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MOLECULAR ANALYSIS OF MICROBIAL DIVERSITY
WITHIN YUCCA MOUNTAIN

By

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A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

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ABSTRACT

Molecular Analysis of Microbial Diversity within Yucca Mountain

by

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Dr. Penny S Amy, Examination Committee Chair
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Yucca Mountain is the proposed site for a high-level nuclear waste repository. As part of the site characterization project, an estimation of microbial diversity was conducted. In this study, the 16S ribosomal DNA sequences of culturable isolates were determined and used for tentatively identification of bacterial strains. The results of identification by 16S rDNA sequence data were also compared to MIDI analysis, an independent phenotypic approach for bacterial identification.

It is accepted that culturable strains only represent a small portion of microbial community in the environment. In order to eliminate bias associated with cultivation, total DNA was extracted from rock samples and subjected to 16S rDNA analysis. 16S rDNAs were amplified from the total DNA pool and cloned. Each cloned fragment in the
constructed clone library was then subjected to sequence determination. Microbial diversity derived from total extracted DNA demonstrated a different vision of diversity from culturable isolates within Yucca Mountain.

Two sample sites with different geology and geochemistry were selected for analysis. While culturable isolates contained mostly gram positive bacteria, gram negative bacteria in this environment were more dominant by DNA analysis. One study site, YM9, contained 100% of gram negative bacteria by total DNA analysis, with 89% being Pseudomonas species.

In order to estimate the reliability of bacterial diversity derived from a series of molecular analyses as a whole, 10 common soil bacteria were seeded into sterile rock and subjected to the same process of sample analysis. The process showed no significant preference when interpreting the major group of gram positive and gram negative bacteria. Pseudomonas, represented by three species in the seeded sample, was not favored by the process. The presence of Pseudomonas species in this environment may be even higher than we detected.

The detection of genetic diversity within a natural environment can be considered the very first step towards understanding the role that bacteria play in an ecosystem. The identity of major bacterial populations in Yucca Mountain will be important for development of strategies
for storage of high-level nuclear waste and the maintenance of the repository integrity.
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This project could not have been completed without the assistance, guidance and support of great many individuals. I would like to first thank Penny Amy, my major advisor, for her constant support, and expert advice during my graduate career. Deep appreciation is also expressed to Larry Hersman, for his advising on several issues of this project, and for providing the great opportunity to accomplish most of my sequencing work at Los Alamos National Laboratory. I would also like to thank my committee members, Dawn Neuman, Daniel Thompson, and Jean Cline. Their comments and support are very much appreciated.

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analysis, and finally, Judy Buckingman, Mona Khalil, and Chiaki Brown for laboratory help.

I would also like to express my special thanks to my parents, Biman Dai and Wanqi Wu, and parents-in-law, Xuezi Yang and Gaoji Liu, for their encouragement and selfless support during years of my graduate study. Finally, I would like to thank my husband, Ning Liu, and my daughter, Katherine Liu, for their love, their patience, their understanding, and their essential support as a family.
CHAPTER 1

INTRODUCTION


Population sizes of bacteria in subsurface or other environments were initially determined by direct microscopic counts or by quantifying total microbial lipids. These estimates were always several orders of magnitude higher than the number of bacteria counted using plate count or most probable number estimations (Kieft et al., 1997). Even though the recoverable microorganisms may represent the active portion of a microbial community in
environment (Haldeman, et al., 1993), the estimated proportion of bacteria cultured from environments ranged from just 0.001 to 1% (Gray and Herwig, 1996; Ward, et al., 1990a).

In recent years, molecular techniques have improved tremendously and provided powerful new tools to microbial ecologists investigating microbial diversity in various environments. Microbiota have now been recovered in many extreme environments, such as underground water (Amy, et al., 1992; Barbaro, 1994), hot springs (Ward, 1990), marine sediments 500 m below the sea floor (Rochelle, 1992); sediment interbeds between basalt layers (Balkwill, 1988; Colwell, 1989; Fredrickson, 1988 and 1991), and even at depths greater than 2750 m below the surface (Boone, et al., 1995).

