crenarchaeota were found to increase with depth, reaching up to 39% of cells detected (Karner, et al. 2001). FISH was also used to determine the placement and abundance of the alphaproteobacteria clade, SAR11, which was found to comprise, on average, 35% of cells in surface waters (0 to 200 m) and 18% in the mesopelagic zone (250 to 3000 m) (Morris, et al. 2002).

The widespread nature of the uncultured bacterial division TM7 has been determined through clone libraries. It has been found in a peat bog (Rheims, et al. 1996), activated sludge (Bond, et al. 1995), arid soils (Dunbar, et al. 2002; Dunbar, et al. 1999), Amazonian soils (Borneman and Triplett 1997), a subsurface aquifer (Cho and Kim 2000), and a TCE contaminated site (Lowe, et al. 2002). FISH data indicate TM7 organisms from activated sludge have various morphologies that included cocci, small rods and sheathed filamentous rods. The filamentous rods were also determined to have a gram-positive cell wall (Hugenholtz, et al. 2001).

The surprising breadth of diversity uncovered by culture-independent 16S rDNA studies implies the possibility of an unprecedented array of novel physiologies and ecological roles when these abundant and important uncultured microorganisms are brought into culture. Phylogenetic study of these novel 16S rRNA genes has revealed major new divisions of uncultured prokaryotes. Currently, the kingdom Bacteria includes over 40 phylogenetic divisions, and only half of these divisions include a cultured representative (Edward F. DeLong and Pace 2001; Hugenholtz, et al. 1998; Pace 1997). The term "the great plate count anomaly" was coined by Staley and Konopka (1985) to describe the orders of magnitude difference between the numbers of cells from natural environments that form colonies on agar media and the numbers countable by microscopic examination (Jannasch and Jones 1959). Even though the impact of culture-independent techniques on the field of microbial ecology has been tremendously valuable and has driven research in this area for over a decade, the information that

can be gleaned from these studies is limited. The physiology and ecological significance of the microorganisms harboring these novel 16S rRNA genes has for the most part been uncertain without cultured representatives.

A great void of knowledge exists about microbial life on the planet since most research is currently being conducted on only a handful of microorganisms. For example, 65% of published prokaryotic research from 1991 to 1997 was dedicated to the study of only eight bacterial genera, *Escherichia*, *Helicobacter*, *Pseudomonas*, *Bacillus*, *Streptococcus*, *Mycobacterium*, *Staphylococcus*, and *Salmonella* (Gálvez, et al. 1998). Even more astounding is the fact that these eight genera represent only 3 phyla, Proteobacteria, Firmicutes and Actinobacteria, which can also be categorized as phylogenetic divisions. This amounts to 65% of prokaryotic research being focused on 3 out of more than 40 bacterial phylogenetic divisions. Medically important microorganisms are the most commonly researched. There are at least five divisions of bacteria that are widespread in the environment but are poorly studied including *Acidobacteria*, *Verrucomicrobia*, the Green non-sulfur (GNS) bacteria, and candidate divisions OP11 and TM7 (Hugenholtz, et al. 1998; Hugenholtz, et al. 2001). OP11 and TM7 have no cultured representatives. *Acidobacteria*, *Verrucomicrobia*, and the GNS bacteria have only a handful of isolates each. The majority of microbial research is being performed on microorganisms that are not important in environmental systems. This leaves scientists with comparatively little information on the physiology of the microbes that are important in natural environmental processes.

Less than 1% of the microorganisms, which are seen microscopically from a variety of environments, including soils, groundwater and marine systems, can be cultured using standard culturing techniques (Amann, et al. 1995). This low culturability achieved using traditional plate methods and the uncultured status of the dominant groups of most environmental microorganisms has spurred research into studying the culturability of these microorganisms. Microbial culturability research first gained recognition through Button's work on the culturability of marine microbes. Bacterioplankton culturability from 2 to 60% has been reported for marine waters around Alaska and The Netherlands using a dilution method of culturing with filtered autoclaved seawater (Button, et al. 1993; Schut, et al. 1993). Culturability from 0.4 to 96% has been reported for the Mediterranean Sea on various nutrient enriched media using the MPN technique and the technique of filtering cells onto polycarbonate membranes and incubating them on nutrient agar or cellulose pads soaked in nutrient medium (Bianchi and Giuliano 1996). Giuliano and colleagues found culturability of 0.0007% for surface water from the Mediterranean Sea diluted by traditional MPN method into aged seawater medium that was previously 0.2 μm filtered and autoclaved (Giuliano, et al. 1999). The length of time from collection of inoculum to inoculation of media was not reported in any of these cases making reports of high culturability questionable in light of the (Ferguson, et al. 1984) bottle containment experiment. Ferguson and colleagues found that marine bacteria culturable on Zobell's agar medium

(MA2216) increased from <0.1% to 41% of the total cells present in seawater after confinement in a 4 L bottle for 30 hours at 25° C.

One of the difficulties in culturing environmental microorganisms results from the oligotrophic nature of these environments and the tendency of researchers to use nutrient rich medium to culture them. Oligotrophic microbes grow on low levels of carbon whereas eutrophic bacteria grow on high levels. There are currently various definitions for what constitutes an oligotroph. Oligotrophic bacteria have been defined by Kuznetsov, et al. (1979) as bacteria that can grow on medium containing 1-15 mg organic carbon per liter. Ishida and colleagues (Ishida, et al. 1982; Ishida and Kadota 1981) defined oligotrophs as bacteria that grow on less than 1mg organic carbon per liter. Fry (1990) describes obligate oligotrophs as not being able to grow on media with an organic carbon level greater than 6 mg/L. The ocean is an oligotrophic environment with dissolved organic carbon (DOC) levels that range from 50-100 μ M or 0.6-1.2 mg/L. Groundwater also has very low natural levels of DOC typically ranging from 0.1 to 10 mg/L (Freeze and Cherry 1979). It was initially thought that seawater and groundwater were too dilute in carbon to support the growth of bacteria (Freeze and Cherry 1979; Stevenson 1978; Wangersky 1977). One of the most widespread and commonly accepted ideas was that these organisms are simply unculturable (Colwell and Grimes 2000). This was based on the observation that starved *E. coli* and *Vibrio* cells can enter a viable-but-unculturable state upon nutrient starvation. However, molecular techniques were able to show that there is a great diversity of

microorganisms in the oceans and groundwater environments. Nutrient rich media has been extensively utilized to culture microbes from the environment. This widespread practice is probably, at least partially, responsible for the lack of dominant environmental microbes in the culture collections.

There is also some evidence that not enough culturing attempts have been made using the nutrient rich media that is widely available to bring some of these uncultured microbes into culture. Suzuki and colleagues found that many novel bacterioplankton could be isolated from seawater using a traditional nutrient rich agar based medium, R2A (Reasoner and Geldreich 1985), although many of the most abundant bacterioplankton were still not detected (Suzuki, et al. 1997). A lack of systematic culturing attempts by researchers may also explain why there are so many uncultured microorganisms.

There are only a few studies prior to this dissertation research that attempted to systematically culture any of these widespread and environmentally numerous uncultured microorganisms. Successful attempts have been made in the isolation of oligotrophic or fastidious organisms using various non-agar-based media. DeBruyn, et al. (1990) described a culture isolation technique that relied on floating polycarbonate filters where colonies grew on the surface of the filter. They successfully isolated the iron-oxidizing autotrophic bacteria, *Thiobacillus ferrooxidans* using this technique. This method has the advantage of isolating microorganisms without the presence of potentially inhibitory agar compounds. This floating filter technique was also used to isolate a thermophilic pyrite-oxidizer

(Boogerd, et al. 1989). Another method, generally referred to as extinction dilution culturing, was successfully used by several research groups to isolate bacterioplankton in seawater based media. This method involves dilution to extinction or dilution to a small but known number of environmental cells per tube in a liquid based media. Baxter and Sieburth (1984) isolated an *Acinetobacter* sp. (strain GO1) by this extinction culture in a medium of 0.01mg glucose carbon per liter of seawater. GO1 had an identical growth rate in unamended seawater or in the presence of glucose. Chisholm, et al. (1992) isolated *Prochlorococcus marinus*, a widespread photosynthetic bacterioplankton. Schut and colleagues isolated *Sphingomonas alaskensis* (strain RB2256) by extinction culture into filtered autoclaved seawater (Schut, et al. 1993; Schut, et al. 1997; Vancanneyt, et al. 2001). '*Marinobacter arcticus*' and '*Cycloclasticus oligotrophus*' were also isolated in the same manner by Button, et al. (1998).

Very recently, in May 2002, two relevant papers were published using unique methods to culture microbes by Janssen, et al. (2002) and Kaeberlein, et al. (2002). Kaeberlein and colleagues stimulated the natural environment of marine sediments in an attempt to culture microbes from these sediments. A diffusion chamber was set up around inocula poured in agar plugs. They estimate that up to 40% of the microbes in the inocula were cultured. However, the inoculum sediment was brought into the lab and incubated for one week in a growth chamber prior to its use as inoculum for the culture experiment. Since they did not report on the microbial diversity of the original sediment sample or the diversity of the

sediment after incubation in the week of incubation in the lab, it is impossible to tell if the inocula they started out with represented the natural microbial assemblage they were attempting to culture. It is possible that the bottle effect reported by Ferguson et al. applies to marine sediments as well. Nevertheless, they were successfully able to culture one unique microorganism using this technique. This microbe, MSC1, was only able to grow in co-culture with several of the isolated species and in the diffusion growth chamber. MSC1 is a member of the phylum *Bacteroidetes* and is only 93% similar to its closest cultured relative, *Lewinella persica*, by 16S rRNA gene comparison. This culture highlights the fact that microorganisms live in assemblages of species that have synergistic relationships to each other and their environment.

Janssen and colleagues used a standard nutrient broth diluted 100 times in order to culture microbes from pasture soil in Australia. The soil was serially diluted in broth for most-probable-number (MPN) counting and spread onto broth solidified with agar or gellan gum for counting by colony detection. The culturability that resulted was 1.4%, 5.2% and 7.5% using broth, agar, and gellan gum media, respectively. They also reported 14.1% culturability when sonicated inoculum was spread on gellan gum medium. The soil inocula were processed within three hours of collection making these culturability results very credible. They chose 30 isolates at random to identify. From these isolates, Ellin408 and Ellin457 were from subdivision 1 of the *Acidobacteria*, which currently has only one other cultured species, *Acidobacterium capsulatum*. Ellin408 and Ellin457 are

only 88.9 and 88.0% similar to *A. capsulatum*, respectively, by 16S rRNA sequence comparison. Isolate Ellin428 is a member of subdivision 2 of the *Verrucomicrobia*, which otherwise has no cultured representatives of this subdivision. It has only 86.4% 16S rRNA sequence similarity to the nearest cultivated *Verrucomicrobia* in subdivision 4. These three isolates represent two of the five understudied and widespread bacterial divisions in the environment (Hugenholtz, et al. 1998; Hugenholtz, et al. 2001).

The goal of this dissertation research was to culture previously uncultured microorganisms, which have been uncovered by 16S rRNA molecular methods. Several hypotheses that may explain why so few microorganisms have been cultured were used to develop our culturing approach:

- (1) Growth only occurs in narrowly defined conditions which have not been successfully reproduced in a laboratory setting.
- (2) Microorganisms that grow slowly and/or achieve low cell densities are not detected.
- (3) Uncultured organisms do in fact grow in culture, but have not been detected because not enough attempts have been made by researchers to specifically target uncultured species for cultivation.
- (4) Trace contaminants in laboratory reagents are toxic.
- (5) Interactions with other organisms are required.

We have taken a dilution culture approach first described by Button and colleagues (Button, et al. 1993) and have designed a high throughput technique (HTC) that

enables us to screen large numbers of cultures. Natural media were used without amendments and natural environmental conditions were replicated as closely as possible to satisfy hypothesis 1. Bacterial cultures were detected at concentrations as low as 2×10^3 cells/ml using a custom-built filter manifold to satisfy hypothesis 2. Over 3000 culture attempts from seawater and groundwater were screened for growth using the HTC technique to satisfy hypothesis 3. Acid washed polycarbonate designated "liveware" was used whenever possible to avoid trace contamination from soaps and glassware metals to satisfy hypothesis 4. More than one microorganism was added to each growth chamber. This allows for the growth of organism that may need interactions with other organism for growth to occur to satisfy hypothesis 5.

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CHAPTER 2:

**HIGH THROUGHPUT METHODS FOR CULTURING
MICROORGANISMS IN VERY LOW NUTRIENT MEDIA YIELD
DIVERSE NEW MARINE ISOLATES**

Stephanie A. Cannon and Stephen J. Giovannoni

Applied and Environmental Microbiology

1752 N Street, N. W.
Washington, DC 20036-2904

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DIVERSE NEW MARINE ISOLATES**

ABSTRACT

Microbial diversity studies based on the cloning and sequencing of DNA from nature support the conclusion that only a fraction of the microbial diversity is currently represented in culture collections. Out of over 40 known prokaryotic phyla, only half have cultured representatives. In an effort to culture the uncultured phylotypes from oligotrophic marine ecosystems, we developed high throughput culturing procedures that utilize the concept of extinction culturing to isolate cultures in small volumes of low nutrient media. In these experiments marine bacteria were isolated and cultivated at *in situ* substrate concentrations, typically three orders of magnitude less than common laboratory media. Microtiter plates and a newly developed procedure for making cell arrays were employed to raise the throughput rate and lower detection sensitivity, permitting cell enumeration from 200 μ l aliquots of cultures with densities as low as 1×10^3 cells/ml. Approximately 2500 extinction cultures from 11 separate samplings of marine bacterioplankton were screened over the course of 3 years. Up to 14% of the cells collected from coastal seawater were cultured using this method, which was 14 to 1400-fold higher than obtained by traditional microbiological culturing techniques. Among the microorganisms cultured were four unique cell lineages that belong to previously uncultured or undescribed marine Proteobacteria clades known from

environmental gene cloning studies. These cultures are related to the clades SAR11 (alpha subclass), OM43 (beta subclass), SAR92 (gamma subclass), and OM60/OM241 (gamma subclass). This method proved successful for the cultivation of previously uncultured marine bacterioplankton that have consistently been found in marine clone libraries.

INTRODUCTION

The term "the great plate count anomaly" was coined by Staley and Konopka (1985) to describe the orders of magnitude difference between the numbers of cells from natural environments that form colonies on agar media and the numbers countable by microscopic examination (Jannasch and Jones 1959). Marine ecosystems are a well-studied example of this phenomenon: only 0.01-0.1% of oceanic marine bacterial cells produce colonies using standard plating techniques (Kogure, et al. 1979). There are numerous explanations for this anomaly. For example, species that would otherwise be "culturable" may fail to grow because their growth state in nature, such as dormancy, prevents adjustment to conditions found in the medium used for the plate counts (Deming and Baross 2000). This hypothesis does not explain the substantial discrepancy between 16S rRNA genes recovered from seawater directly by cloning, and those of the readily cultured marine taxa (Lanoil, et al. 2000; Suzuki, et al. 1997). Another explanation for the "the great plate count anomaly" is that many of the microbial species that dominate in natural settings are not adapted for growth in media containing high

concentrations of complex organic carbon. Many microorganisms may need oligotrophic or other fastidious conditions to be successfully cultured. There are many examples of microbial strains that are common in nature but can only be cultivated by specialized techniques (Baxter and Sieburth 1984; Boogerd, et al. 1989; Button, et al. 1998; DeBruyn, et al. 1990; Ferris, et al. 1996; Koops and Möller 1992; Nold, et al. 1996; Partensky, et al. 1999; Schut, et al. 1993; Schut, et al. 1997; Vancanneyt, et al. 2001; Wirsen, et al. 2002).

Button and colleagues pioneered an approach that has been successful in isolating novel oligotrophic, heterotrophic cells from marine ecosystems (Button, et al. 1993). This method uses unamended environmental water as the medium and is often referred to as extinction culturing to distinguish it from dilution culturing, which also uses natural water, but involves complex microbial communities (Ammerman, et al. 1984; Carlson and Ducklow 1996; Li and Dickie 1985). Their approach was to dilute natural communities of microorganisms to a known number, ranging from 1-10 cells per tube, and then examine these potential cultures for microbial growth by means of flow cytometry, which is effective for counting very dilute populations of cells. Using this method bacterioplankton culturability from 2 to 60% was reported for marine waters around Alaska and The Netherlands (Button, et al. 1993). This work resulted in the description of two new oligotrophic bacterioplankton, *Sphingomonas alaskensis* and "Cycloclasticus oligotrophus" (Button, et al. 1998; Schut, et al. 1993; Vancanneyt, et al. 2001; Wang, et al. 1996). However, this extinction culturing method is relatively laborious. The isolates that

have been obtained by this method are of considerable scientific interest, but they are few in number.

The goal of this study was to develop high throughput culturing (HTC) methods that would enable a large number of extinction cultures to be identified so that the efficacy of this approach could be assessed with a larger sampling of isolates. Over the course of 3 years and 11 separate samplings of marine bacterioplankton, 2484 extinction wells were examined for growth. The results indicate that these newly developed HTC techniques yield isolates of many novel microbial strains, including members of previously uncultured groups that are believed to be abundant in coastal seawater.

MATERIALS AND METHODS

High throughput culturing (HTC) technique

A series of protocols and techniques were developed to allow the efficient screening of a large number of extinction culture attempts for growth and subsequent identification (Fig. 2.1). Slight variations of the method were performed during the development of these HTC techniques over the course of three years, but the overall approach remained constant. Microtiter plates were used to culture cells and cell arrays were made to allow efficient screening of the plates for growth. The cultures acquired were designated with high throughput culture collection (HTCC) numbers.

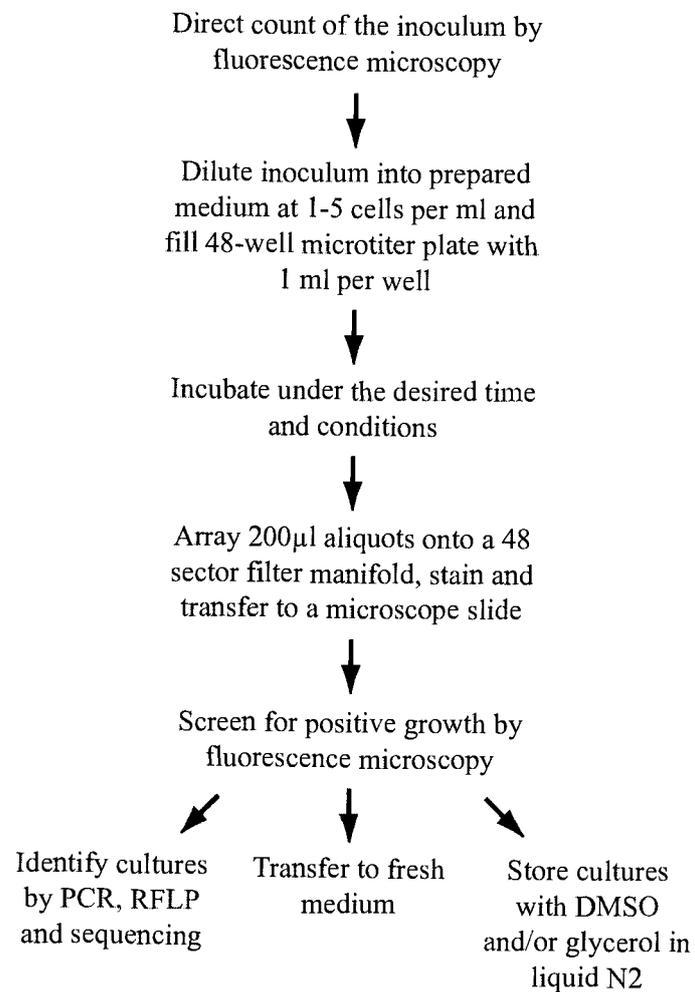


FIG. 2.1. Flowchart of high throughput culturing (HTC) procedures.

Media preparation

Water for media was collected on the south side of the southern jetty in Newport, Oregon, at high tide with a bucket on 3-19-98 and 8 km ($44^{\circ} 39.1\text{N } 124^{\circ} 10.6\text{W}$) offshore from the mouth of Yaquina Bay, Oregon, with a Niskin bottle deployed at 5 m on 6-7-00. On the same day the water was collected, it was filtered through a $0.2 \mu\text{m}$ Supor membrane and immediately autoclaved. In order to restore the bicarbonate buffer lost during autoclaving, the seawater was sparged with sterile CO_2 for at least 6 hours, followed by sterile air for at least 12 hours. Acid washed polycarbonate containers were used for media and live samples whenever possible. Dissolved organic carbon (DOC) concentrations of the seawater media were $107.1 \mu\text{M}$ (SD 1.1) for the 3-19-98 collection, determined using a Shimadzu TOC-500, and $91.6 \mu\text{M}$ (SD 1.6) for the 6-7-00 collection, determined using a Shimadzu TOC-5000A (Shimadzu Co., Kyoto, Japan). Before each use the liquid media were checked for sterility by directly counting cells stained with DAPI (4',6-diamidino-2-phenylindole) as described by Turley (1993) except that 1% formaldehyde was used.

Inocula collection, dilution and incubation

Water samples for inocula were collected on the south side of the southern jetty in Newport, Oregon, at high tide with a bucket and at 8 km ($44^{\circ} 39.1\text{N } 124^{\circ} 10.6\text{W}$) and 25 km ($44^{\circ} 39.1 \text{N } 124^{\circ} 24.7\text{W}$) offshore from the mouth of Yaquina Bay, Oregon, with a Niskin bottle deployed at 5 m. The water was held in darkness at ambient sea surface temperatures until the processing of samples began, within 1

to 4 hours after collection from the Jetty and within 9 hours after collection off the boat to avoid bottle effects (Ferguson, et al. 1984). To determine the bacterioplankton cell densities of the inocula, direct cell counts were done by DAPI staining, where at least 300 cells were counted per filter on triplicate filters. To determine viable cell counts (i.e. culturability) by traditional methods, inocula of 50 or 100 μ l of seawater was applied to spread plates of MA2216 (Difco Laboratories, Detroit, MI), Marine R2A (R2A) (Suzuki, et al. 1997), and a 1/10 dilution of Marine R2A (1/10R2A). Inocula samples were diluted into the prepared seawater medium and distributed as 1 ml aliquots into 48-well non-tissue culture treated Polystyrene plates (Becton Dickinson, Franklin Lakes, NJ) to a final average inoculum ranging from 1.1 to 5.0 cells per well. At least 1 control plate was made for each sample collection by distributing 1 ml aliquots of uninoculated medium. The 48-well plates and agar plates were incubated in the dark at 16°C. The extinction cultures were incubated for 3 weeks and the agar cultures were incubated until colonies were large enough to count, about 1 week for MA2216 and up to 8 weeks for 1/10 R2A.

Detection of growth using cell arrays

A cell array was made from each 48-well plate to examine wells for growth. Two hundred microliters from each well in the plate was filtered into the corresponding chamber of a 48-array filter manifold of custom design manufactured by HyTek Plastics, Corvallis, Oregon. Cells were then DAPI stained and vacuum filtered onto a 48 x 60 mm 0.2 μ m white polycarbonate membrane (cut

from 8 x 10 inch sheets, Whatman Nuclepore, Newton, MA). The membrane was laid on an oiled 75 x 50 mm slide (Corning Glass Works, Corning, NY) and covered with a 48 x 60 mm cover glass (Erie Scientific, Portsmouth, NH). The diameter of each sector of the array was 2 mm, which enabled the detection of a culture with a cell titer as low as 1×10^3 cells/ml when 200 μ l of sample was filtered. The array was then scored for growth by fluorescence microscopy. Cell titers were estimated by counting five random fields within each positive sector.

Culturability statistics

Percent culturability was determined using the equation for estimation of culturability ($V = -\ln(1-p)/X$), and the theoretical number of pure cultures was estimated using the equation ($u = -n(1-p) \ln(1-p)$) described by Button, et al. (1993) where (u) is an estimation of the expected number of pure cultures, (n) is the number of inoculated wells, (V) is estimated culturability, (p) is the proportion of wells positive for growth (wells positive for growth/total inoculated wells), and (X) is the initial inoculum of cells added per well. To calculate the error, first, the exact lower and upper 95% confidence limits for the binomial proportion (p) were determined using the SAS package version 6.12 (SAS Institute Inc.). Next, these exact limits were put into the culturability equation and pure culture equation in place of the term (p) to give the exact lower and upper 95% confidence limits for percent culturability and the theoretical number of pure cultures.

RFLP analysis and sequencing of HTCC isolates

A subset of 56 HTCC cultures were identified by RFLP and rRNA gene sequencing methods. One hundred or 200 μl of culture was put through two cycles of freezing and thawing to promote cell lysis, and concentrated in a 10K MW Vivaspin concentrator (Vivascience, Stonehouse, United Kingdom). Some samples were also treated with 150 μl of GES lysis buffer (5M guanidine thiocyanate, 100mM EDTA and 0.5% sarkosyl) while in the concentrator. The lysates were then rinsed three times with 200 μl of Ultrapure water (Specialty Media, Phillipsburg, NJ) to remove medium salts and lysis buffer. The final volumes of the concentrated samples ranged from 10 to 30 μl . Two to 3 negative controls (the same procedure with no added culture) were run with each set of concentrated samples.

16S rRNA genes were amplified by nested PCR. Two to 5 μl of each concentrated sample was added to the first PCR, which had a 20 μl reaction volume, and 2-5 μl of the first PCR was added to the second PCR, which had a 60-100 μl reaction volume. Twenty-five to thirty-three cycles were used for each PCR, for a total of 50-66 cycles of amplification. The PCR cocktail for both reactions contained 0.025 U Taq/ μl (Promega, Madison, WI or MBI Fermentas, Hanover, MD), 5% acetamide, 1.5 mM Mg^{2+} , 200 nM of each primer, 220 μM dNTP and 1X PCR buffer (Promega, Madison, WI or MBI Fermentas, Hanover, MD). The PCR cocktail was treated with UV irradiation to reduce the contamination levels present in the reagents (Blitchington, et al. 1992; Moore, et al.

1991). The length of UV treatment needed was empirically determined by amplifying a set of negative and positive controls. The amplification conditions for both PCRs were 94°C denaturation for 30 seconds, 50-55°C (depending on primers used) annealing for one minute and 72°C extension for two minutes. The second PCR primer set had at least one primer that amplified from a position internal to the set of primers used in the first PCR. The primers used were 8F (5'-AGR GTT TGA TCM TGG CTC AG-3'), 519F (5'-CAG CMG CCG CGG TAA TWC-3'), 1395R (5'-ACG GGC GGT GTG TRC-3'), 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'), and 1522R (5'-AAG GAG GTG ATC CAN CCR CA-3'), which are variations of commonly used primers that target bacteria or prokaryotes (Lane 1991). The nested set of primers most frequently used was 519F/1492R and 519F/1395R but other variations of the listed primers were also used. Three negative controls and positive controls with 1×10^8 , 2000, 200, and 20 copies of the 16S rRNA gene from the clone SAR242 were run in each PCR set. All primers used have no mismatches to the SAR242 sequence, except for 1492R, which does not match the first and third bases on the five prime end (non priming end). The concentration of the positive control DNA was measured in a Shimadzu UV160U spectrophotometer (Shimadzu Co., Kyoto, Japan). The 20 copy positive control could be routinely amplified with a total of 50-66 cycles of nested PCR.

Restriction fragment length polymorphism (RFLP) of the PCR product was done with the restriction enzymes *Mbo*I and *Hae*III (MBI Fermentas, Hanover, MD) (Vergin and Giovannoni 2001). HTCC cultures were determined to be a mix

of more than one species if RFLP bands from each digest added up to two or more times the length of the expected PCR product. The cultures with fragments that added up to the expected PCR product length were grouped based on matching RFLP patterns and at least one culture from each RFLP group was sequenced and phylogenetically analyzed.

Before sequencing, the PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA). The concentration of the purified product was measured in a Shimadzu UV160U or BioSpec-1601 spectrophotometer (Shimadzu Co., Kyoto, Japan). The purified PCR product was then sequenced by an ABI 373A or 377 automated sequencer (Applied Biosystems, Foster City, CA).

Phylogenetic analysis

HTCC culture sequences were aligned and masked in ARB (Strunk, et al. 1996). Phylogenetic analyses were performed using ARB and PAUP* (Swofford 2001). Phylogenetic trees were inferred by neighbor-joining using the Jukes and Cantor model to estimate evolutionary distances. Bootstrap values were obtained in PAUP* from a consensus of 1000 neighbor-joining trees. Short sequences of HTCC isolates were added to the tree using the parsimony insertion tool in ARB. The percent similarity of sequences was determined using the distance matrix tool in ARB; ambiguous bases were not included.

Recovery of HTCC isolates from frozen storage

The probability of recovering HTCC isolates from frozen storage has not been systematically investigated and not all cultures were saved for further study.

However, isolates from three of the four significant phylogenetic clades in this study, HTCC202 (OM43 clade), HTCC230 and HTCC234 (SAR92 clade), and HTCC223 and HTCC227 (OM60/OM241 clade), have been successfully transferred from the initial well, propagated and stored. Cells were stored in 7% dimethyl sulfoxide (DMSO) and/or 10% glycerol.

DAPI stained cell images

Images were obtained using a Hamamatsu ORCA-ER cooled interline CCD camera (5Mz) mounted on a Leica DMRB microscope. IPLab Spectrum 3.5 image analysis software was used to acquire images.

Nucleotide sequence accession numbers

The sequences of the HTCC isolates used in the phylogenetic analyses have been deposited in GenBank under accession numbers AY102012 to AY102033.

RESULTS

High throughput culturing (HTC)

Our general approach to high throughput culturing is outlined (Fig. 2.1). This method, which allows a large number of culture attempts to be efficiently screened for growth and identified, was successful in bringing four major uncultured or undescribed groups of bacterioplankton into culture. These four groups include SAR11 (alpha subclass) (Giovannoni, et al. 1990), OM43 (beta subclass) (Rappé, et al. 1997), SAR92 (gamma subclass) (Britschgi and Giovannoni 1991), and OM60/OM241 (gamma subclass) (Rappé, et al. 1997).

Culturability statistics

Two hundred and fifty-three extinction culture wells were scored positive for growth out of 2484 wells screened during 3 years and 11 sample collections. A culturability range of 0.4 to 14.3% was calculated for the different sample collections (Table 2.1). The average culturability for the six samples collected between late-May and mid-July was 8.8% and for the five samples collected between early-October and early-April was 1.2%. Comparisons of culturability were made between the HTC method and traditional plating on nutrient rich agar media; the culturability ranged from 1.4 to 120 times higher using HTC methods (Table 2.1). In addition, the first 143 cultures grown from water collected during the summer of 1998 were spotted onto MA2216 and R2A agar plates to determine if they had the ability to grow on these media. Only 3 grew on MA2216 and a fourth grew on R2A; none of these 4 cultures grew on both agar media (data not shown).

Detection of growth and cell densities

The cell densities of the HTCC cultures ranged from 1.3×10^3 to 1.6×10^6 cells/ml with a mean of 1.1×10^5 cells/ml and a median of 3.0×10^4 cells/ml. The minimum density for a culture to be detectable was 1×10^3 cells/ml. This range of cell densities is the result of as few as 10.0 to as many as 23.3 doublings during the three-week incubation period, assuming only one cell from the initial inoculum grew in the well (Table 2.2). The 253 wells that showed cell growth fall into four categories of cell density (Table 2.2). The maximum cell concentration attained

TABLE 2.1. Extinction culturability statistics compared to traditional culturability counts

Inoculation sample ^a	Inocula sample cells/ml	Ave. cells per well	Total wells inoculated	Positive wells ^b	Culture designations	Percent culturability ^c	% culturability on nutrient rich agar ^d		
							1/10R2A	R2A	MA2216
5-21-98 J	1.1 x 10 ⁶	1.1	144	7	HTCC 1-7	4.5 (1.8, 9.3)	--	--	--
6-5-98 J	1.5 x 10 ⁶	1.5	192	37	HTCC 8-44	14.3 (10.0, 19.7)	--	--	--
7-6-98 8km	3.7 x 10 ⁶	3.7	192	62	HTCC 45-106	10.5 (8.0, 13.5)	--	--	--
7-6-98 25km	1.5 x 10 ⁶	1.5	192	37	HTCC 107-143	14.3 (10.0, 19.7)	--	--	--
6-17-99 J	5.6 x 10 ⁶	3.0	192	21	HTCC 144-164	3.9 (2.4, 5.9)	--	--	--
10-29-99 J	1.9 x 10 ⁶	3.0	192	10	HTCC 165-174	1.8 (0.9, 3.3)	--	--	--
12-21-99 J	8.1 x 10 ⁵	5.0	384	10	HTCC 175-184	0.5 (0.3, 1.0)	--	--	--
1-26-00 J	1.1 x 10 ⁶	5.0	192	11	HTCC 185-191, 193-196	1.2 (0.6, 2.1)	0.01	0.01	0.02
4-5-00 J	9.0 x 10 ⁵	5.0	192	20	HTCC 197-216	2.2 (1.3, 3.4)	--	0.15	0.12
7-12-00 J	1.9 x 10 ⁶	3.0	228	33	HTCC 217-233, 236-251	5.2 (3.6, 7.3)	0.98	0.15	0.12
10-9-00 8km	1.3 x 10 ⁶	3.0	384	5	HTCC 252-256	0.4 (0.1, 1.0)	0.29	0.09	0.02

a. Samples were collected on the date indicated from the jetty (J), 8 km or 25 km out from the mouth of Yaquina Bay, OR.

b. Wells were scored for growth after 3 weeks of incubation at 16° C.

c. Ninety-five percent confidence interval shown in parenthesis.

d. Inocula was the same as that used for the microtiter plates; (--) indicates not determined.

TABLE 2.2. Cell densities and inferred doublings attained after 3 weeks of incubation

Final cells/ml	Number of cultures^a	Inferred doublings
$1.0-9.9 \times 10^3$	66	10.0-13.3
$1.0-9.9 \times 10^4$	120	13.3-16.6
$1.0-9.9 \times 10^5$	62	16.6-19.9
$1.0-9.9 \times 10^6$	5	19.9-23.3

a. Out of 253 cultures.

b. This inference is based on the assumption that only one inoculated cell in each well grew.

(1.6×10^6 cells/ml) is similar to the natural bacterial numbers in seawater, which ranged from 8.1×10^5 to 5.6×10^6 cells/ml for the 11 inoculum samples collected.

Imaging of the DAPI stained isolates revealed unicellular organisms that were generally of small size. The SAR11 clade isolate HTCC150 was a small, curved rod (ca. 1-0.8 μm by 0.3-0.2 μm). The OM43 clade isolates HTCC163 and HTCC175 were short rods (ca. 0.8-0.5 μm by 0.5 μm). The SAR92 clade isolates HTCC148, HTCC151 and HTCC154 were short rods (ca. 1-0.7 μm by 0.7-0.5 μm). OM60/OM241 clade isolate HTCC160 was an irregularly shaped coccus that occasionally formed doublets and more rarely chains of three (ca. 0.7 μm by 0.7 μm). These measurements are subject to sizeable error since these small cells are at or approach the resolution of visible light microscopes. The cells have been stained with a DNA staining dye, and have been fixed with formaldehyde. The images shown are from the original extinction dilutions that yielded the four previously uncultured/undescribed groups (Fig. 2.2).

Phylogenetic analysis and culture identification

Uncultured or undescribed groups, SAR11, OM43, SAR92, and OM60/OM241, accounted for the majority of cultures that were identified out of a subset of 56 cultures (Table 2.3). All cultured cells from thirteen 48-well plates (56 cultures) were chosen to represent five different sampling months to minimize biases that might emerge as a result of seasonal variation in bacterioplankton abundance. Forty-seven of the 56 cultures were identified; of the nine cultures that were not identified, seven were found to be unknown mixtures of several cell types

DAPI images of HTCC isolates

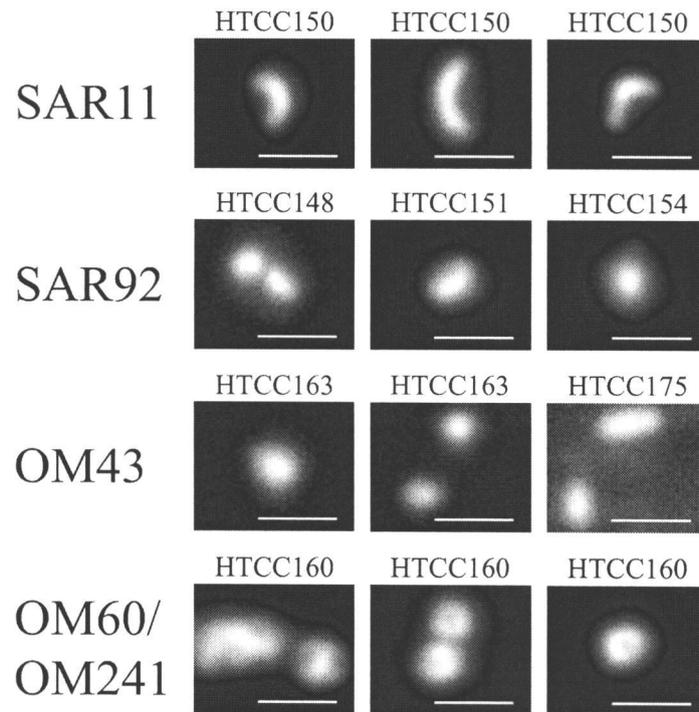


FIG. 2.2. Fluorescence microscopy images of several of the novel isolates. The cells were stained with DAPI. Size bars are 1 μm .

TABLE 2.3. Phylogenetic identification and pure culture statistics for 56 cultures

Inoculation date	Wells screened	No. of Cultures detected	Theoretical # of Pure Cultures ^a	Culture Identification						
				SAR11	OM43	SAR92	OM60/OM241	Other ^c	Mixed culture	Not identified
5-21-98	96	7	6.7 (2.8, 12.8)	--	--	5	--	--	2	--
6-17-98	96	11	10.3 (5.5, 16.8)	2	1	4	--	2	3 ^b	--
10-29-99	96	10	9.5 (4.8, 15.9)	--	8	--	--	1	1	--
1-26-00	192	11	10.7 (5.5, 18.2)	--	7	2	--	1	1	--
7-12-00	144	17	16.0 (9.8, 23.7)	--	--	3	3	8	1	2
Total	624	56	53.4 (41.2, 67.4)	2	16	15	3	11	8	2

a. Statistical estimation of the theoretical number of pure cultures acquired with 95% confidence interval. The total 53.4 was determined independently using 624 wells and 56 cultures in the pure culture equation.

b. One SAR11 culture was mixed with an unknown cell type (RFLP analysis) and is also included under the heading "Mixed culture."