Unsaturated tuff strata at Yucca Mountain, Nevada, are being evaluated by DOE as a potential site for the nation's first high-level radioactive waste repository (Vaniman and Bish, 1993; Hersman, 1997). Yucca Mountain is located in the Topapah Springs Range on the Nevada Test Site, approximately 125 miles northwest of Las Vegas (Figure 1). The planned repository is to be located 350 meters or more below the ridge line of Yucca Mountain in the Topapah Springs member of the Paintbrush Tuff and 225 meters above the present static water table in unsaturated, densely welded, rhyolitic tuff (Geesey, 1993). Due to oligotrophic
conditions of this volcanic tuff, especially the low water/rock ratio, microorganisms existing in the subsurface of Yucca Mountain are expected to be few, both in diversity and quantity. In a study to characterize the microbiology of the Yucca Mountain subsurface, direct microscopic cell counts ranged from $3.2 \times 10^4$ to $2.0 \times 10^5$ cells g$^{-1}$ (dry weight), near microscopic detection limits. Plate counts, conducted on R2A agar, were consistent with the low abundance, $1.0 \times 10^1$ to $3.0 \times 10^3$ CFU g$^{-1}$ (dry weight). Total phospholipid fatty acid concentration (0.1 to 3.7 pmol g$^{-1}$) again indicated a low microbial biomass, and so did the concentration of diglyceride fatty acid or dead cells biomass (0.2 to 2.3 pmol g$^{-1}$) (Kieft, et al., 1997).

The low abundance of microbial cells and low rate of their activities may still have significant impact on alteration of radionuclide transportation in the long term. In addition, there are concerns about the subsequent changes of water and nutrient flux as well. Research has demonstrated that water and organic C were major limiting factors to microbial growth and activity in Yucca Mountain (Kieft, et al., 1997). The hydration alterations associated with thermal elevation and rehydration on cooling might have important impacts on the water budget of a repository in unsaturated rock, which may in turn affect the microbial activities in situ (Kieft, 1993). It has been reported that microorganism has the ability to degrade
construction materials (Diercks, 1991); to initiate microbially influenced corrosion which may cause degradation of waste containers (Jones, 1992; Lee, 1994; Pitonzo, 1996); to alter the geochemistry of surrounding environments by metabolic activities; and to influence the permeability of subsurface strata (Shaw, 1985; Hersman, 1997). Subsurface bacteria also have the potential to affect radionuclide transport through sediments and fractured rocks (Hersman, 1997; McCabe, 1990; West, et al., 1991). Determination of indigenous microbial diversities in Yucca Mountain will be important in assessing microbial potential for direct or indirect transport of radionuclides (Hersman, 1997), and their impact on the long-term stability of the subsurface nuclear waste repository (Horn, et al., 1996; Horn and Meike, 1995; Pitonzo, et al., 1996).

**Sampling Sites and Sample Collection:**

As part of the site characterization of Yucca Mountain, an exploratory study facility (ESF) was initiated in early 1995 by drilling a 7.6 m diameter tunnel into the eastern face of Yucca Mountain. Samples were collected at nine locations at different depths along the ESF tunnel, from the entrance to repository depth (Table 1 & Figure 2). Because of the tunnel structure and relative impermeable characteristic of the rock, the sampling process (using sterile tools to chip the surface rock on the tunnel wall
immediately before sampling) enhances the opportunity of obtaining uncontaminated rock samples. For the purpose of this study, two sample sites, YM3 and YM9 (Figure 2), were selected in an attempt to test the hypothesis that microbial distribution and diversity is highly related to the geology and geochemistry of the strata in Yucca Mountain.

**Culturable Isolates from Yucca Mountain (Chapter 2)**

One of reliable ways to assess bacterial activities and their potential to affect the environment depends on culturable isolates. Since most of the identification systems such as fatty acid methyl esters (MIDI system), BIOLOG system and API strips, fail to recognize all species from native environments, especially at subsurface sites (Amy, et al., 1992; Haldeman, et al., 1993; Khalil, et al., 1996), application of molecular techniques using 16S rDNA sequence data represented a significant improvement on standard identification techniques for this study.

Each morphologically distinct colony of culturable bacteria was analyzed for 16S rDNA sequence. The comparison of the 16S rDNA sequences to the rDNA database was used to classify species. The results were also compared to that of the MIDI method, an independent phenotypic identification system using fatty acid profiles of cells. Although both systems were consistent and gave
similar results for most of the isolates, fatty acid profiles failed to identify some isolates. The DNA sequence determination proved to be a more reliable approach in this study.