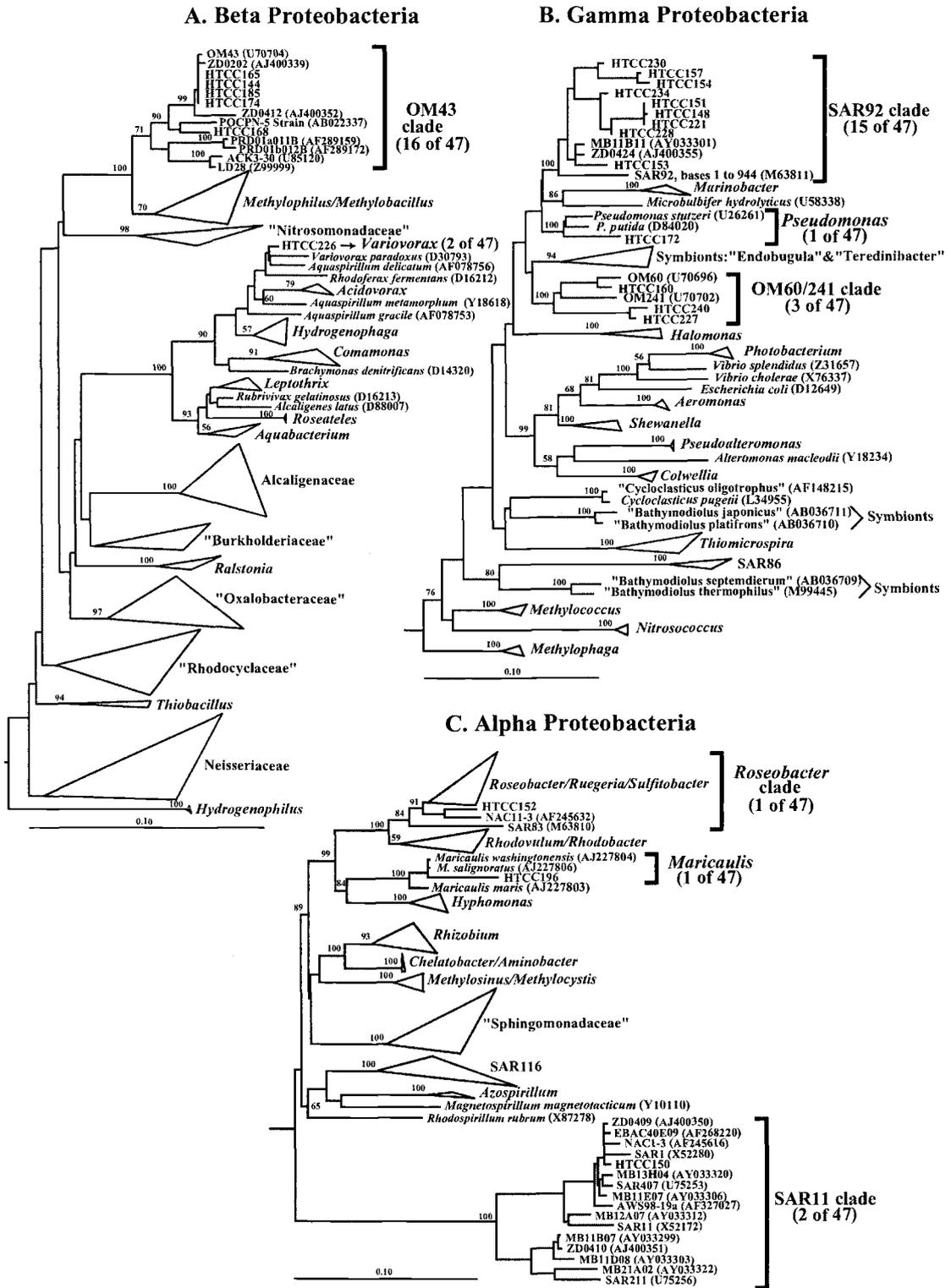
c. "Other" indicates cultures that fall into previously cultured groups.

based on RFLP analysis and two did not amplify with the conditions used. There were a total of eight mixed cultures; HTCC149 was found to be a mix of cells from the SAR11 clade and unknown cells. The failure of two cultures to amplify is probably attributable to problems with the DNA extractions and/or low cell densities in the cultures. A considerable effort was made to ensure that these lineages did not fail to amplify because of mismatches to amplification primers. Theoretical statistical estimation for the number of pure cultures versus mixed cultures that should be acquired was consistent with the RFLP analysis (Table 2.3).

Of the 47 identified cultures, four were alpha Proteobacteria (Fig. 2.3C). Two belonged to the SAR11 clade and one each from the genus *Maricaulis* and the *Roseobacter* clade. Eighteen isolates were identified as beta Proteobacteria (Fig. 2.3A). These included members of two clades, 16 isolates from the OM43 clade and two related to the genus *Variovorax*. Nineteen cultures were gamma Proteobacteria (Fig. 2.3B). These included three sub-groups, SAR92 clade (15 isolates), the OM60/OM241 clade (three isolates) and one from the genus *Pseudomonas*. Six isolates were members of the phylum Bacteroidetes.

The 16S rRNA sequence for the SAR92 clone (M63811) was found to be a chimera. From sequence positions 1 to 944 SAR92 is a gamma Proteobacteria; from positions 1120 to 1354 it is an alpha Proteobacteria. The identity of the sequence from 945 to 1119 is ambiguous. The gamma portion of the SAR92 clone sequence represents a previously uncultured phylogenetic clade we have termed the SAR92 clade.

FIG. 2.3. Neighbor joining trees showing phylogenetic relationships of the 16S rRNA genes of high throughput cultures compared to representative species and environmental clones. Scale bars indicates 0.1 change per nucleotide. Bootstrap values below 50 are not shown. Short sequences, approximately 600bp, of HTCC isolates were added to the trees using the parsimony insertion tool in ARB. HTCC230 and HTCC234 are close to full length and were put in the original tree. In parentheses next to HTCC isolates is the number of total cultures from the subset of 47 identified cultures that are included in that clade. However, not all the HTCC sequences used in the tree are part of the subset of 47 identified cultures. (A) Alpha Proteobacteria phylogenetic tree. Beta and gamma Proteobacteria were used to root the tree; 1051 characters were used to infer the tree. (B) Beta Proteobacteria phylogenetic tree. Gamma Proteobacteria were used to root the tree; 789 characters were used to infer the tree. (C) Gamma Proteobacteria phylogenetic tree. Beta Proteobacteria were used to root the tree; 1042 characters were used to infer the tree.



Percent similarities of the sequenced HTCC cultures from the four previously uncultured/undescribed phylogenetic clades were determined, where several of the sequences were close matches to clones in GenBank (Table 2.4) (Bano and Hollibaugh 2002; Béjà, et al. 2000b; Suzuki, et al. 2001). Sequences of oligotrophic isolates from the OM43 and OM60/OM241 clades were recently deposited into GenBank as strain POCPN-5 (AB022337) and KI89C (AB022713), respectively, by Katanozaka and Yoshinaga (unpublished). HTCC isolates from the four distinct phylogenetic clades, SAR11, OM43, SAR92 and OM60/OM241, are more similar to cloned sequences from these clades than to previously cultured species with the exception of HTCC168, which is 99.8% similar to the isolate POCPN-5.

DISCUSSION

Culturability statistics and detection of growth

The goal of this study was to evaluate a culturing format for the high throughput isolation of uncultured strains of bacterioplankton that are commonly found in gene clone libraries from marine environments. The use of microtiter dishes and a novel technique for making cell arrays enabled us to achieve a higher throughput rate, shorten incubation times, and raise sensitivity for the detection of cells with low growth rates relative to previous studies that employed the concept of extinction culturing in natural media.

TABLE 2.4. 16S rRNA sequence similarities of HTCC isolates from previously uncultivated clades to nearest neighbors in GenBank

Clade	HTCC Culture	<i>E. coli</i> position^a	Clone	% similarity
SAR11	150	524 to 1362	ZD0409	99.8
OM43	144	712 to 1386	OM43	100
	165	710 to 1386	OM43	100
	168	711 to 1371	POCPN-5 ^b	99.8
	174	713 to 1362	OM43	100
	185	719 to 1378	OM43	100
SAR92	148	716 to 1384	MB11B11	96.9
	151	716 to 1383	MB11B11	96.9
	153	713 to 1360	MB11B11	99.4
	154	716 to 1360	Artic97A-6	99.7
	157	716 to 1351	Artic97A-6	99.5
	221	716 to 1346	MB11B11	96.7
	228	707 to 1266	SAR92	96.5
	230	28 to 1537	MB11B11	97.0
	234	28 to 1537	MB11B11	97.0
OM60/OM241	160	713 to 1383	OM60	100
	227	705 to 1373	MERTZ-2CM-38	97.0
	240	716 to 1360	MERTZ-2CM-38	96.9

a. *E. coli* position of the bases used to determine % similarity.

b. POCPN-5 is a cultured strain, not a clone.

The percentage of cells that could be cultured by the HTC approach was several orders of magnitude higher than was obtained by culturing on agar plates. Ferguson and colleagues found that the percentage of microbial cells in seawater that could be cultured on a rich nutrient agar medium (MA2216) increased from <0.1% to 13% after 16 hours and to 41% after 32 hours of confinement in a 4 liter bottle at ambient sample collection temperature (Ferguson, et al. 1984). Our results cannot easily be explained by this “bottle effect” because 1) the cells were diluted into the 48-well plates between 1 to 4 hours after collection from the jetty and within 9 hours after collection from the boat; 2) readily culturable genera such as *Pseudomonas* and *Vibrio* were rarely detected in our cultures; and 3) four previously undescribed lineages were grown using our culture method.

Culturability was observed to be higher in the summer months (8.8%) than in the winter months (1.2%). There are at least two plausible explanations for this observation. First, bacterioplankton cells may be in a dormant state during the winter and either fail to grow or need longer incubation times for growth to be detected. Alternatively, the predominant strains or species of cells present in the winter could be organisms that are unable to grow under the laboratory conditions we provided, which were more similar to summer environmental conditions. The seawater media used for these experiments was collected during the spring and summer months, and our incubation temperature of 16° C is closer to the summer temperature range of 10.0 to 14.7° C versus the winter range of 9.5 to 10.7° C for the eleven samples collected. Also, the summer months off the Coast of Oregon

are dominated by upwelling events that bring cool nutrient-rich water to the surface, which subsequently induces large algal blooms. During the winter, the water off the coast is diluted by the Columbia River water plume, mixed by frequent storm events and not subject to algal blooms. The bacterioplankton that predominate during the summer may be better adapted to the higher nutrient levels and/or nutrient types provided and therefore more amenable to cultivation by the methods we used.

The majority of the cultures identified were pure cultures based on RFLP analysis. Theoretical estimates of the number of pure cultures expected were consistent with the number and proportion of pure cultures observed by RFLP analysis; eight of 54 cultures studied in this manner were mixed cultures. This would indicate that most cultures were the result of only one of the inoculated cells growing in the well. However, RFLP analysis would miss mixed cultures with differential cell lysis or where the primers used for PCR amplification fail to amplify all cell types in a culture. In addition, a dominating cell type may preferentially amplify and thus appears as a pure culture in an RFLP analysis.

Phylogenetic analysis and culture identification

Phylogenetic identification of the isolates provided striking evidence that extinction culturing in microtiter dishes, using natural seawater and low thresholds of detection, results in the cultivation of microbial groups that appear in environmental clone libraries but have not been previously detected in culture. The SAR11 and SAR92 clades, which were isolated in this study (transiently, in the

case of SAR11) have previously been detected only by environmental rRNA gene cloning. Ribosomal RNA gene sequences from isolates of other previously uncultivated clades, OM43 and OM60/OM241 (strains POCPN-5 and KI89C, respectively), were recently deposited into GenBank by other investigators.

Some of the isolates that were cultured belong to phylogenetic clades that are highly abundant in marine clone libraries. Clones in the SAR11 clade are abundant in clone libraries made from surface marine waters around the world (Giovannoni and Rappé, 2000). The OM43 clade is a sister clade to a group of marine methylotrophs that includes *Methylophilus* and *Methylobacillus*, which are commonly found in clone libraries from coastal sites, but not the open ocean (Rappé, et al., 2000). *Methylophilus* and *Methylobacillus* are classified as Type I methylotrophs, which use the ribulose monophosphate (RuMP) pathway for carbon assimilation. The OM60/OM241 clades are frequently found in coastal marine clone libraries and the SAR92 clade is found in open ocean as well as coastal clone libraries. In subsequent work (unpublished results) several strains obtained by these procedures were scaled up to 20 liter volumes for further study.

Several other major uncultured groups that are thought to be abundant in surface seawater, such as the SAR86 and SAR116 clusters, did not appear among the HTCC isolates. Further innovations of the HTC approach will be needed to close the gap between culture collections and the microbial species dominating marine bacterioplankton communities. The approach we describe can be used to target specific bacterial groups for cultivation by screening cultures for the

microorganisms of interest by means of fluorescence *in situ* hybridization, so that uncultured “targets” can be sought in a deliberate manner. The SAR86 cluster has been recently linked to a bacterial rhodopsin gene that facilitates light-mediated proton translocation (Béjà, et al. 2000a). Incubation of extinction cultures under varying conditions, including different sources of carbon, light, and other matrices of variables, may lead to the culturing of microorganisms that have specialized growth requirements. To examine arrays of variables, it would be necessary to increase the rate at which cultures are examined. Work in progress has partially achieved these goals by the application of automation tools, such as robotic liquid handling and the automated scanning of cell arrays (J.-C. Cho, C. S. Alexander, S. Dunlap, S. A. Connon, M. S. Rappé and S. J. Giovannoni, unpublished results).

Culturing organisms remains an important step in the process of understanding the biology and ecology of microbial species. Cultures can be used to obtain complete genome sequences, and to identify properties of organisms that could not be identified by genome sequence alone. Cultures also provide a means to test hypotheses emerging from genome sequences. Combined with proteomics or microarrays, cultures of environmentally significant organisms can be used to examine the adaptations of organisms to environmental change. For example, cultures of key heterotrophic bacterioplankton may enable oceanographers to study how nutrient limitation and other oceanographically relevant variables affect the growth of individual species, and thereby help identify the role dominant species play in geochemical cycles.

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CHAPTER 3:**THE BACTERIAL COMMUNITY COMPOSITION IN
TRICHLOROETHENE CONTAMINATED PROPANE SPARGED VERSUS
AIR SPARGED GROUNDWATER AT MCCLELLAN AIR FORCE BASE,
SACRAMENTO, CA**

Stephanie A. Connon, Adisorn Tovanabootr (*Post Mortem*), Mark Dolan, Kevin Vergin, Stephen J. Giovannoni, and Lewis Semprini

**THE BACTERIAL COMMUNITY COMPOSITION IN
TRICHLOROETHENE CONTAMINATED PROPANE SPARGED VERSUS
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ABSTRACT

An in situ cometabolic air sparging (CAS) study was carried out at McClellan Air Force Base (MAFB), Sacramento California, in a trichloroethene (TCE) and *cis*-dichloroethene (*cis*-DCE) contaminated aquifer. The major objectives of this study were 1) survey the bacterial population shifts that occurred in the groundwater during CAS and air sparging and 2) determine how they were related to propane utilization and chlorinated aliphatic hydrocarbon (CAH) transformation. Two identical sets of monitoring wells located approximately 100 feet apart were sparged. One test zone received air sparging only, and the other test zone received 2% propane in air. Length heterogeneity polymerase chain reaction (LH-PCR) fragment analysis showed a shift in the bacterial community of propane sparged groundwater to a dominance of an organism(s) that had a fragment size of 385 base pairs (385bp). A clone library made from the bacteria sampled in propane sparged groundwater included 3 clones of a TM7 division bacterium that had a 385bp LH-PCR fragment; no other bacterial species with this fragment size were detected. Direct counts of the groundwater showed an increase in the microbial concentration after propane sparging when compared to pre-sparged and air-sparged groundwater. The 385bp LH-PCR fragment was positively correlated with

propane removal rates. Propane removal rates and the 385bp LH-PCR fragment both decreased as nitrate levels in the groundwater decreased.

INTRODUCTION

TCE is a chlorinated aliphatic hydrocarbon (CAH) that has been widely used as a degreasing agent for aircraft engines. This has led to extensive groundwater contamination at numerous military bases around the country and it is listed as a top priority pollutant by the Environmental Protection Agency (Federal Register 1989). TCE undergoes reductive dehalogenation under anaerobic conditions to form *cis*-DCE, 1,1-DCE, *trans*-DCE and vinyl chloride (VC), which are also common co-contaminates at these sites (Lee, et al. 1998; McCarty 1997; Vogel, et al. 1987; Vogel and McCarty 1985). Traditionally, CAH contaminated groundwater has been remediated by 1) pumping and treating, ex-situ, via air stripping or chemical means or 2) in situ air sparging in order to volatilize the CAHs (Norris, et al. 1994).

CAS is an innovative form of conventional in situ air sparging that has the potential to remediate CAH contaminated groundwater more effectively and less expensively. CAS has the advantage of reducing the off-gas of CAH emissions during sparging through enhanced biodegradation of contaminants (Marley and Bruell 1995) using cometabolic growth substrates such as methane (Travis and Rosenberg 1997) propane or butane. Another advantage of CAS is the avoidance of VC production since VC is a potent carcinogen that only accumulates in a

reducing environment (Bouwer 1994; Code of Federal Regulations 1985; Eder 1991; McCarty and Semprini 1994).

Propane was used as a cometabolic substrate at the CAS demonstration site located at McClellan Air Force Base, Sacramento, California. The demonstration aquifer was contaminated with a mixture of primarily TCE and *cis*-DCE, with levels as high as 2.68 mg/L and 2.27 mg/L, respectively. A microcosm study conducted using soil cores taken from the site indicated that propane was an effective cometabolic substrate for the bioremediation of a complex mixture of CAHs when compared to methane and butane (Tovanabootr and Semprini 1998). Propane also contributed to better long term TCE removal activity after the propane substrate was consumed. Details of the cometabolic sparging tests conducted at MAFB are provided by Tovanabootr and colleagues (Tovanabootr, et al. 2000; Tovanabootr, et al. 2001). The test zone receiving propane began to show propane utilization in situ 4-6 weeks after propane sparging began, and TCE and *cis*-DCE levels began to decrease in proportion to propane usage 6 weeks after sparging began (Tovanabootr, et al. 2000). The CAS demonstration during this phase of testing lasted 187 days.

We conducted a bacterial characterization study prior to and during the CAS demonstration to detect shifts in the bacterial community composition. The bacterial community of the groundwater was analyzed using LH-PCR and a clone library of 16S rRNA genes to survey the bacterial community shifts that occurred in the groundwater during CAS and air sparging. We investigated how the

community shifts were related to rates of propane utilization, nitrate levels, and TCE and *cis*-DCE removal. Microbial abundance in the groundwater was also analyzed before and after CAS and air sparging.

MATERIALS AND METHODS

Study site

Two identical test zones were located approximately 100 feet apart at MAFB (Fig. 3.1.). Monitoring wells were located in both the A-zone and C-zone at depths of 113 ft below ground surface (bgs) and 117 ft bgs. The water table started at a depth of approximately 100 ft bgs. The A-zone was sparged only with air and the C-zone received 2% propane in air (Tovanabootr, et al. 2000; Tovanabootr, et al. 2001). Sparging was initiated on day 36 of the study and groundwater was periodically sampled from the monitoring wells until day 76. From day 80 to 120, weekly sparging events of propane and air were conducted with continued sampling of the groundwater. From day 120-160 sparging was increased to twice a week. No sparging was performed from day 160-187. The sparging was conducted using a passive approach with active sparging occurring for only a 5-10 hour period. This intermittent sparge approach was used in an attempt to minimize volatilization of the CAHs from the groundwater to the vadose zone.