**Analysis of Directly Extracted DNA (Chapter 3)**

A major concern in microbial ecology is the inability to recover the majority of microbial diversity from environmental samples via plating or enrichment techniques (Amann, et al., 1995; Brockman, et al., 1992; Colwell, 1989; Haldeman, et al., submitted; Olsen and Bakken, 1987; Torsvik, et al., 1990). It is recognized that bacteria can only be cultivated in the laboratory when their physiological niche has been duplicated. The culturing step is highly selective for a small portion of the total community (Amann, et al., 1995; Olsen and Bakken, 1987; Torsvik, et al., 1990; Ward, 1990). In addition, it has been reported that disturbing surrounding environments, and handling and storage of collected samples cause shifts in microbial community composition and activities (Fredrickson, et al., 1995; Haldeman, et al., 1994a, 1995). The shift in microbial community may include the recovery of dormant or previously non-culturable microbial types (Haldeman, et al., 1994a).

To eliminate the selective bias of culturing, total DNA of the microbial community was separately extracted
from two rock samples and subjected to 16S ribosomal RNA gene amplification using PCR (Polymerase Chain Reaction). The amplified DNA fragments were then cloned into plasmid vectors and transformed into bacteria to generate large numbers of each cloned gene. A 16S rDNA clone library was constructed and sequence determination of each cloned fragment was used to identify the bacterial populations and their diversities at each site. Since diversity analysis from total DNA extraction is independent of the selective culture process, it represents an attempt to secure a more complete microbial community in Yucca Mountain. Native DNA analysis of microbial diversity at the two geological locations supported the hypothesis that microbial diversity is highly related to local environment.

Analysis of Possible Bias in Molecular Analysis of Environmental Bacteria (Chapter 4)

Molecular techniques, used to determine the bacterial phylogeny and diversity from whole community DNA extractions, have been demonstrated to be powerful tools. The techniques detect many novel populations, which were not recovered as culturable isolates, in various environments (Giovannoni, et al., 1990; Kuske, et al., 1997; Ward, et al., 1990;). A previous study of microbial diversity from total extracted DNA in Yucca Mountain demonstrated a different outcome from the culturable
isolates (Wu, 1998). While all of the culturable isolates were identified as gram positive bacteria in the YM3 sample, total DNA analysis showed that 56.6% of the DNA molecules came from gram negative bacteria. A similar phenomena was also noticed in YM9 sample. To conclude that gram negative bacteria are actually dominant in the rock, although mostly in the form of dormant, viable but non-culturable, or dead cells, it is necessary to test the reliability of molecular techniques applied to this study.

An experiment was designed to detect potential technical bias. An equal amount of 10 strains of common soil bacteria were seeded onto the sterile rock (baked in a muffle oven to remove any organic material) and subjected to the same processes used on YM3 and YM9 rock samples for DNA extraction, purification, PCR amplification, and cloning. The proportion of each strain in the clone library was then used to estimate possible bias of the analysis.

Discussion (Chapter 5)

In the last section of this dissertation, the research of all approaches on microbial diversity in Yucca Mountain, and their implications are summarized. The potential biases of different analytical processes are also discussed.
Reference


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Annual International High-Level Radioactive Waste Management Conference, Las Vegas, NV, April 29-May 3


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Table 1. Yucca Mountain Sampling Designation, Identification Numbers and Approximate Station Locations

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<td>SPC00507890</td>
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<td>YM3**</td>
<td>SPC00503951</td>
<td>5+37</td>
</tr>
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<td>YM4</td>
<td>SPC00507891</td>
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* Stations represent the distance into the Exploratory Studies Facility (ESF) from the portal and are measured in hundreds + tens of meter.
** Sample sites selected for this study.
Figure 1. Location of Yucca Mountain Relative to the Nevada Test Site and Vicinity
Figure 2. Diagram of Yucca Mountain Showing the ESR Tunnel, Major Geologic Formations, and Sample Collection Sites

* The two sample sites selected for this study are marked.
CHAPTER 2

16S rDNA SEQUENCING FOR CLASSIFICATION OF BACTERIAL ISOLATES FROM YUCCA MOUNTAIN AND CORRELATION WITH FATTY ACID PROFILES

This chapter has been prepared for submission to *Microbial Ecology* and is presented in the style of that journal.

The complete citation is:

Wu, Y., D. L. Haldeman, M. Khalil, P. S. Amy, and L. Hersman. 16S rDNA Sequencing for Classification of Bacterial Isolates from Yucca Mountain and Correlation with Fatty Acid Profiles

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Abstract

Bacterial isolates from Yucca Mountain, the proposed site of an underground high-level nuclear waste repository, were recovered on low nutrient medium, R2A, and subjected to identification and classification using 16S rDNA sequence information. Analysis of microbial diversity based on these culturable isolates was conducted for two sites with different geological and geochemical characteristics. The dominant culturable isolates were identified as *Arthrobacter* in both sites. While only 10.1% of culturable isolates in site YM9 were confirmed as gram negative bacteria, site YM3 contained no culturable gram negatives bacteria. MIDI analysis for identification of the isolate was used as a phenotypic method independent of genetic macromolecular data. MIDI results were compared to identification results from 16S rDNA sequences.

Introduction

Virtually all biological research depends on the accurate identification of species. This is particularly true in community ecology which is concerned with the study of species number, diversity, and patterns of abundance. In contrast to animals and plants, the morphology of microorganism is generally not definitive as a basis for
sound classification of species. In addition, bacteria do not have sexual reproduction which is tied to the definition of species in many eukaryotic organisms. This makes bacterial classification a challenge.

Currently, there are several commercial bacterial identification systems available. Since most of them were designed originally for clinical use, unknown bacterial identification is always restricted by appropriate databases. Most current databases contain primarily the pathogenic species important to human beings and animals (2).

In the subsurface environment, because of geographical location, geological material and isolation time, endolithic bacteria were often found to be capable of surviving for extended periods of time with little or no water and/or carbon sources (7). Using available identification systems, such as API strips, the BIOLOG system (both based on specific bacterial metabolic activities) and MIDI analysis (based on cellular fatty acid methyl ester profiles), there is often a failure to identify subsurface isolates. This is partly due to limited databases and partly due to our lack of knowledge about environmental, particularly subsurface, microorganisms. Bacteria recovered from depths up to 450 m at Rainier Mesa, a subsurface environment with structural similarity to Yucca Mountain, were often unidentified by
standard characterization methods (2, 4). Approximately 50% of the isolates recovered from the Exploratory Studies Facility (ESF) in Yucca Mountain were not identified by the same methods (6). Identification systems based on morphological, developmental, and nutritional characteristics did not always correlate well with identification systems defined by macromolecular sequences comparison (15).

With rapid developments within biotechnology, sequence comparison of genetic markers has become more practical as a tool for phylogenetic purposes and identification of bacteria (10). The stability of genetic information, quantitative inference of relationships contained in sequences, rapid and extended databases and convenient access through the Internet make this approach powerful. Of the macromolecules commonly used for phylogenetic analysis and taxonomy, 16S ribosomal DNA has proven to be the most useful biomarker. Because of the universal existence of ribosomal DNA, conservative regions with intersections of variable segments, 16S ribosomal DNA contains valuable evolutionary information for interpreting both phylogeny and taxonomy.

The use of rRNA gene sequences to characterize microorganisms has now gained wide acceptance. However, Woese reminds us that although phylogenies derived from genetic sequence data are powerful, they must not be over-
interpreted due to the process of molecular evolution (18). He cautions that one should compare the results from sequence data with an independent methodology. The importance of a polyphasic taxonomic approach, which integrates the use of both phylogenetic and phenotypic characteristics, is appreciated by many microbiologists. The combination of tests has been applied to tentatively identify environmental bacteria and test for evolutionary relationships (3, 12, 17).

The purpose of this study was two fold. First, use the power of sequencing techniques to identify culturable isolates from Yucca Mountain subsurface rock. Second, determine if a correlation could be made between the phylogeny and phenotype of bacteria by comparing the identification profiles for fatty acid and 16S ribosomal DNA sequence analyses.

Materials and Methods

Sample Sites and Sample Collection. Two sample sites with different geological characteristics were selected for this study. YM3, a highly fractured rock stratum expected to contain relatively greater bacterial diversity, and YM9, a rock bed with low permeability, expected to yield lower bacterial numbers and diversity, were chosen. Rock samples were collected aseptically and transported to the lab on
ice. Samples were ground using sterile mortars and pestles, mixed with 0.12 M sodium pyrophosphate solution and shaken for 1 h. R2A (DIFCO), a low nutrient medium designed for culturing bacteria from natural environments, was used to enumerate culturable bacteria from the slurry. After 14 days incubation at room temperature (25°C), colonies were evaluated by colony morphology. Each distinct colony type was re-streaked twice on R2A plates to establish purity.