Site Map

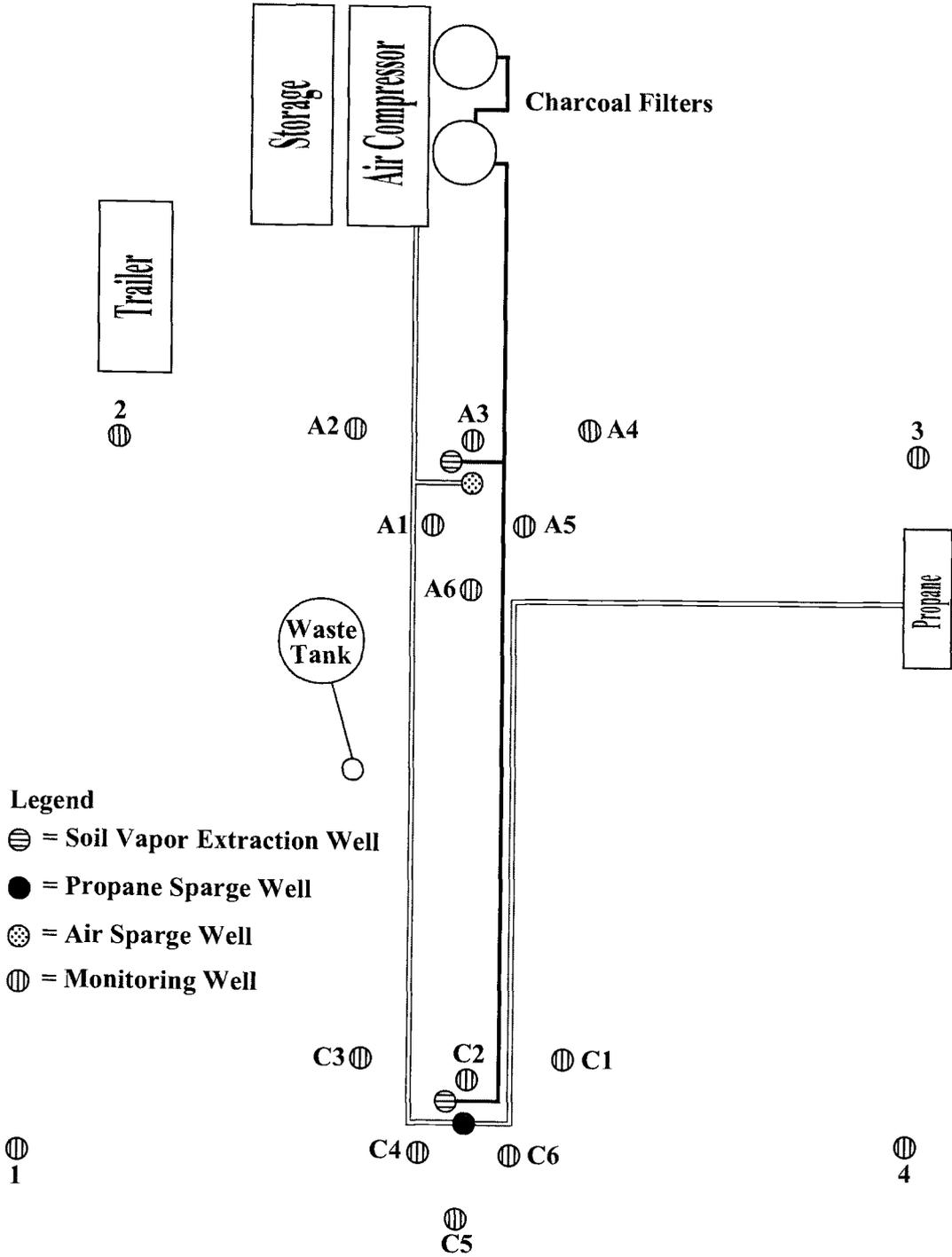


FIG. 3.1. Layout of study site at McClellan Air Force Base, Sacramento CA, showing location of sampling wells.

Analytical procedures for the measurement of propane, TCE, *cis*-DCE, dissolved oxygen (DO) and nitrate

Groundwater samples were tested for the presence of propane, TCE, *cis*-DCE, DO and nitrate concentrations. DO was measured on site using a dissolved oxygen probe and a DO meter. Propane, TCE and *cis*-DCE groundwater concentrations were determined by a purge and trap method, and nitrate concentrations were determined by ion chromatography (Tovanabootr and Semprini 1998). The collection of nitrate data began on day 100 of this study. Propane removal rates were estimated based on the first three propane measurements made after propane was added and had reached maximum levels in the groundwater sampled. For accuracy, only measured propane levels above 0.01mg/L were used and propane rates were not determined for propane sparge tests where only two measurements were available.

Microbial direct counts

Groundwater samples from the A-zone well A1-113 and C-zone wells C2-113 and C4-113 were collected for direct counts before propane sparging (day 20) and after sparging began (day 128). A3-113 groundwater was only collected on day 20 and A2-113 groundwater was only collected on day 128. Direct cell counts were done by staining with 4',6-diamidino-2-phenylindole (DAPI) as described by (Turley 1993), except that 1% formaldehyde was used. At least 300 cells were counted per filter and triplicate filters were counted for each sample.

Sample collection for DNA purification

DNA of microbial cells collected from the groundwater samples were purified for bacterial community analysis by LH-PCR. Groundwater samples from the A-zone at 113 ft bgs (A1-113, A2-113 and A3-113) and the C-zone at 113 ft bgs (C2-113, C3-113, C4-113 and C5-113) were collected before propane sparging (day 20) and after onset of propane uptake (days 96, 128, 174, and 187). Groundwater samples from the A-zone at 117 ft (A1-117, A2-117 and A3-117) and C-zone at 117 ft bgs (C2-117, C3-117, C4-117 and C5-117) were collected before propane sparging (day 21) and after sparging began (day 126, and 187). One-liter samples of groundwater from each well were filtered onto 0.2 μm Supor membranes, placed into 10 ml cryovials (Midwest Scientific Inc., Valley Park, MO) with 5ml of GES (5M Guanidine thiocyanate, 100mM EDTA, 0.5% Sarkosyl) and immediately stored in liquid nitrogen dewars. Samples in cryovials were then thawed and incubated at 37° C for 30 min in a Hybridiser HB-1 (Techne Corporation, Princeton, NJ) to insure complete cell lysis before the DNA was extracted. The DNA was purified by a standard phenol chloroform protocol followed by ethanol precipitation (Sambrook, et al. 1989).

Bacterial community analysis by LH-PCR

LH-PCR was used to measure the change in the bacterial community composition of the groundwater (Suzuki, et al. 1998). The primers 8F (5'-(6-FAM)AGAGTTTGATCMTGGCTCAG-3') (Genset Corp., La Jolla, Calif.) and 338R (5'-GCWGCCWCCCGTAGGWGT-3'), were used to amplify the 5' end

of the 16S rRNA genes from each extracted DNA sample. The LH-PCR reaction mixture contained 0.025 U Taq/ μ l (Promega, Madison, WI or MBI Fermentas, Hanover, MD), 5% acetamide, 1.5 mM Mg²⁺, 200 nM of each primer, 220 μ M dNTP and 1X PCR buffer (Promega, Madison, WI or MBI Fermentas, Hanover, MD). The amplification conditions were 94°C denaturation for 30 s, 55°C annealing for 1 min and 72°C extension for 1 min, except the final extension, which was run for 20 min. The number of cycles of LH-PCR varied from 16 to 26 in order to restrict the final amplified product concentration to a range of 0.5 to 1 ng/ μ l, since a low final concentration of amplified product reduces PCR bias (Suzuki and Giovannoni 1996; Suzuki, et al. 1998). Relative amounts of amplicons were estimated using a DNA mass ladder on a 1% agarose gel stained with ethidium bromide. The LH-PCR samples were run on an ABI 373A or 377 gel based automated sequencer (Applied Biosystems, Foster City, CA) in GeneScan mode and analyzed using Applied Biosystems GeneScan analysis software.

Clone library construction

A clone library was made from a sample collected on day 128 from monitoring well C4-113 of the propane sparged test zone and consisted of 91 clones. The primers 8F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1522R (5'-AAGGAGGTGATCCANCCRCA-3') were used to amplify the 16S rRNA gene for cloning. The PCR reaction mixture was identical to that used for the LH-PCR above. The amplification conditions were 35 cycles of 94°C denaturation for 30 sec, 55°C annealing for 1 min, and 72°C extension for 2 min. Amplification

products were cloned into the pGEM-T-easy vector (Promega, Madison, WI) according to the manufacturer's instructions. The library was screened for two specific LH-PCR fragments of interest (385bp and 366bp). Clones, representing the LH-PCR fragments of interest, were sequenced on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA).

Phylogenetic analysis

Sequences were aligned and masked in ARB (Strunk, et al. 1996). Phylogenetic analyses were performed using ARB and PAUP* (Swofford 2001). Phylogenetic trees were inferred by neighbor-joining using the Jukes and Cantor model to estimate evolutionary distances. Bootstrap values were obtained in PAUP* from a consensus of 1000 neighbor-joining trees. The percent similarity of sequences was determined using the distance matrix tool in ARB; ambiguous bases were not included. The short sequence from clone MAFB-C4-52 was added to the tree using the parsimony insertion tool in ARB. All sequenced clones will be deposited in GenBank.

RESULTS

An increase was seen in the abundance of bacteria in the groundwater after propane sparging. Groundwater from wells C2-113 and C4-113 showed a dramatic 25-fold and 19-fold increase, respectively, in microbial cell numbers after propane sparging, while well A1-113 showed only a slight increase in cell numbers after air sparging (Table 3.1.). There were technical difficulties in obtaining a sample from

Table 3.1. DAPI direct counts

Monitoring well	Sparge type	Before sparge day 20 (cells/ml)	After sparge day 128 (cells/ml)
C2-113	propane	7.6×10^4	1.9×10^6
C4-113	propane	4.5×10^4	8.6×10^5
A1-113	air	2.0×10^5	2.8×10^5
A2-113	air	--	9.4×10^4
A3-113	air	9.6×10^4	--

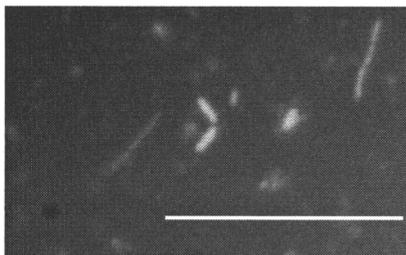
well A3-113 after air sparging; a sample was obtained from well A2-113 instead. The addition of propane in the groundwater at MAFB caused a general increase in bacterial abundance.

Direct count data collected from groundwater showed that large, brightly stained filamentous rods, approximately 0.5 μm wide and up to 11 μm long, were seen only in the wells that had received propane. An accurate count of these filamentous rods could not be assessed due to severe clumping of these cells. Representatives of typical microbes seen in groundwater collected from C2-113 before propane sparging (day 20) and representatives of the large filamentous rods seen after propane sparging (day 128) are shown (Fig. 3.2). The small rods and cocci, generally 1-2 μm in length, seen in the pre-sparged C-zone and A-zone groundwater, continued to be observed in the C-zone after propane sparging and are visible as faint cells around the filamentous rods in the image (Fig. 3.2).

LH-PCR of the 16S rRNA gene was used to detect the overall bacterial composition differences between air-sparged and propane-sparged groundwater and the changes that occurred in a specific well before and after propane sparging. A hyper-variable region between the 8F and 338R primer allows the discrimination of different organisms based on the natural variation in the length of this amplified fragment (Suzuki, et al. 1998). Propane sparging shifted the bacterial community in the groundwater toward a dominant LH-PCR fragment of 385bp; LH-PCR electropherograms for groundwater from wells C2-113, C4-113 and A1-113 are shown (Fig.3.3.). The peak that represents the 385bp LH-PCR fragment occurred

Cell Images

**C2-113
day 20
before
propane
sparging**



**C2-113
day 128
after
propane
sparging**

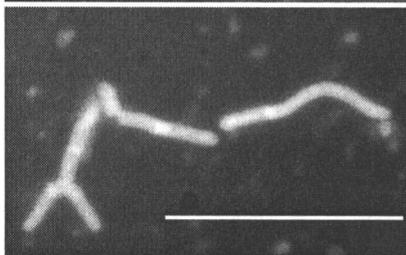
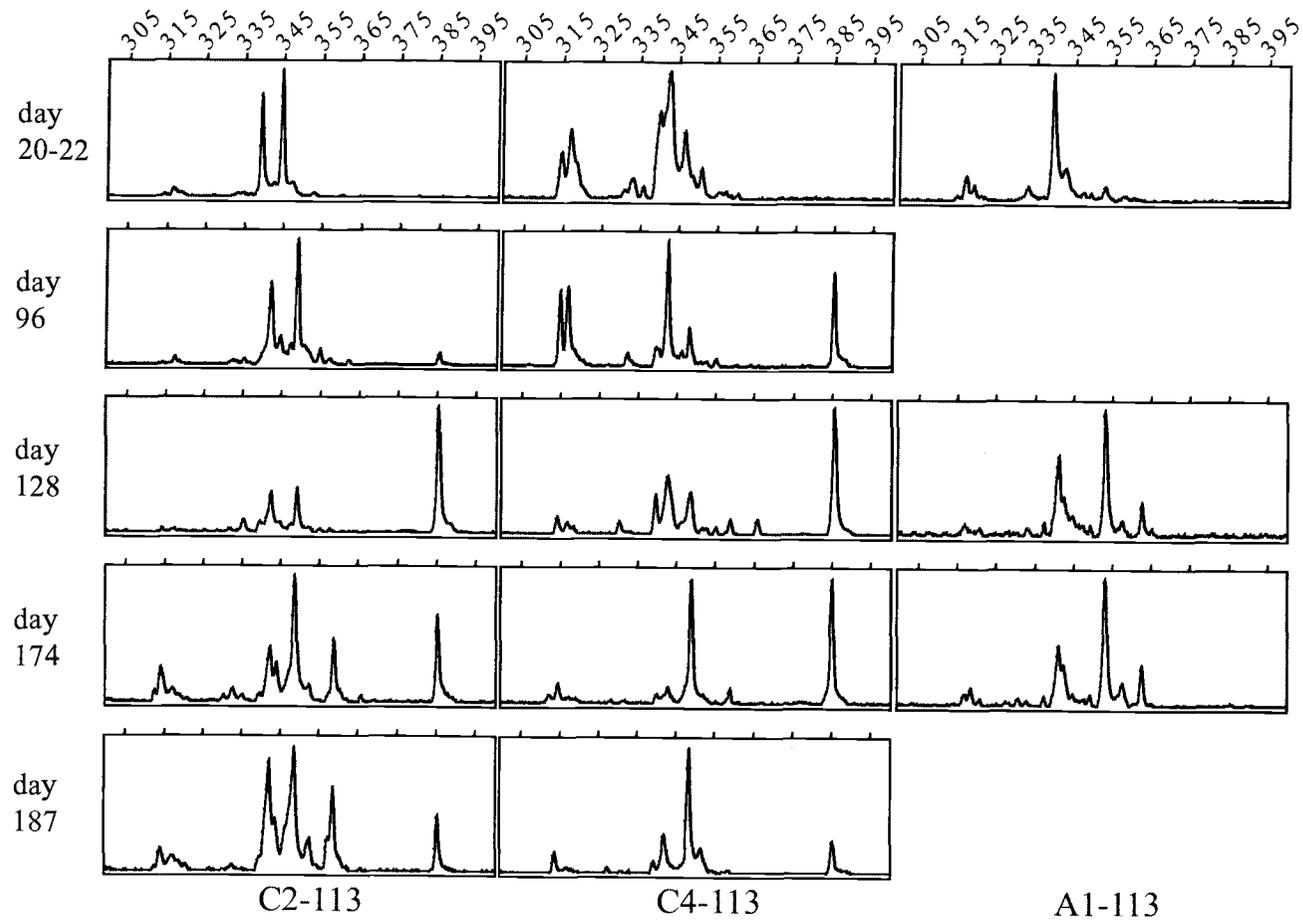


FIG. 3.2. Groundwater was 10% (v/v) formalin fixed on site and stored at 4°C for up to one week prior to staining with 5 μ g/ml DAPI and filtering down onto 0.2 μ m black polycarbonate membranes for counting. Images were taken with a Princeton Instruments MicroMax 1300Y 5MZ cooled interline CCD camera mounted on a Leica DMRB epifluorescence microscope using a 100X PL Fluotar 1.3 NA oil objective. Bar is 10 μ m.

Figure 3.3. The LH-PCR electropherograms show the amplified fragments represented as peaks. The fragment size is shown on the x-axis. The y-axis is relative peak intensity where the sum of the peak areas for each sample represents the total LH-PCR product. The shift in the bacterial community is shown over time for wells C2-113, C4-113 and A1-113. Propane sparging was initiated in the C-zone on day 36 of this study. Data was not collected for well A1-113 on day 96 and 187.

Fig 3.3.



exclusively in groundwater samples from propane sparged wells. The only other fragment size that correlated with propane sparging was a 366bp fragment, which was seen in several, but not all, groundwater samples that received propane; it was never seen in A-zone samples or from pre-propane sparged C-zone samples. This 366bp fragment is visible in the sample taken from well C4-113 on day 128 of the study (Fig. 3.3.). All other fragments that occurred in the propane sparged C-zone samples occurred at least once in an A-zone sample and/or a pre-sparged C-zone sample (data not shown).

The LH-PCR fragment of 385bp was detected in all wells that received propane except wells C5-113 and C5-117, which received very little propane (Table 3.2.). The distribution of propane was quite variable in the C-zone due to aquifer heterogeneities as discussed by Tovanabootr, et al. (2001). Table 3.2. shows the highest single measurement of propane and the largest percentage of the 385bp fragment measured in each C-zone well during the course of this study, which ended on day 187, 21.5 weeks after the first propane sparging began. The percentage of the 385bp fragment was determined by dividing the 385bp fragment peak area by the total peak area. The maximum level of propane measured in wells C5-113 and C5-117 was 95 and 3.5 times less, respectively, than well C3-117, which had the lowest maximum measurement of propane (0.95 mg/L) that still exhibited a 385bp LH-PCR fragment. The highest proportion of the 385bp fragment, 83.3% of the total LH-PCR fragments, was measured in well C2-117, which also displayed the highest maximum concentration of propane (3.57 mg/L).

Table 3.2. Highest propane concentration and greatest percentage of the 385bp fragment measured in each C-zone well during the 187 day study.

	C2-113	C2-117	C3-113	C3-117	C4-113	C4-117	C5-113	C5-117
%385*	55.6	83.3	36.6	6.9	36.7	12.6	0	0
Propane (mg/L)	1.18	3.57	1.49	0.95	1.91	2.14	0.01	0.27

*385 base pair fragment not detected in the A-zone groundwater samples.

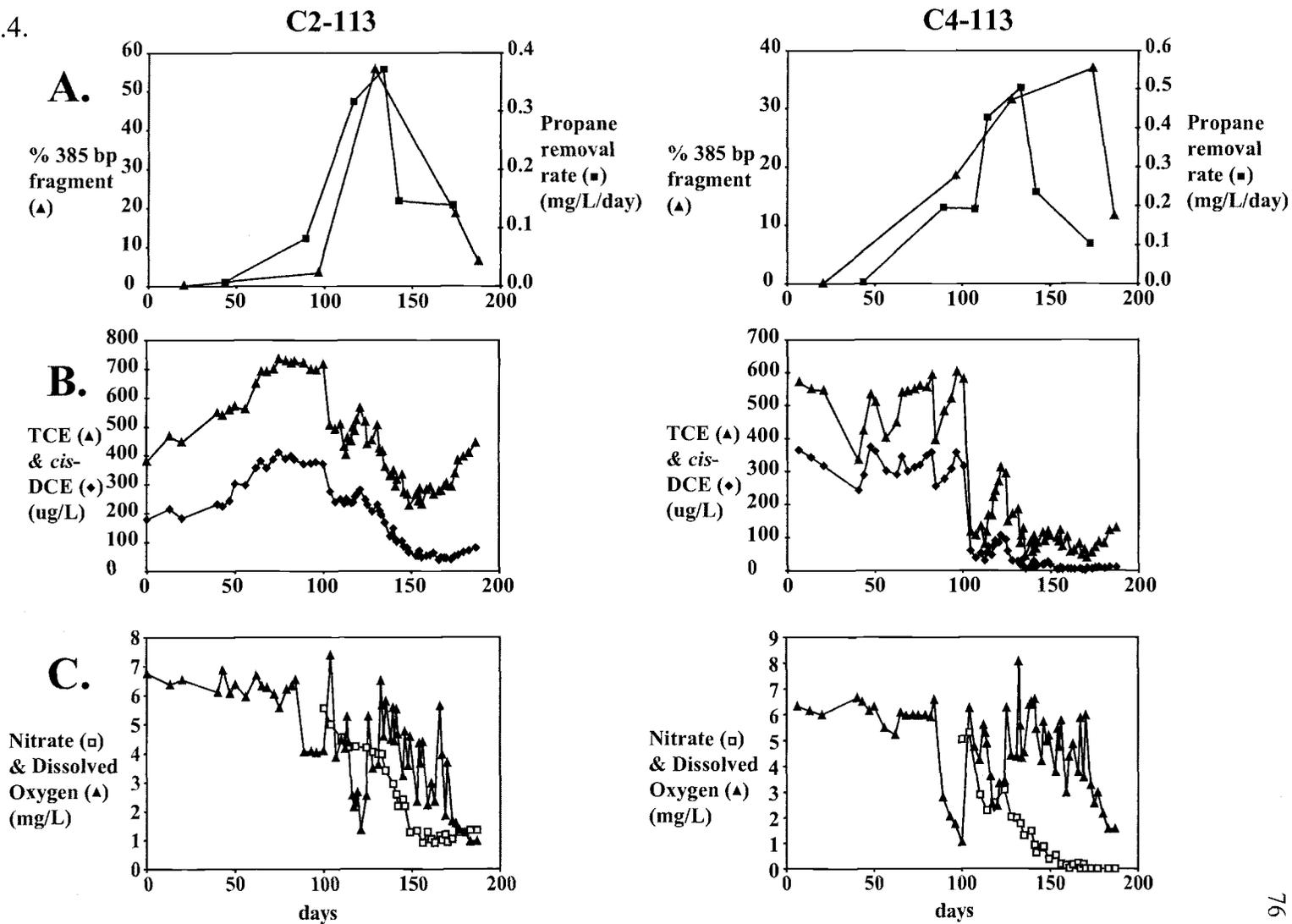
The proportion of the 385bp fragment in the LH-PCR product increased as the propane removal rate increased and diminished when propane removal slowed (Fig. 3.4.A.). TCE, *cis*-DCE and nitrate levels were all inversely correlated with the increase of the 385bp LH-PCR fragment and the propane removal rate. TCE, *cis*-DCE and nitrate levels begin to drop as the propane removal rate and the percentage of the 385bp fragment increased (Fig. 3.4.B. and C.). However, as nitrate levels continued to drop the proportion of the 385bp LH-PCR fragment and propane removal rate also decreased and the TCE concentration reach steady concentrates and then began to increase. Interestingly, while the TCE concentration rebounded due to fresh inputs of groundwater, the *cis*-DCE and nitrate levels showed no increase in well C4-113 and very little increase in C2-113 (Fig. 3.4.C.).

Dissolved oxygen (DO) levels varied between 5.2 and 6.8 mg/L until day 89, when the DO levels dropped dramatically nine days into the bi-weekly sparging events, which started with the second sparge event on day 80. This was an indicator that microbial respiration of oxygen had increased, which occurred as propane removal rates also increased. The DO levels then fluctuated between 1.0 and 8.1 mg/L throughout the rest of the study, but remained at higher levels, overall, in well C4-113 than in C2-113 (Fig. 3.4.C.).

A clone library consisting of 91 clones was made from well C4-113 (day 128) and screened for the 385 and 366bp LH-PCR fragments, since they were the

FIG. 3.4. Propane utilization rates, percentage 385bp LH-PCR fragment, and concentration of TCE, cis-DCE and nitrate for wells C2-113 and C4-113 over the course of the 187 day CAS demonstration.

FIG. 3.4.



only fragments that could be correlated to propane sparging. Three clones had an LH-PCR fragment size of 385bp, as determined by ABI GeneScan analysis, and had identical *Hae*III and *Mbo*I restriction digest patterns. One clone had a fragment size of 366bp as determined by ABI GeneScan analysis. Clones MAFB-C4-28 (385bp) and MAFB-C4-52 (366bp) were sequenced and phylogenetically analyzed (Fig. 3.5.). The sequences showed that the actual sequence lengths, between and including the two LH-PCR primers, were 384bp for MAFB-C4-28 and 365bp for MAFB-C4-52. Both clones represent TM7 candidate division of bacteria (Hugenholtz, et al. 1998; Rheims, et al. 1996) and are members of subdivision 3 of the TM7 bacterial division as defined by Hugenholtz, et al. (2001). MAFB-C4-28 (385bp) has a sequence similarity of 92.8% to its closest relative in GenBank, clone NoosaAW89 (AF269022), which was acquired from a sewage treatment plant. Clone MAFB-C4-52 (366bp) is phylogenetically most closely related to clone MAFB-C4-28 (385bp) and has a sequence similarity of 97.1%.

Sixty-eight of the 91 clones in the clone library were represented more than once in the library and were also sequenced and analyzed to determine their phylogenetic relationships to known sequences in GenBank (Table 3.3.). These 68 clones included 47 Betaproteobacteria, 10 Alphaproteobacteria, 4 Gammaproteobacteria, 4 OP11, and 3 TM7. Several clones are closely related to previously cultured microorganisms that are known to degrade toxic or recalcitrant compounds, which are shown in Table 3.3.

FIG. 3.5. Neighbor joining TM7 tree showing phylogenetic relationship of the 16S rRNA gene clones, MAFB-C4-28 and MAFB-C4-52, to TM7 subdivision 3. Scale bars indicates 0.10 change per nucleotide. Bootstrap values below 70 are not shown. MAFB-C4-52 was added to the tree using the parsimony insertion tool in ARB. TM6 division bacteria were used to root the tree; 795 characters were used to infer the tree.

FIG. 3.5.

TM7 Division

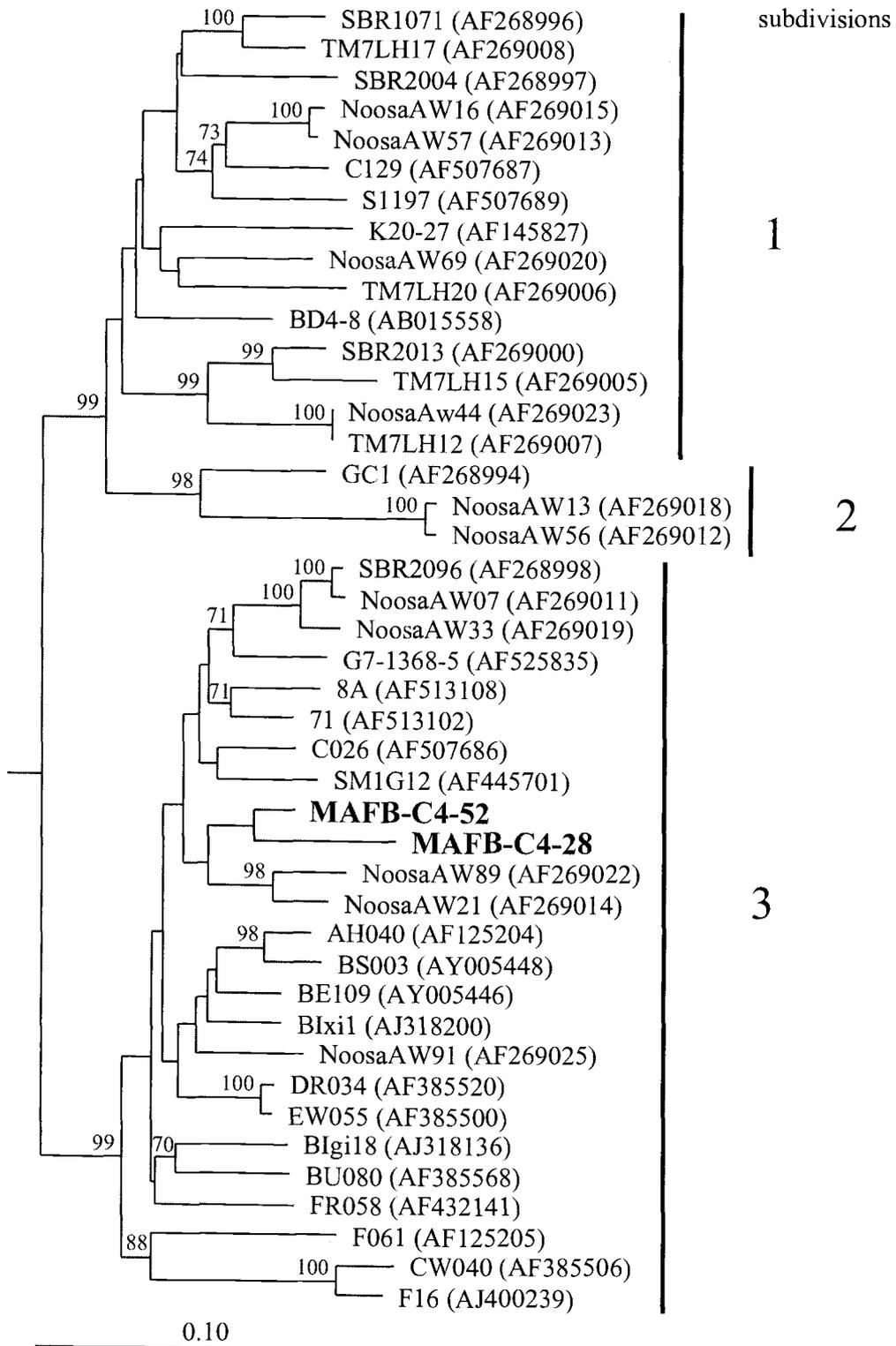


Table 3.3. Sequence based projection of LH-PCR fragment length and known carbon degradation abilities for isolates in the same phylogenetic clade as clone.

MAFB-C4 clones ^a	Sequence based determination of LH-PCR fragment ^b	Phylogenetic clade of clones	Toxic or generally bioresistant compounds degraded by isolates in each phylogenetic clade ^c
Betaproteobacteria			
3, 25, 70, 73, 83, 89, 24, 85, 67	342	Herbaspirillum/Oxalobacter	6-methylnicotinic acid chlorophenols
1, 30, 37, 55, 57, 64	342	Ramlibacter	--
9, 15, 27, 62, 72	342	PM1 (isolate)	methyl <i>tert</i> -butyl ether (MTBE)
10, 82, 94	342	HTA10 (clone)	--
38, 60, 92	342	JS666 / Polaromonas	<i>cis</i> -dichloroethene (<i>cis</i> -DCE)
2, 4, 17, 19, 33, 45, 50, 53, 56, 63, 65, 69, 71, 87, 95	340	MAFB-C4-17 (clone this study)	--
5, 35, 43, 79, 86, 96	340	Ideonella	--
Alphaproteobacteria			
13, 20, 21, 22, 36, 44, 48, 58	329	SM2C02 (clone)	--
14, 84	322	Holospora (endosymbiont)	--
Gammaproteobacteria			
29, 31	342	Acinetobacter	aliphatic polyesters chlorinated guaiacols
32, 34	348	Legionella	--
28, 61, 76	384	TM7 subdivision 3	--
OP11			
11, 12, 68, 80	365	subdivision 5	--

a. Numbers are clone names, which are otherwise appended by MAFB-C4-. Only clones represented more than once in the library are listed.

b. LH-PCR fragment length as determined by running on ABI equipment may differ by up to 2 base pairs from actual sequence length.

c. Carbon compounds, many known to be toxic or generally bioresistant, which can be utilized or degraded by at least one isolate in the clade.

-- Not determined.

Comparison of LH-PCR and Clone Library

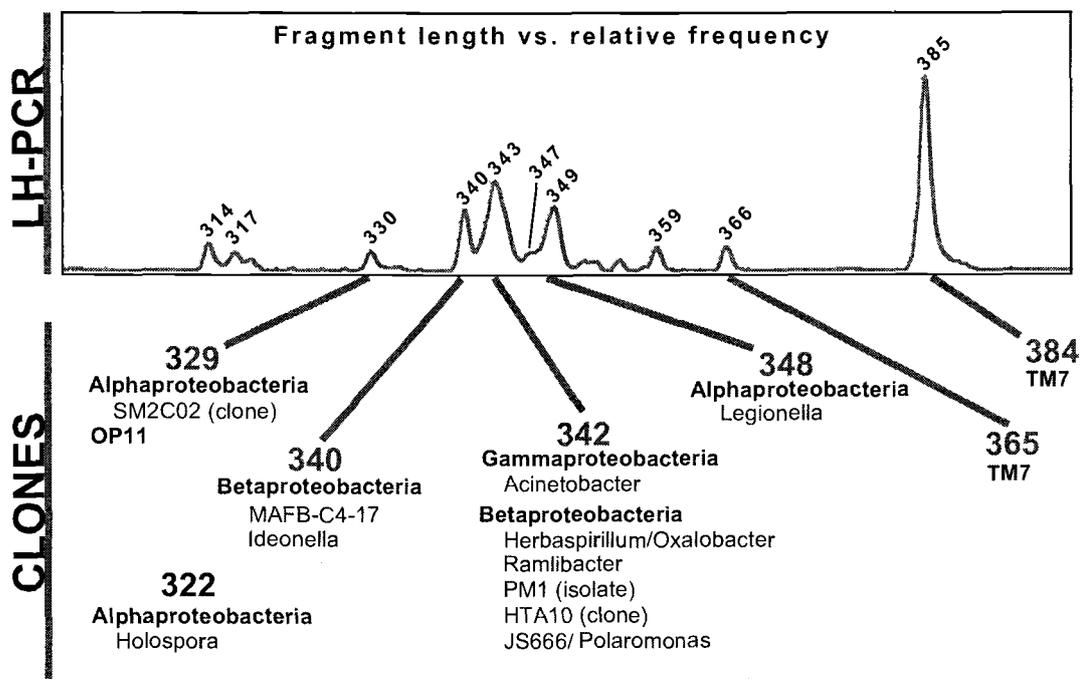


Fig. 3.6. Comparison of LH-PCR and clone library. The sequenced determined LH-PCR fragment sizes of the clones are correlated to the most likely LH-PCR fragment peak on the electropherogram. Only clones represented more than once in the library were analysed with the exception of the clone represented by the 366bp peak, MAFB-C4-52, which was only detected once in the library. The phylogenetic clades that these clones represent are listed under the sequenced determined fragment sizes.

The number of bases between and including the LH-PCR primers 8F and 338R for each sequenced clone was determined and compared to the LH-PCR fragments from the same sample (Fig. 3.6.). Six prominent fragments in the LH-PCR corresponded to clones with sequence determined fragment sizes within one base pair of an LH-PCR fragment. The 385bp LH-PCR fragment from this sample was 31.5% of the total LH-PCR fragments. Other dominant LH-PCR fragments represented in the clone library included 343bp (23.7%), 349bp (13.9%) and 340bp (8.4%), 366 (3.1%) and 330bp (2.7%).

DISCUSSION

All microbial community analyses in this study were performed on the groundwater or aqueous born fraction of the microbial community. Microorganisms that adhere tightly to the soil matrix may not be represented. Some sediment was released during pumping of the groundwater, and sparging itself could possibly release attached bacteria, so the attached portion of the microbial community cannot be strictly excluded in these analyses.

An increase in the size and numbers of microorganisms in the propane-sparged groundwater is an indication that this carbon source was being utilized. Biofouling due to nutrient amendments and subsequent increase in microbial biomass is a common problem in the field of groundwater bioremediation (Baveye, et al. 1998). While the microbial numbers dramatically increased after the addition

of propane, this increase wasn't enough to interfere with pumping of groundwater for sample collection.

Community structure before and after treatment

The addition of propane in the groundwater at MAFB caused a strong bacterial community shift in species composition to a TM7 division bacterium with an LH-PCR fragment of 385bp, which was not detected in the A-zone that received only air. The only other fragment size that could be exclusively correlated with propane-sparging was the 366bp fragment, which also corresponded to a TM7 division bacterium that was closely related to the TM7 division bacterium with the 385bp fragment. It is possible that other members of the bacterial community in the propane-sparged groundwater increased in abundance with propane sparging, but it cannot be determined from these data, since all other fragment sizes were also amplified from pre-sparged C-zone or in A-zone groundwater.

The proportion of the 385bp LH-PCR fragment from groundwater sampled from well C4-113 on day 128 was 31.5% of the total fragments, but only 3.3% of the clones (3 out of 91) had this LH-PCR fragment size. PCR bias of the LH-PCR analysis was minimized by restricting the final concentration of the amplified product, since this effectively reduces the bias in the template proportions and was specifically tested on the LH-PCR primer pair used, 8F and 338R (Suzuki and Giovannoni 1996; Suzuki, et al. 1998). The clone library was made with the same forward primer, but the reverse primer, 1522R, was used instead of 338R. No TM7 sequences could be found in GenBank that included the 1522R primer site,

indicating that this primer is not likely a good match for the TM7 bacterial division, which could explain its low yield in the clone library.

The TM7 division of bacteria currently has no cultured representatives and was named after clone TM7 found in a peat bog (Rheims, et al. 1996). TM7 division bacterial 16S rRNA genes are found in many diverse environments including activated sludge (Bond, et al. 1995), arid soils (Dunbar, et al. 2002; Dunbar, et al. 1999), Amazonian soils (Borneman and Triplett 1997), and a subsurface aquifer (clones WJGRT-17 and WJGRT-8) (Cho and Kim 2000). Of particular interest to the present study is the fact that TM7 clones have been recovered from a TCE contaminated site (clone d152) (Lowe, et al. 2002). They are also found in mouse feces (clone F16) and the oral cavity (clone AH040) (unpublished GenBank sequences). Hugenholtz, et al. (2001) presented an in-depth investigation of the TM7 bacterial division. They also reported fluorescence *in situ* hybridization data that indicate TM7 organisms from activated sludge had various morphologies that included cocci, small rods and filamentous rods and the filamentous rods were determined to have Gram-positive cell walls.

The LH-PCR fragment, which migrated at 343bp, was second in abundance to the 385bp fragment. It included members of at least six phylogenetic groups as determined by the clone library. The LH-PCR fragment size determined by the migration can differ by as much as 2 base pairs to the known length determined by the sequence itself (Anne Bernhard, personal communication). This can explain the discrepancy in the size of the fragments from the clone library sequences and

the size of the LH-PCR fragments. Most of the LH-PCR fragments were 1bp longer than the most probable corresponding sequences determined from the clone library.

Bacteria detected in the study site by molecular analysis

Of the 91 clones recovered in the clone library, 68 were represented more than once based on T-RFLP analysis and were further investigated. The 16S rDNA clones discussed below are members of phylogenetic clades that include isolates with known physiologies.

Three of the 47 Betaproteobacterial clones were included in the JS666/Polaromonas clade and had a sequenced determined LH-PCR fragment size of 342bp. The sequenced clone, MAFB-C4-60, is 98.2% similar to the 16S rRNA gene from isolate JS666, which is known to use *cis*-DCE as a sole source of carbon and energy (Coleman, et al. 2002). After sparging ended, TCE, *cis*-DCE, nitrate and DO levels continued to be measured (Tovanabootr, et al. 2001). TCE levels increased to pre-propane-sparged levels, but *cis*-DCE levels continued to remain low. It is possible that clone MAFB-C4-60 represents an organism that can aerobically utilize *cis*-DCE as a carbon and energy source, thus explaining these continued low *cis*-DCE levels in the absence of a co-oxidative substrate.

Nine Betaproteobacterial clones belonged to the *Herbaspirillum*/*Oxalobacter* clade and had a sequenced determined LH-PCR fragment size of 342bp. *Herbaspirillum* sp. have a respiratory metabolism and fix nitrogen (Elbeltagy, et al. 2001; Kirchhof, et al. 2001). Nitrogen fixation has also been

found to be important to TCE co-oxidation. TCE removal by a nitrogen-fixing methane oxidizing mixed community was found to consistently outperform a non nitrogen-fixing methane oxidizing community that was supplied nitrogen (Chu and Alvarez-Cohen 2000). Members of this monophyletic group also include microorganisms with the ability to utilize carbon compounds that are considered toxic to humans. Isolate K1 can degrade chlorophenols (Mannisto, et al. 2001). Isolates Mena 23/3-3c (Tinschert, et al. 1997) and MCI3289 (Ueda and Sashida 1998) utilize 6-methylnicotinic acid, a nicotine derived pesticide, as a sole carbon source. The two sequenced clones, MARF-C4-24 and MAFB-C4-83, both have a 342bp LH-PCR fragment size based on sequence analysis.

Six Betaproteobacterial clones were closely related to *Ideonella dechloratans*. The sequenced clone, MAFB-C4-43, has a 98.5% 16S rRNA gene sequence similarity to *I. dechloratans* and a 340bp LH-PCR fragment size based on sequence analysis. *I. dechloratans* has a strictly respiratory metabolism and can grow aerobically or anaerobically with chlorate or nitrate as an electron acceptor.

Three Betaproteobacterial clones were in the PM1 clade named for the only previously cultured bacteria in this clade. The sequenced clone, MAFB-C4-9, has a sequenced determined LH-PCR fragment size of 342bp and has a sequence similarity of 95.8% to isolate PM1, which aerobically uses methyl *tert*-butyl ether (MTBE), a gasoline additive, as a sole carbon source (Bruns, et al. 2001).

Two Gammaproteobacterial clones were in the Acinetobacter clade. The sequenced clone, MAFB-C4-29, had a sequenced determined LH-PCR fragment size of 342bp and was 99.1% similar to *A. junii*, which was found to degrade aliphatic polyesters (Suyama, et al. 1998) and several chlorinated guaiacols (Gonzalez, et al. 1993).

Two Gammaproteobacterial clones represented members of the Legionella clade, a common groundwater bacteria (Riffard, et al. 2001). The sequenced clone, MAFB-C4-32, had sequence determined LH-PCR fragment sizes of 348bp.

Two Alphaproteobacterial clones represented members of the clade that includes *Holospira*, which is an intracellular endosymbiont of *Paramecium caudatum*. However, the 16S rRNA sequence similarity of MAFB-C4-84 to *Holospira obtusa* was only 88.5%.

The decrease in both the propane removal rates and the proportion of the 385bp fragment were correlated with a decrease in nitrate levels (Fig3.4.C). Nitrogen limitations may have been responsible for this decrease in the rate of propane removal and subsequent decrease in TCE transformation. The groundwater flow at the site continually brought nitrate, TCE and *cis*-DCE, which complicates the analysis of the nitrate, TCE and *cis*-DCE concentration trends. As nitrate concentrations decreased below 2.0 mg/L in groundwater from wells C2-113 on day 146 and C4-113 on day 131, TCE concentrations began to increase. Another consideration is the fact that propane sparging was discontinued from day 160 to 188, which was the end of this portion of the study. However, the increase

in TCE was clearly occurring before day 160. It is possible that this increase in TCE was the result of nitrate limitation by the microorganisms responsible for TCE remediation.

The *cis*-DCE levels and nitrate levels did not rebound in the same manner as the TCE after propane sparging was discontinued. C2-113 groundwater showed a slight increase in *cis*-DCE levels but no increase was detected in C4-113 groundwater. While C2-113 groundwater had measurable nitrate levels throughout the study with a low of 0.91 mg/L, the nitrate in C4-113 was undetectable even as TCE levels were increasing. It is interesting to speculate if microorganisms represented by the 16S rRNA gene clone, MAFB-C4-60, may have played a role in keeping the *cis*-DCE levels reduced. This clone represents a microbe that is closely related to the isolate JS666, which can use *cis*-DCE as a sole carbon and energy source in the presence of oxygen.

CONCLUSION

The strong correlation of the 385bp fragment with propane removal rates suggests that a TM7 division bacterium may be utilizing propane or a metabolic by-product of propane oxidation by another microorganism. The sharp drop in TCE concentrations in response to stimulation of the microbial community by propane-sparging also suggests that the microorganism(s) responsible for propane utilization are likely co-oxidizing TCE. It is possible that correlations to propane utilization by other microorganisms went undetected due to the nature of the LH-PCR

analysis. The primers used for the LH-PCR do not amplify archaeal 16S rRNA genes and multiple microorganisms can be represented by one fragment size, potentially obscuring correlation patterns.

The clone, MAFB-C4-60, represents a bacteria that is a candidate for the metabolism of *cis*-DCE at the site. At the end of this CAS study, when propane sparging was ceased and oxygen levels remained high, the TCE concentration rebounded while *cis*-DCE levels remained low. The sequenced clone, MAFB-C4-60, has 98.2% 16S rRNA sequence identity with isolate JS666, which is known to use *cis*-DCE as a sole carbon source in the presence of oxygen.

An available nitrogen source is important for CAS to be effective. Nitrate concentrations dramatically decreased after propane removal rates peaked and the subsequent decrease in propane utilization rates most likely resulted from low nitrate levels. It is not surprising that one of the dominant clone groups at this time represented bacteria that are related to the *Herbaspirillum* genus, which are known nitrogen fixers.

While some of the clones corresponded to isolates with known physiologies, many of the clones, including those represented by the TM7 group, have no cultured representatives. Isolates of the dominant species of bacteria detected during propane utilization and subsequent bioremediation are needed to address unanswered questions that resulted from this study. For example, was the TM7 microorganism responsible for TCE and *cis*-DCE cometabolism, and are microorganisms present that can use *cis*-DCE as a carbon and energy source.

Extrapolation of the physiological function of bacteria, whose clones are closely related to cultured isolates, is not optimal. It is not possible to know conclusively the physiological role of these bacteria, without isolating them from the site, for further study.

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CHAPTER 4:**HIGH THROUGHPUT CULTURES FROM PROPANE SPARGED VERSUS
AIR SPARGED GROUNDWATER AT MCCLELLAN AIR FORCE BASE,
SACRAMENTO, CA**

Stephanie A. Connon, Lewis Semprini and Stephen J. Giovannoni

**HIGH THROUGHPUT CULTURES FROM PROPANE SPARGED VERSUS
AIR SPARGED GROUNDWATER AT MCCLELLAN AIR FORCE BASE,
SACRAMENTO, CA**

ABSTRACT

Uncultured species of microorganisms have been shown to be important agents of bioremediation in contaminated ecosystems. High throughput culturing (HTC) techniques were used to isolate microorganisms from trichloroethene (TCE) and *cis*-dichloroethene (*cis*-DCE) contaminated groundwater collected at McClellan Air Force Base (MAFB), Sacramento California, during an aerobic bioremediation cometabolic air sparging (CAS) study. 73.3% of the HTC isolates were members of previously uncultured or undescribed species, while only 17.4% of isolates collected on traditional minimal agar medium fall into this category. Eight novel groups of bacteria were cultured using the HTC method, and 2 novel groups were cultured using traditional minimal agar medium for a total of 9 novel groups of isolates. Four of the previously uncultured HTC isolates were Proteobacteria, including the MHP14 clade (alpha subclass), the 4-Org1-14 clade (alpha subclass), the HTCC333 (beta subclass) and HTCC410 (beta subclass). One novel group of isolates fell within the Betaproteobacterial *Herbaspirillum/Oxalobacter* clade. The HTC method also produced isolates from 3 previously cultured, yet currently undescribed Proteobacterial groups, including the PM1 clade (beta subclass), Boom-7m-04 clade (beta subclass) and OM43 clade (beta subclass). Traditional minimal agar medium produced two previously

uncultured or undescribed isolates. One agar isolate is an uncultured member of the Flexibacteraceae clade. The other agar isolate is very closely related to the undescribed isolate Boom-7m-04.

INTRODUCTION

Uncultured microorganisms have been detected in many environments by 16S rRNA based molecular approaches. These studies support the conclusion that only a fraction of microbial diversity is currently represented in culture collections. Out of over 40 known prokaryotic phyla, only about half have cultured representatives. Culturing microorganisms is a crucial step that allows the study of their physiology. Since existing culture collections incompletely represent microbial diversity, relying on them for the study of microbial processes such as bioremediation and waste cleanup could result in an incomplete or misleading picture of microbial activity. In activated sludge, for example, it has been widely thought that Gammaproteobacteria, such as *Acinetobacter*, dominate the microbial community, since they are often the dominant cultures acquired. However, Wagner and colleagues determined that *Acinetobacter* represented less than 10% of the microbial community in activated sludge, as determined by fluorescence *in situ* hybridization, yet were 32-33% of the cultures collected on nutrient rich agar medium (Wagner, et al. 1994).

No culture isolation studies have previously been done at sites where propane was used as a cometabolic substrate for *in situ* TCE bioremediation.

However, two culture isolation studies have been carried out on groundwater undergoing cooxidation of TCE by phenol, toluene and methane. In one of those studies isolates were acquired during a field study at Moffet Federal Airfield, California, which assessed a microbial community of phenol and toluene degraders in groundwater where TCE cooxidation was occurring (Fries, et al. 1997a; Fries, et al. 1997b). That study employed amplified rDNA restriction analysis and found that three Betaproteobacterial groups (*Comamonas-Variovorax*, *Azoarcus* and *Burkholderia*) and three Gram positive groups (*Bacillus*, *Nocardia* and an unidentified group) were dominant at the site. When restriction patterns of the recovered isolates were compared to the six genera identified in the groundwater samples, it was found that members of five of these six groups had been cultured. At the U.S. Department of Energy Savannah River Site, South Carolina, an isolation study was carried out to determine the feasibility of using methane as a cooxidative substrate for TCE bioremediation. Methanotrophic cultures from the genera *Methylosinus* and *Methylocystis* were isolated after methane enrichment of groundwater collected from the site (Bowman, et al. 1993). Most of these isolates exhibited soluble methane monooxygenase activity and degraded TCE, indicating that stimulation of the indigenous subsurface microbial community by methane and oxygen additions could be effective for bioremediation.

TCE and *cis*-DCE are widespread groundwater contaminants, especially at air force bases around the country where TCE was used as an airplane engine degreaser. The contaminated groundwater site at McClellan Air Force Base

(MAFB) has TCE and *cis*-DCE levels as high as 2.68 mg/L and 2.27 mg/L, respectively. A groundwater zone (C-zone) selected for CAS treatment was sparged with 2% propane and air, and a control zone (A-zone) was sparged only with air. CAS with propane was shown to promote the bioremediation of TCE and *cis*-DCE (Tovanabootr, et al. 2000; Tovanabootr, et al. 2001; Chapter 3, this dissertation).

An ongoing CAS study at MAFB afforded a unique opportunity to test the HTC methods previously developed in marine systems (Connon and Giovannoni 2002) on a groundwater microbial community that was utilizing propane and bioremediating TCE and *cis*-DCE. The major objectives of this study were 1) to measure the percentage of groundwater microorganisms that are culturable using HTC techniques; 2) to determine which species could be cultured by HTC methods and how they compared to the species that could be cultured on more traditional agar plating medium; and 3) to determine if the cultures acquired were dominant members of the microbial community as determined by 16S rRNA molecular methods.

MATERIALS AND METHODS

Sampling site

Two demonstration sites were located approximately 100 feet apart at MAFB. Groundwater in the A-zone was sparged only with air, while the C-zone was sparged with 2% propane in air. Sparging was initiated on day 36 of the CAS

demonstration, and both zones were sparged intermittently for 21 weeks (Tovanabootr, et al. 2000; Tovanabootr, et al. 2001).

Media preparation

Groundwater was collected for HTC culturing medium on May 23, 1999, from monitoring well 244, located near the test site on MAFB. Chlorinated solvents present in the groundwater were removed by sparging with air for several hours. It was then filtered through a 0.2 μm Supor membrane, autoclaved, and sparged with CO_2 , followed by air sparging as described by Connon and Giovannoni (2002) and in Chapter 3 of this dissertation. The groundwater medium was not amended with any nutrients.

Traditional mineral medium agar plates were used in an attempt to culture propane oxidizers. This medium, previously described by Wiegant and deBont (1980), consisted of 0.5mM MgSO_4 , 0.1 mM CaCl_2 , 23.5 mM NaNO_3 , 757 μM $(\text{NH}_4)_2\text{SO}_4$, and was phosphate buffered to a pH of 7.5 with K_2HPO_4 and NaH_2PO_4 for a final PO_4 concentration of 631 mM. The following trace elements were added: 22.6 μM FeSO_4 , 1.52 μM MnCl_2 , 0.514 μM ZnSO_4 , 1.0 μM H_3BO_3 , 0.45 μM Na_2MoO_4 , 0.1 μM NiCl_2 , 0.1 μM CuCl_2 , and 0.1 μM CoCl_2 . Fifteen grams of agar were added per liter of medium to make agar plates.

Culture collection and identification using HTC methods

Groundwater was sampled from wells C2-113, C4-113, A1-113 and A2-113 on September 17, 1999, 92 days after propane and air sparging began. This corresponds to sampling day 128 as outlined by Tovanabootr, et al. (2000). An

HTC approach was used to culture microorganisms from the groundwater as outlined in Cannon and Giovannoni (2002). Inoculum water was collected in 1 L polycarbonate bottles that had been washed with 10% HCl followed by a NANOpure (Barnstead, Dubuque, Iowa) water rinse. They were stored in a cooler with blue ice packs for transport to Corvallis, Oregon. Direct cell counts of the inocula were performed immediately before diluting into the prepared medium by staining with 4',6-diamidino-2-phenylindole (DAPI) as described by Turley (1993), except that 1% formaldehyde was used. At least 300 cells were counted per filter and triplicate filters were counted for each sample. Cells were diluted to either 3 or 10 cells per ml and 1 ml of the dilution was added to the wells of 48-well plates within 12 hours of collection. Two 48-well plates were made with 10 cells per well for each of the A-zone and C-zone wells. Two 48-well plates were made with 3 cells per well for each of the C-zone wells. Two control 48-well plates were also made with only medium added. One set of 48-well plates was incubated in an acrylic desiccant cabinet (Nalge Nunc International, Rochester, NY) that contained 2% propane and several trays of water added for humidity. The propane was added through a syringe port. This propane chamber was opened and recharged with propane every week. Propane levels in the chamber never fell below 0.75%, except briefly each week when the propane/air mixture was renewed. Propane was measured by methods outlined by Tovanabootr and Semprini (1998). The other set of 48-well plates was wrapped in PARAFILM and incubated in ambient air. All plates were incubated at 16°C in the dark for 7 weeks. Growth in the wells was

detected and cultures were identified by methods outlined in Cannon and Giovannoni (2002). Unique isolates were identified by RFLP analysis using *HaeIII* and *MboI* or *HaeIII* and *Bsh1236I* (MBI Fermentas, Hanover, MD). Only unique isolates were subsequently studied further by rRNA gene sequencing.

Traditional collection, storage and identification of propane oxidizers

Mineral medium agar plates were also used to culture propane oxidizers from groundwater collected from wells C2-113 and C4-113 on day 128. The same groundwater inocula described above were spread onto the agar plates. The plates were inoculated in duplicate with 100, 10 and 1 μl of inocula; 1 μl was diluted into 9 μl of sterile groundwater before spreading. Plates were incubated in the propane chamber, and colonies were counted after 7 weeks of growth. Colonies were streaked for isolation at least 3 times before the colonies on the plate were rinsed with 1 ml sterile mineral medium broth to collect cells for sequence identification and cryopreservation. Twenty-three colonies were randomly chosen (10 from C2-113 and 13 from C4-113) for subsequent isolation and identification. The names are indicative of the colony color: a "W" prefix indicates isolates that formed white or clear colonies, "P" indicates coral pink colonies and "Y" indicates yellow colonies.

A Qiagen DNeasy kit was used to extract the genomic DNA from 200 μl of the cell suspensions collected from the agar plates. The primers 8F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1522R (5'-AAG GAG GTG ATC CAN CCR CA-3') were used to amplify the 16S rRNA gene from each isolate. The PCR

cocktail contained 0.025 U Taq/ μ l (Promega, Madison, WI or MBI Fermentas, Hanover, MD), 5% acetamide, 1.5 mM Mg²⁺, 200 nM of each primer, 220 μ M dNTP and 1X PCR buffer (Promega, Madison, WI or MBI Fermentas, Hanover, MD). Thirty-five cycles were run with denaturation for 30 s at 94°C, annealing for 1 min at 55°C and extension for 2 min at 72°C. The isolates were screened with the restriction enzymes *Hae*III and *Mbo*I and an isolate representing each unique restriction digestion pattern was sequenced on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA).

Glycerol at a final concentration of 5% was added to the remaining cell suspension and 200 μ l aliquots were frozen to -80 °C in 1.5 ml cryovials (Nalge Nunc International, Rochester, NY) using Mr. Frosty (Nalge Nunc Int.) which controls the rate of freezing to -1 °C per min. The cells were then transferred to liquid nitrogen storage dewars.

Clone library

A clone library was made from a sample collected on September 17, 1999, from well C4-113 and is described in Chapter 3. The library consisted of 91 clones and was screened with *Hae*III and *Mbo*I enzyme digestions. All clones represented more than once in the library by the restriction digests were sequenced with an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA).

Phylogenetic analysis

Sequences were aligned and masked in ARB (Strunk, et al. 1996). Phylogenetic analyses were performed using ARB and PAUP* (Swofford 2001).

Phylogenetic trees were inferred by neighbor-joining using the Jukes and Cantor model to estimate evolutionary distances. Bootstrap values were obtained in PAUP* from a consensus of 1000 neighbor-joining trees. Short sequences were added to the tree using the parsimony insertion tool in ARB. The percent similarity of sequences was determined using the distance matrix tool in ARB; ambiguous bases were not included. The Betaproteobacterial and Alphaproteobacterial trees were made using 189 and 226 sequences, respectively, and most of the described genera from these two classes of Proteobacteria were included in the tree. Genera without a representative 16S rRNA gene sequence in the GenBank database and genera represented by poor sequence data were not included. The type species for each genus was used whenever possible. The sequences of the HTCC and agar isolates will be deposited in GenBank.

RESULTS

Growth was detected in 112 out of 576 HTC extinction culture attempts on A- and C-zone groundwater. The groundwater in the C-zone was showing active bioremediation of TCE and *cis*-DCE when the inocula were collected for this study (Chapter 3). Culturability ranged from 0.6 to 5.0% in these zones when the HTC method was employed (Table 4.1.). No significant difference in culturability was detected for the ambient air vs. propane growth chamber treatments, either with the A- or C-zone groundwater. Bacteria were also isolated from the C-zone using traditional mineral medium agar plates in the presence of 2% propane and two

TABLE 4.1. HTC culturability statistics compared to traditional counts

Inoculation sample ^a	Ave. cells per well	Total wells inoculated	Positive wells ^b	Culture designations	Percent culturability ^c	% culturability on agar ^d
Propane atmosphere growth chamber						
C2-113	3.0	48	3	HTCC 301-303	2.2 (1.0, 3.4)	1.5
	10	48	11	HTCC 304-314	2.6 (1.8, 3.4)	
C4-113	3.0	48	3	HTCC 315-317	2.2 (1.0, 3.4)	11.1
	10	48	12	HTCC 318-329	2.9 (2.1, 3.7)	
A1-113	10	48	3	HTCC 330-332	0.6 (0.2, 1.0)	--
A2-113	10	48	19	HTCC 333-351	5.0 (3.8, 6.2)	--
Ambient air atmosphere						
C2-113	3.0	48	3	HTCC 352-354	2.2 (1.0, 3.4)	--
	10	48	14	HTCC 355-368	3.4 (2.5, 4.3)	
C4-113	3.0	48	5	HTCC 369-373	3.7 (2.1, 5.3)	--
	10	48	18	HTCC 374-391	4.7 (3.6, 5.8)	
A1-113	10	48	5	HTCC 392-396	1.1 (0.6, 1.6)	--
A2-113	10	48	16	HTCC 397-412	4.1 (3.1, 5.1)	--

a. Groundwater samples were collected from the well indicated on day 128 of the cometabolic sparging demonstration at MAFB, which was 92 days after propane and air sparging began.

b. Wells were scored for growth after 7 weeks of incubation at 16° C.

c. Ninety-five percent confidence interval shown in parenthesis.

d. Inocula was the same as that used for the microtiter plates; (--) indicates not determined.

other potential carbon sources, carbon dioxide and agarose. The culturability was 1.5% and 11.1% from groundwater collected from wells C2-113 and C4-113, respectively (Table 4.1).

All HTCC and minimal agar medium isolates were identified by phylogenetic analysis of the 16S rRNA gene and by comparing these sequences with sequences in GenBank to determine the closest matches (Table 4.2.). Novel isolates recovered by HTCC methods were determined to be members of the Betaproteobacteria and Alphaproteobacteria. Novel isolates recovered by traditional agar plating were determined to be members of the Betaproteobacteria and the Cyclobacterium clade within the Flexibacteraceae family. Phylogenetic analyses are shown for the novel isolates of Alphaproteobacteria and Betaproteobacteria (Fig 4.1 and 4.2.).

The HTC method was evaluated for its overall ability to culture previously uncultured isolates. Based on phylogenetic analysis and the “rule-of-thumb” that a bacterium can be considered a novel species if the 16S rRNA sequence has less than 97% similarity to any described species (Wayne, et al. 1987), eight groups of uncultured or undescribed bacteria were detected. Sixty-six of the 90 identified HTCC isolates (73.3%) were included in these eight groups. Four of these eight novel groups were previously uncultured Proteobacterial clades, the MHP14 clade (alpha subclass), the 4-Org1-14 clade (alpha subclass), the HTCC333 clade (beta subclass) and the HTCC410 clade (beta subclass). HTCC isolates that grouped within the *Herbaspirillum/Oxalobacter* clade were considered to be novel isolates

Table 4.2. Sequenced isolates and their 16S rRNA gene sequence similarities to nearest cultured neighbor or nearest clone

Isolate	No. of isolates ^a	No. of nucleotides ^b	Phylogenetic clade ^c	Closest match in GenBank	% sequence similarity
BetaProteobacteria					
HTCC333	21	1439	*HTCC333	<i>Ideonella</i> sp. B511 (AB049106)	96.5
HTCC410	1	1414	*HTCC410	<i>Aquabacterium parvum</i> (AF035052)	96.3
HTCC303	2	765	*PM1	MAFB-C4-9	100
HTCC304	1	1335		isolate PM1 (AF176594)	99.5
HTCC349	1	1392	*OM43	marine isolate HTCC165 (AY102013)	97.2
HTCC379	2	1411	*Boom-7m-04	Boom-7m-04 (Z73450)	99.2
W8	2	867		Boom-7m-04 (Z73450)	99.0
HTCC302	35	1424	*Herbaspirillum/Oxalobacter	MAFB-C4-83	98.9
HTCC315	1	1432		isolate COL (AF214642)	96.5
HTCC392	2	792	Rhodofera	<i>Rhodofera antarcticus</i> (AF084947)	98.4
HTCC347	4	788	Variovorax	<i>Variovorax paradoxus</i> (AB008000)	98.4
HTCC332	2	1436	Ramlibacter	MAFB-C4-1	97.2
HTCC356	1	667	Acidovorax	<i>Acidovorax</i> sp. smarlab 133815 (AY093698)	100
W4	1	874		<i>Acidovorax</i> sp. smarlab 133815 (AY093698)	99.2
HTCC329	2	640	Hydrogenophaga	<i>Hydrogenophaga taeniospiralis</i> (AF078768)	98.9
HTCC319	2	475	Ralstonia	<i>Ralstonia pickettii</i> (X67042)	97.1
HTCC376	1	1407	Limnobacter	<i>Limnobacter thiooxidans</i> (AJ289885)	99.2
Alphaproteobacteria					
HTCC309	1	806	*MHP14	clone 92 (AJ412676)	98.2
HTCC396	1	899	*4-Org1-14	clone 4-Org1-14 (AF143833)	94.3
HTCC353	1	421	Bradyrhizobiaceae	<i>Blastobacter denitrificans</i> (AF338176)	100
HTCC381	2	653		<i>Bradyrhizobium elkani</i> (U35000)	100
HTCC354	2	771	Afipia	<i>Afipia broomeae</i> (U87759)	99.4
HTCC407	1	900		<i>Afipia broomeae</i> (U87759)	98.7
W10	1	845		<i>Afipia broomeae</i> (U87759)	99.6
HTCC339	1	421	Methylobacterium	<i>Methylobacterium thiocyanatum</i> (U58018)	99.1
HTCC335	1	416	Novosphingobium	<i>Novosphingobium</i> isolate F0917 (AF235994)	99.3
HTCC399	1	896		<i>Novosphingobium subterraneae</i> (AB025014)	98.1
HTCC345	1				
Y8	2	926	Xanthobacter	<i>Xanthobacter flavus</i> (X94199)	99.8
W7	1	588	Agrobacterium/Rhizobium	<i>Agrobacterium albertimagni</i> (AF316615)	98.8
W5	1	939	Mesorhizobium	<i>Mesorhizobium loti</i> (U50165)	98.1
Actinobacteria phylum					
HTCC345	1	453	Arthrobacter	<i>Arthrobacter</i> sp. SMCC ZAT262 (AF197055)	99.3
Y1	6	902	Mycobacterium	<i>Mycobacterium senegalense</i> (AF480596) and <i>M. fortuitum</i> (AF480580)	98.0
P5	1	878		<i>Mycobacterium senegalense</i> (AF480596) and <i>M. fortuitum</i> (AF480580)	98.2
P2	2	927	Rhodococcus	<i>Rhodococcus</i> sp. RHA4 (U16317)	99.9
W1	3	926	Nocardioides	<i>Nocardioides</i> sp. C157 (AF253509)	97.7
W6	1	878		<i>Nocardioides</i> sp. V4-BE-17 (AJ244657)	97.7
Flexibacteraceae					
P3	2	627	*Cyclobacterium	clone SM1E06 (AF445684)	96.7

a. Number of isolates that have the same RFLP pattern as the sequenced isolate.

b. Ambiguous bases were removed from analysis.

c. Isolates from the clades that have a * preceding the name are less than 97% similar to any described species.

FIG. 4.1. Neighbor joining Betaproteobacteria tree showing phylogenetic relationships of the 16S rRNA genes of high throughput cultures compared to representative species and environmental clones. Scale bars indicates 0.10 change per nucleotide. Bootstrap values below 70 are not shown. Short sequences were added to the trees using the parsimony insertion tool in ARB and are indicated by an asterisk (*). Gammaproteobacteria were used to root the tree; 891 characters were used to infer the tree.

Fig 4.1.

Betaproteobacteria

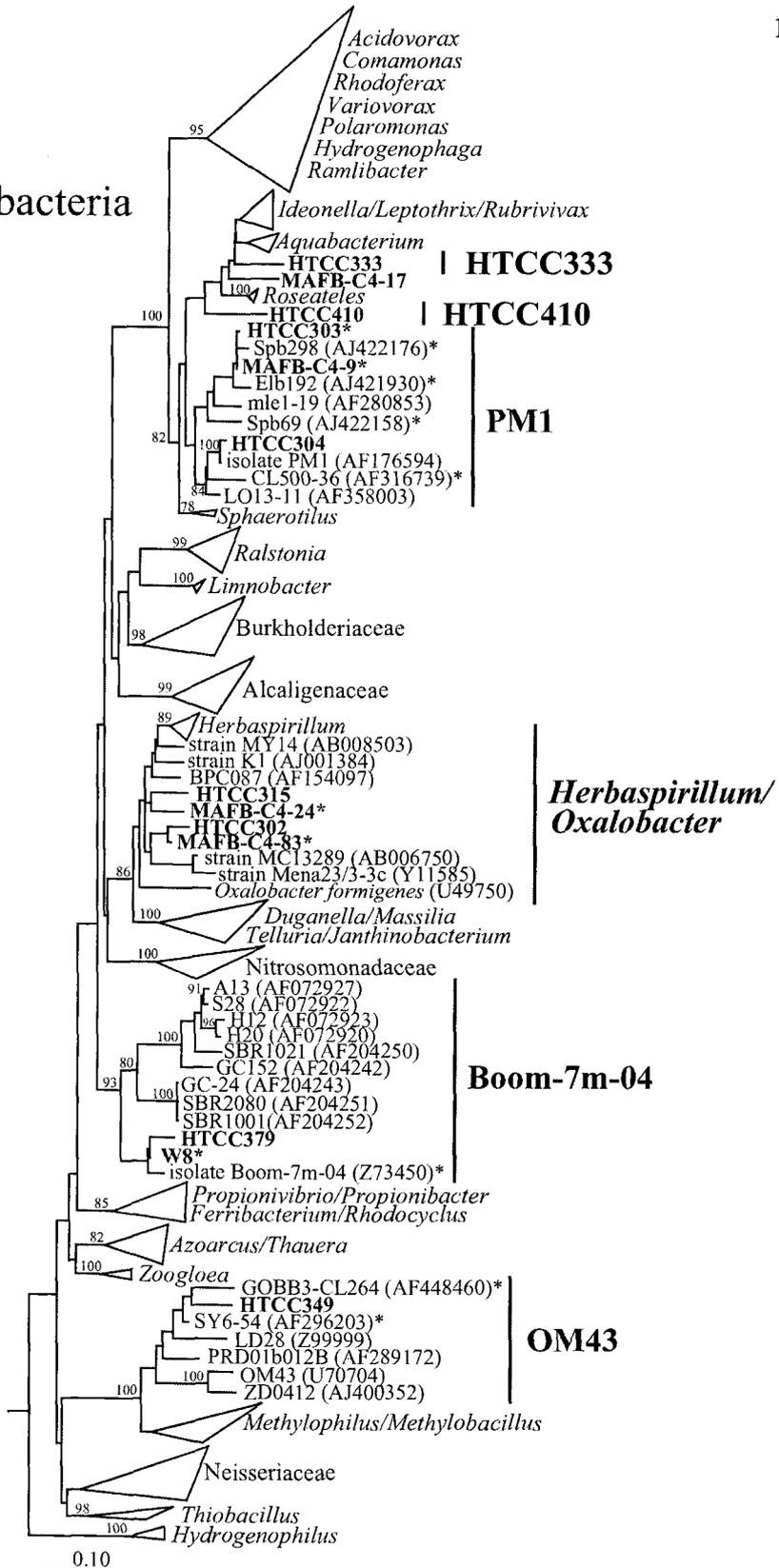
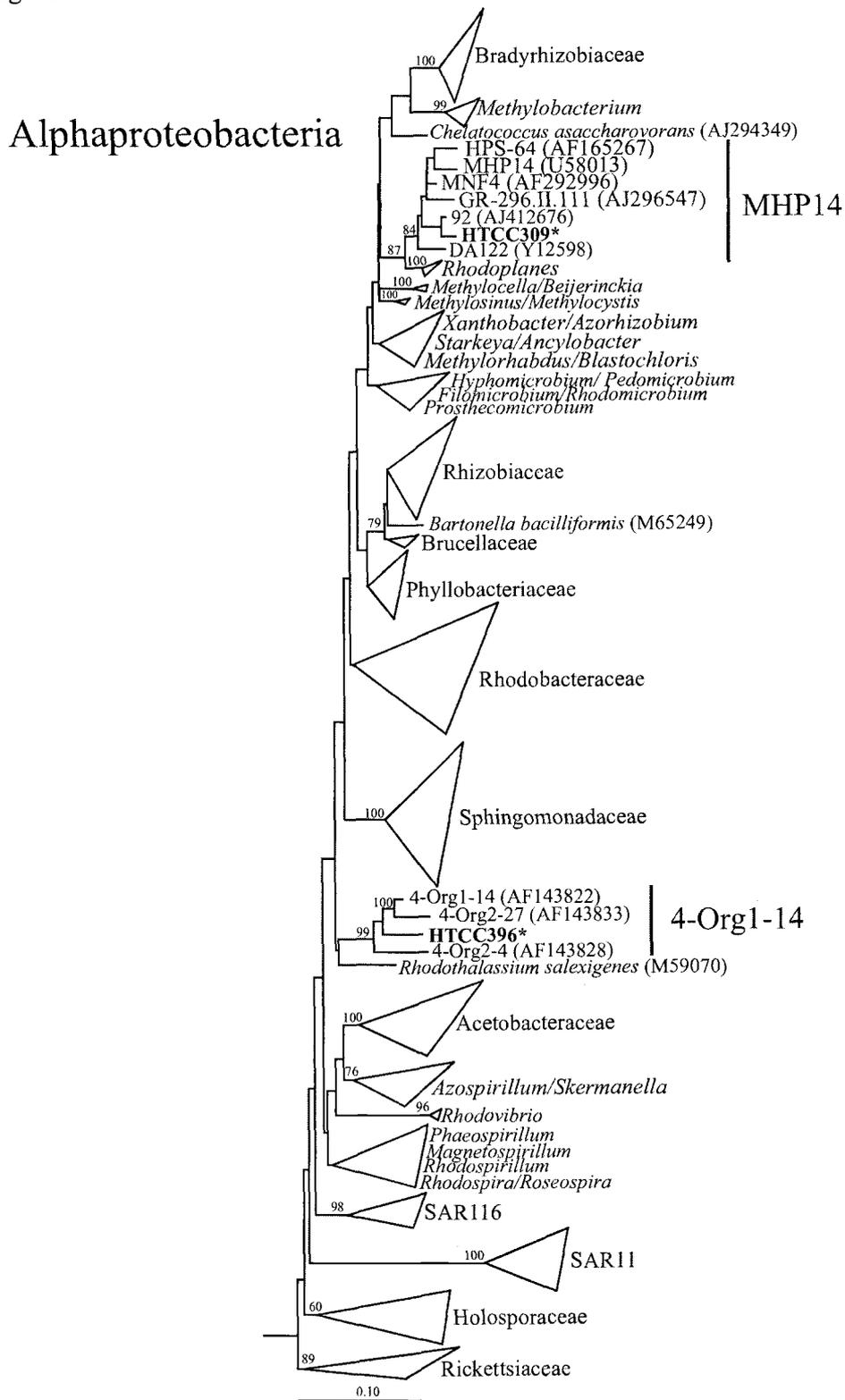


FIG. 4.2. Neighbor joining Alphaproteobacteria tree showing phylogenetic relationships of the 16S rRNA genes of high throughput cultures compared to representative species and environmental clones. Scale bars indicates 0.10 change per nucleotide. Bootstrap values below 70 are not shown. Short sequences were added to the trees using the parsimony insertion tool in ARB and are indicated by an asterisk (*). Betaproteobacteria and Gammaproteobacteria were used to root the tree; 683 characters were used to infer the tree.

Fig 4.2.



since their 16S rRNA gene sequences were less than 97% similar to any described species. The HTC method also produced isolates from 3 previously cultured, yet currently undescribed, *Proteobacteria*, including the PM1 clade (beta subclass), Boom-7m-04 clade (beta subclass) and OM43 clade (beta subclass).

Four of the 23 agar isolates (17.4%) were determined to be previously uncultured or undescribed microorganisms. Isolates P3 and P4 are uncultured isolates within the Flexibacteraceae family. Their closest cultured relative is *Cyclobacterium marinum* with an 84.5% 16S rDNA sequence similarity. The 16S rRNA gene sequence of isolates W8 and W9 are 99.2% similar to the undescribed Betaproteobacterial isolate Boom-7m-04.

Although the species cultured from the C-zone and A-zone were clearly different, whether the cultures were grown in a propane or air atmosphere didn't have a noticeable effect on the diversity of species cultured under the conditions used. The dominant cultures acquired from the C-zone, *Herbaspirillum/Oxalobacter* clade isolates (Table 4.3.), and the A-zone, HTCC333 clade isolates (Table 4.4.), were isolated at similar frequencies from both propane and ambient air conditions. The rest of the isolates recovered from the A-zone and C-zone were not recovered often enough for it to be clear whether or not they dominated in either the propane or ambient air growth conditions.

Although there was very little overlap in the cultures identified from the two zones, the dominant isolates from both zones were representatives of the class Betaproteobacteria. The only other group of microorganisms isolated from both

these zones by the HTC method was of the class Alphaproteobacteria. The *Afipia* clade, an Alphaproteobacterium, was the only phylogenetic group that was represented in both the C-zone (Table 4.3.) and A-zone (Table 4.4.) by HTCC isolates. The *Afipia* clade was also represented by the minimal agar medium isolate W10.

The C-zone HTCC cultures grew to higher cell densities than those detected in the A-zone. The mean and median for all combined C-zone collected cultures was 1.3×10^6 cells/ml and 1.1×10^6 cells/ml, respectively. The mean and median for all A-zone cultures was 7.1×10^5 cells/ml and 6.2×10^4 cells/ml, respectively. Much of this difference can be attributed to the difference in cell densities reached by the two different species that dominated the cultures acquired in the A-zone vs. the C-zone (Table 4.3. and 4.4.). The C-zone collected cultures, grown with both propane and air, were dominated by cultures from the *Herbaspirillum/Oxalobacter* clade, which grew to a high cell density with a mean and median of 1.9×10^6 and 1.3×10^6 cell/ml, respectively. The A-zone collected cultures, grown with both propane and air, were dominated by cultures from the HTCC333 clade, which grew to a lower cell density with a mean and median of 2.0×10^5 cells/ml and 3.5×10^4 cells/ml, respectively.

A comparison was made among the HTCC isolates, the minimal agar medium isolates, and clones that appeared more than once in the environmental rDNA library (Table 4.3.). Microorganisms represented more than once in the clone library were considered to be dominant members of the microbial

Table 4.3. Comparison of HTCC isolates, minimal medium isolates, and clones from the C-zone

Phylogenetic Clade	HTCC isolates ^{a, b}	Minimal medium isolates ^a	Clones represented more than once in the library ^a
Betaproteobacteria			
Ramlibacter	--	--	1, 30, 37, 55, 57, 64
HTA10 (clone)	--	--	10, 82, 94
JS666/Polaromonas	--	--	38, 60, 92
Acidovorax	<u>356</u>	W4	--
Hydrogenophaga	<u>329, 369</u>	--	--
Ideonella	--	--	5, 35, 43, 79, 86, 96
PM1 (isolate)	303, 304, 383	--	9, 15, 27, 62, 72,
MAFB-C4-17 (clone this study)	--	--	2, 4, 17, 19, 33, 45, 50, 53, 56, 63, 65, 69, 71, 87, 95
Herbaspirillum/ Oxalobacter	301, 302, 305, 306, 307, 311, 312, 313, 314, 315, 316, 317, 320, 321, 324, 325, 327, 328, <u>352, 359, 360, 363, 364, 365,</u> <u>367, 368, 371, 372, 375, 380,</u> <u>382, 384, 385, 386, 389, 390</u>	--	3, 24, 25, 67, 70, 73, 83, 85, 89
Ralstonia	319, <u>355</u>	--	--
Limnobacter	<u>376</u>	--	--
Boom-7m-04 (isolate)	<u>377, 379</u>	W8, W9	--
Alphaproteobacteria			
Bradyrhizobiaceae	<u>353, 381, 387</u>	--	--
Afipia	<u>354, 362</u>	W10	--
MHP14 (clone)	309	--	--
Xanthobacter	--	Y7, Y8	--
Agro/Rhizobium	--	W7	--
Mesorhizobium	--	W5	--
Holospora	--	--	14, 84
SM2C02 (clone)	--	--	13, 20, 21, 22, 36, 44, 48, 58
Gammaproteobacteria			
Acinetobacter	--	--	29, 31
Legionella	--	--	32, 34
TM7			
subdivision 3	--	--	28, 61, 76
OP11			
subdivision 5	--	--	11, 12, 68, 80
Actinobacteria phy.			
Mycobacterium	--	P5, Y1, Y2, Y3, Y4, Y5, Y6	--
Rhodococcus	--	P1, P2	--
Nocardioides	--	W1, W2, W3, W6	--
Flexibacteraceae			
Cyclobacterium	--	P3, P4	--
Mixed culture			
	308, 318, 322, 323, 326, <u>357,</u> <u>358, 361, 366, 370, 373, 374,</u> <u>378, 388, 391</u>		
Not determined			
	310		

a. The names that are bolded were recovered from well C2-113; unbolded names are from C4-113.

b. The cultures underlined were grown under ambient air conditions in Parafilm sealed plates; not underlined indicates they were grown in the propane chamber.

Table 4.4. A-zone HTCC isolates

Phylogenetic Clade	HTCC isolates ^a
Betaproteobacteria	
Rhodoferax	<u>392, 411</u>
Variovorax	330, 347, <u>394, 395</u>
Ramlibacter	332, <u>405</u>
HTCC333	333, 336, 337, 338, 340, 341, 342, 343, 344, 346, 348, 350, 351, <u>398, 400, 401, 403, 404,</u> <u>406, 408, 412</u>
HTCC410	<u>410</u>
OM43	349
Alphaproteobacteria	
Methylobacterium	339
Afipia	<u>407</u>
Novosphingobium	335, <u>399</u>
4-Org1-14 (clone)	<u>396</u>
Actinobacteria	
Arthrobacter	345
Mixed cultures	<u>331, 334, 393, 397, 402, 409</u>

a. The cultures underlined were grown under ambient air conditions in Parafilm sealed plates; not underlined indicates they were grown in the propane chamber.

community. The clones and the HTCC isolates were both dominated by representatives of the Betaproteobacteria. Two dominant clone groups, the PM1 clade (5 clones) and the *Herbaspirillum/Oxalobacter* clade (9 clones), were both cultured by HTC methods. None of the isolates collected on mineral agar medium, which were dominated by *Actinobacteria*, were from any of the 13 dominant clone groups (Table 4.3.). Thus, the HTC isolation method was determined to be superior for acquiring cultures from dominant bacterial groups.

A TM7 bacterium dominated the propane sparged groundwater, as determined by length heterogeneity PCR (LH-PCR) (Chapter 3, this dissertation), but was not detected in any of the cultures acquired at the site.

DISSCUSSION

Novel Betaproteobacteria isolates

The novel Betaproteobacterial isolates that were cultured are shown in the tree in Fig. 4.1. Betaproteobacteria dominated the HTCC isolates acquired from both the A-zone and C-zone groundwater, but the specific types of Betaproteobacteria isolated were different for the two sites. The A-zone isolates were dominated by the clade named for the isolate HTCC333. No sequences deposited in GenBank were phylogenetically related to this group based on bootstrap analysis. The C-zone was dominated by novel isolates that can be included in the *Herbaspirillum/Oxalobacter* clade.

Herbaspirillum/Oxalobacter clade

HTCC315 and HTCC302 along with clones MAFB-C4-24 and MAFB-C4-83 from Chapter 3 are members of the *Herbaspirillum/Oxalobacter* clade. Thirty-five of the 36 *Herbaspirillum/Oxalobacter* clade HTCC isolates are represented by HTCC302 based on RFLP analysis. The 16S rRNA gene sequences of these two HTCC isolates showed that they are 95.9% similar to each other. The sequence similarity for the 16S rRNA gene of HTCC302 is 98.9% similar to clone MAFB-C4-83, 96.1% to *Herbaspirillum* sp. BA161 (AF164063) which is a nitrogen fixer isolated from banana root (Cruz, et al. 2001), and 96.0% to *H. seropedicae* (Y10146) (Sievers, unpublished). HTCC315 is 96.5% similar to nitrogen-fixing bacterial strain COL (AF214642) (Reinhardt, et al. unpublished) and to hydrocarbon seep clone BPC087 (AF154097) (O'Neill, et al. unpublished) but is only 94.4% similar to clone MAFB-C4-24. These HTCC isolates probably represent two novel groups within the *Herbaspirillum/Oxalobacter* clade, since there is no bootstrap support for branching with any other isolate in this clade or to each other. These 36 HTCC isolates were determined to be dominant microorganisms in the groundwater by 16S rRNA gene clone library analysis.

HTCC333 and HTCC410 isolates within the family Comamonadaceae

HTCC333 and HTCC410, isolated from the A-zone, are each distinct subclades within the larger phylogenetic clade that includes *Ideonella*, *Aquabacterium*, *Leptothrix*, *Roseateles*, *Sphaerotilus*, and *Rubrivivax*. There is no bootstrap significance to their association with any of these genera, to each other,

or to the 15 clones that are represented by MAFB-C4-17, which also represents a separate subclade within this larger clade. Twenty-one isolates are represented by the HTCC333 sequence based on RFLP analysis. HTCC333 represents the dominant culture acquired in the A-zone by the HTC method. The 16S rRNA gene sequence of HTCC333 is most similar to *Ideonella* sp. B511 (AB049106) with a 96.5% sequence identity, but it is also 96% similar to *Aquabacterium* and *Sphaerotilus*. HTCC410 is most similar to *Aquabacterium parvum* (AF035052) with a 96.3% similarity. It is also 95.3% similar to *Rubrivivax gelatinosus* (D16213). These two HTCC isolates do not phylogenetically group with any genus in the family Comamonadaceae and closely related clones could not be found in GenBank. Both these isolates are potentially new genera in the Comamonadaceae family.

PM1 isolates within the family Comamonadaceae

Three isolates, HTCC303, HTCC383 and HTCC304, and 5 clones from the PM1 clade were acquired from the C-zone at MAFB after propane sparging. HTCC303 and HTCC383 had identical RFLP patterns. No isolates from this group were recovered on agar plates or in the A-zone. These isolates are one of the three groups of HTC isolates from the C-zone that were determined to be environmentally dominant by 16S rRNA gene clone library analysis.

The PM1 clade was named for the isolate PM1, which is the only previously recovered isolate in this clade. PM1 was collected from a methyl tert-butyl ether (MTBE) degrading bacterial enrichment and is able to use MTBE as a

sole carbon source (Bruns, et al. 2001). This clade also includes clones collected from bacterial enrichments of methane oxidizers (Morris, et al. 2002), polluted rivers (unpublished sequences deposited by Bruemmer et al.), a wastewater treatment bioreactor (LaPara, et al. 2000), and a pristine deep-water lake (Urbach, et al. 2001). The PM1 clade phylogenetically groups within the Comamonadaceae family of the Betaproteobacteria by 16S rRNA gene sequence analysis.

The 16S rRNA gene sequence of HTCC304 is very closely related to PM1 with a similarity of 99.5%. HTCC304 was also 97.4% similar to clone LO13-11 (AF358003), which was recovered from the ^{13}C labeled DNA fraction of a methanotroph population grown on $^{13}\text{CH}_4$ (Morris, et al. 2002). HTCC304 is only 95.8% similar to clone MAFB-C4-9.

The 16S rRNA gene sequence of HTCC303 is identical to the environmental clone MAFB-C4-9, from this study. HTCC303 is also 99.5% similar to clone Spb298 (AJ422176) and 98.6% similar to clone Elb192 (AJ421930), which are both from a study of polluted rivers in Germany (Bruemmer, et al. unpublished).

OM43 clade

HTCC349, isolated from the A-zone, is a member of the OM43 clade. The 16S rRNA gene sequence of this isolate is 97.2% similar to clone GOBB3-CL233 (AF448465) collected from a marine microbial community by Kisand and Wikner (unpublished), and 96.8% similar to clone SY6-54 (AF296203) collected from a 100M depth in Lake Soyang, South Korea (Han and Ahn, unpublished).

Methylobacillus flagellatum is the most closely related described species, which is 94.1% similar to HTCC349. Several undescribed strains of OM43 from marine environments have been acquired, including strain POCPN-5 (AB022337) and KI89C (AB022713), respectively, by Katanozaka and Yoshinaga (unpublished), and several strains of marine HTCC isolates were acquired from the coastal waters in Oregon (Connon and Giovannoni 2002). HTCC349 is the first non-marine species from this clade detected in culture. The OM43 clade is a sister clade to a group of methylootrophs that includes the genus *Methylophilus* and *Methylobacillus* classified as Type I methylootrophs, which use the ribulose monophosphate (RuMP) pathway for carbon assimilation (Rappé, et al. 2000).

Boom-7m-04 clade

HTCC377, HTCC379, W8 and W9 were isolated in the C-zone. The 16S rRNA gene sequence of HTCC379 is 99.2% similar to Boom-7m-04, an isolate from a 7 m depth in a subsurface clay environment (Boivin-Jahns, et al. 1996). The W8 sequence is identical to HTCC379. W8 was cultured on the minimal agar medium with propane as the sole added carbon source, however, carbon dioxide was also available. W9 was also acquired in the same manner as W8 and was identical to W8 based on RFLP analysis. This undescribed group forms a monophyletic clade within the Betaproteobacteria.

Novel Alphaproteobacteria isolates

Alphaproteobacteria were also cultured using the HTC method. These cultures were acquired from both the A-zone and the C-zone groundwater. Two groups of Alphaproteobacteria were also detected in the clone library. All Alphaproteobacterial isolates were phylogenetically analyzed to determine their placement within this group, but only the novel isolates are shown in the Alphaproteobacterial tree (Fig. 4.2).

MHP14 clade

HTCC309, collected in the C-zone, is 98.3% similar to clone 92 (AJ412676), acquired from a denitrifying reactor (Etchebehere, et al. 2002). HTCC309 is currently the only culture represented in the MHP14 clade, which was named for peat bog clone MHP14 (McDonald, et al. 1996) since it was the first clone from this clade that was recovered. This clade forms a monophyletic group that is most closely related to the genus *Rhodoplanes* in the Hyphomicrobiaceae family. Other clones in this group come from a wide diversity of environments and include HPS-64 (AF165267) from pasture soil (Nusslein and Tiedje 1999), MNF4 (AF292996) from a ferromanganous micronodule (Stein, et al. 2001), GR-296.II.111 (AJ296547) from uranium mining mill tailings (Selenska-Pobell, et al. unpublished), and DA122 (Y12598) from grassland soil (Felske, unpublished). The most closely related described species to isolate HTCC309 is *Rhodoplanes roseus*, which has a 16S rDNA similarity of 95.1%.

4-Org1-14 clade

HTCC396, collected in the A-zone, is most closely related to 4-Org1-14 (AF143822) with a 16S rRNA sequence similarity of 94.5%. Clone 4-Org1-14 was acquired from a hydrocarbon-degrading consortium (Chang, et al. 2000). Other clones in this group also come from this same study and include 4-Org2-27 (AF143833) and 4-Org2-4 (AF143828). This clade probably represents a novel group within the Alphaproteobacteria, since there is no bootstrap support for branching with any other clade. The 16S rDNA of HTCC396 and the clones in this clade are no more than 88.5% similar to the closest cultured relative, *Rhodothalassium salexigenes*.

Novel Flexibacteraceae isolates

P3 and P4 were isolated in the C-zone on agar medium and they are novel isolates within the Flexibacteraceae family (data not shown). Clones SM1E06 (AF445684) and ccspost2184 (AY133105) are the most similar sequences in GenBank with a sequence similarity of 96.7% and 94.6%, respectively, to the 16S rDNA from isolate P3. Clone SM1E06 was recovered from travertine deposits in Yellowstone (Bonheyo, et al. unpublished). The 16S rDNA clone ccspost2184 was recovered from a TCE-contaminated site (Carroll and Zinder, unpublished). The 16S rDNA from isolate P3 is 84.5% similar *Cyclobacterium marinum*, which is the most closely related described species.

CONCLUSION

While TM7, clearly a dominant bacterium in the C-zone based on LH-PCR (Chapter 3, this dissertation), was not brought into culture, the HTC approach was very successful in culturing eight previously uncultured or undescribed groups, including two groups detected in the clone library. Further modification in the HTC culturing method may lead to culturing of more of the dominant species from this groundwater system. Some avenues of research, which could be pursued, are additions of nitrogen sources as well as TCE and *cis*-DCE to the medium.

Culturing microorganisms that are dominant at sites undergoing bioremediation is important to fully understand the bioremediation process. Their role in bioremediation cannot be fully understood without investigating their physiology in culture. Cultures can also be used to acquire genome sequences that would reveal much about the physiological capabilities of these species. This is especially advantageous for the study of microorganisms that are difficult to study in the lab due to fastidious culture requirements.

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CHAPTER 5:

CONCLUSIONS AND FUTURE DIRECTIONS

Stephanie A. Connon

CONCLUSIONS AND FUTRUE DIRECTIONS

High throughput culturing (HTC) methods have been successful at bringing previously uncultured environmental microorganisms into culture. Phylogenetic identification of the isolates provided striking evidence that HTC methods resulted in the cultivation of microbial groups that appear in environmental clone libraries but have not been previously detected in culture. Using the HTC approach up to 14% of the microorganisms counted by direct microscopy were cultured, while traditional culturing methods typically isolate less than 1%. A total of eleven novel groups of bacteria were cultured from seawater and groundwater. Dominant members of the microbial community were also cultured using the HTC method.

There are three main reasons that almost certainly explain why this culturing approach worked and are addressed by the first three hypotheses 1) growth only occurs in narrowly defined conditions which have not been successfully reproduced in a laboratory setting; 2) microorganisms that grow slowly and/or achieve low cell densities are not detected; and 3) uncultured organisms do in fact grow in culture, but have not been detected because not enough attempts have been made by researchers to specifically target uncultured species for cultivation. First, natural media were used without amendments and natural environmental conditions were replicated as closely as possible. The vast majority of seawater isolates collected in the first year of the study were unable to grow on nutrient rich agar media. This may indicate that these species are finely

adapted to the environmental conditions that they have evolved under. Second, a low threshold of sensitivity allowed us to detect bacterial cultures at concentrations as low as 2×10^3 cells/ml using a custom-built filter manifold. We were able to detect cultures that were growing at low cell densities, which are more typical of the cell concentrations found in natural environments. The mean cell density for seawater and groundwater cultures recovered with the HTC method ranged from 1.1×10^5 cells/ml to 1.3×10^6 cells/ml while the median ranged from 3.0×10^4 cells/ml to 1.1×10^6 cells/ml. Third, the HTC approach enabled us to screen a large number of cultures. Over the course of 3 years, 2,484 culturing attempts were screened for microbial growth from sample collections off the coast of Oregon and 576 attempts from trichloroethene (TCE) contaminated groundwater at McClellan Air Force Base (MAFB). This large number of isolates greatly improved the chances of recovering a novel species.

The fourth hypothesis that was considered when designing the HTC methods was that trace contaminants in laboratory reagents are toxic. The use of acid washed polycarbonate lab ware may have contributed to the success of the HTC method. While no experiments were specifically designed to test this hypothesis it was taken into account when developing the HTC method.

The fifth hypothesis that drove the development of the HTC methods was that interactions with other microorganism are required for growth. However, it was not determined during the course of this research how important this may be to the isolation of novel species. It is certainly possible that important

microorganisms could be cultured in a co-culture using the HTC method since more than one cell is generally added to each well in the 48-well plate. This is an area for future exploration to determine the importance of co-cultures for the isolation of novel species using the HTC method.

Novel uncultured seawater isolates included two previously uncultured Proteobacterial clades, SAR11 (alpha subclass) and SAR92 (gamma subclass). SAR11 was transiently cultured in this study but was successfully isolated by Mike Rappé using the HTC approach. Two previously cultivated yet undescribed clades, OM43 (beta subclass) and OM60/OM241 (gamma subclass) clades were also isolated from seawater. These four novel seawater isolates belong to phylogenetic clades that are highly abundant in marine clone libraries.

Groundwater isolates included 4 previously uncultured Proteobacterial clades the MHP14 clade (alpha subclass), 4-Org1-14 clade (alpha subclass), HTCC333 (beta subclass) and HTCC410 (beta subclass). One novel group of groundwater isolates fell within the *Herbaspirillum/Oxalobacter* clade (beta subclass). The HTC method also produced groundwater isolates from 3 previously cultured, yet currently undescribed Proteobacteria, including the PM1 clade (beta subclass), Boom-7m-04 clade (beta subclass) and OM43 clade (beta subclass). Two of the groundwater clades isolated, *Herbaspirillum/Oxalobacter* and PM1 clades, were determined to be dominant members of the microbial community by clone library analysis.

However, several major uncultured groups that are thought to be abundant in surface seawater, such as the SAR86 and SAR116 clusters, did not appear among the HTCC seawater isolates. The TM7 group, an uncultured bacterial division determined to be dominant in propane sparged groundwater by LH-PCR, was also not cultured. Further innovations of the HTC approach will be needed to close the gap between culture collections and the microbial species dominating environmental communities. It is important to adequately replicate, within the lab, the natural environments in which they thrive. The specific physical characteristics of the environment can be analyzed for carbon and nutrient levels. Incubation of extinction cultures under varying conditions, including different sources of carbon, light, and other matrices of variables, may lead to the culturing of microorganisms that have specialized growth requirements.

Culturing approaches can also be guided by molecular methods analyses. The HTC approach we describe can be used to target specific bacterial groups for cultivation by screening cultures for the microorganisms of interest by means of fluorescence *in situ* hybridization (FISH). Uncultured “targets” can be sought in a deliberate manner. This is a powerful approach that can be readily applied to screening the isolates since up to 48 cultures can be screened by FISH on a single slide. Many variations of the growth conditions including media, temperature, light quality and intensity, and incubation times can be screened for a specific microorganism of interest in an efficient manner.

Culturing microorganisms responsible for the in situ bioremediation of toxic compounds is critical to the study of these processes. It is especially important to have cultures in order to model the processes in the lab for a more accurate assessment of the in situ bioremediation capacity of a contaminated site. It was apparent that a TM7 bacterium was closely correlated with propane utilization rates in the groundwater site at McClellan Air Force Base and was suspected of cometabolism of TCE. While this microorganism was not cultured under the HTC conditions used, this study highlights the need to bring into culture microorganisms suspected of performing bioremediation rather than relying on isolates that are easy to culture using traditional methods. The potential propane oxidizers isolated on the traditional agar medium were not detected in the clone library and thus it is not likely that they were significant players in the bioremediation of TCE or *cis*-DCE.

Culturing organisms remains an important step in the process of understanding the biology and ecology of microbial species. Microbial processes are the foundation of life on our planet and play a dominant role in the earth's ecosystems. The elucidation of the microbial physiology of widespread environmental microorganisms is crucial to be able to gain an accurate description of these ecosystems. For example, oceanographic models of carbon and nutrient cycles are currently input with physiological data from bacterial species that make up less than 1% of the bacterioplankton population. Studies of the dominant bacterioplankton, such as SAR11, will allow oceanographers to enter more meaningful data into these models.

Cultures can be used to obtain complete genome sequences, and to identify properties of organisms that could not be identified by genome sequence alone.

Cultures also provide a means to test hypotheses emerging from genome sequences. Combined with proteomics or microarrays, cultures of environmentally significant microorganisms can be used to examine the adaptations of these organisms to their unique environments.

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