16S rDNA Amplification and Purification. A pair of universal bacterial primers for 16S rDNA, 16s-27f (5'-GTG CTG CAG AGA TTT GAT CCT GGC TCA G-3'), 16S-1492r (5'-CAC GGA TCC TAC GGG TAC CTT GTT CGA CTT-3') (Bandi, et al., 1994) were used to amplify 16S rDNA of 1.5 kb in length. 16S rDNA of each isolate was first amplified after direct lysis. An amount of cells, obtained by touching a 0.2 ml disposable pipette tip to the colony, was mixed with 50 μl of 10 mM EDTA and heated at 95°C for 5 min. The lysis mixture (1 μl) was added to PCR reaction tubes for 16S rDNA amplification. Isolates that could not be amplified for its 16S rDNA in this way were subjected to genomic DNA extraction using a QIAamp Tissue Kit (QIAGEN Inc., Germany) with a modified protocol for bacterial DNA extraction. Bacterial cells were first suspended in 180 μl of lysozyme buffer (4 mg/ml lysozyme; 20 mM Tris-HCl, pH 8.0; 2 mM
EDTA; 1.2% Triton X-100) in a microcentrifuge tube. After incubating at 37°C for 30 min, 25 μg proteinase K was added along with 200 μl buffer AL (provided with the Kit). The mixture was incubated at 70°C for 30 min, followed by an additional 30 min at 95°C. Ethanol (210 μl) was added and mixed thoroughly, then the protocol of QIAamp Tissue Kit was followed with spin columns. DNA was eluted from the column with 400 μl preheated (60°C) distilled water for each isolate. Extracted DNA (1 μl) was then used for the PCR reaction. PCR amplification was conducted for 35 cycles on a GeneAmp PCR System 2400 (Perkin Elmer). Cycling parameters included denaturing at 94°C for 30 s, annealing at 52°C for 45 s, and extension at 72°C for 1.5 min.

Desired PCR products were first separated in a 1% TAE (Tris-Acetate-EDTA) agarose gel. The desired bands were excised after staining with ethidium bromide (0.5 g/ml) for 20 min. The excised gel bands were then incubated with 400 ml NaI (6 N) at 55°C for 10 min or until the gel dissolved. "Glassmilk" (4 ml) was added and the mixture was rotated for 10 min at room temperature (21°C). After centrifugation at 15,000 rpm for 15 s, the "Glassmilk" was collected in the bottom after discarding the supernatant. Ice-cold New Wash Solution (300 μl) (BIO 101, Inc., Vista, CA) was used to wash the "Glassmilk" three times, and the
DNA was eluted from "Glassmilk" with 25 μl of deionized water. The cleaned PCR products were then ready for sequencing.

16S rDNA Sequence Determination. DNA sequencing was conducted using a DNA Sequencing Kit — Dye Terminator Cycle Sequencing Ready Reaction (Perkin Elmer, Applied Biosystems Division, Foster City, CA) with primer 16S-27f (5'-GTG CTG CAG AGA TTT GAT CCT GGC TCA G-3'). Sequencing was done on an ABI 377 DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) using a 4.25% Acrylamide Burst — Pak Custom Gel (Owl Scientific, Inc., Woburn, MA). The collected sequence data were analyzed using ABI 200 Base Caller (Perkin Elmer, Foster City, CA).

Phylogeny and Tentative Identification of Bacterial Isolates by 16s rDNA Sequence Data. Each sequence was searched for its closest matching sequences available in the GenBank and RDP (Ribosomal Database Project) databases using a similarity search program, "BLAST" (Basic Local Alignment Search Tool), on line (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov; 1). Alignment of obtained sequences, along with some published sequences of representatives inside and outside of the Actinomycete sublines of descent included in the RDP (11),
was conducted using Clustal W (Vision 3.1.1) (16). Sequence regions that could not be aligned with confidence were excluded. Bacterial phylogenies were constructed by maximum-parsimony analysis using PAUP (Version 4.0 Beta Version, Swofford). Bootstrap analyses were performed on 100 replicate data sets, with random-order sequence additions. In addition to that, the closest matching genus to each isolate was compared with the genus identified by phospholipid fatty acid profiles produced by the MIDI system.

Results

Approximately 400 bp were obtained for each sequencing reaction using primer 16S-27f. Table 1 gives the results of tentative identification of culturable isolates by the MIDI system and by 16S rDNA sequences. For the YM3 sample, "BLAST" search results of partial sequences of 16S ribosomal DNA from 19 isolates showed that all of them matched gram positive bacteria. Among them, 18 isolates were matched gram positive high "G+C" bacteria or the Actinomycete group. They matched closely to genera *Rhodococcus, Nocardioides, Streptomyces, Microbacterium, Micrococcus,* and *Arthrobacter.* Seven out of 19 (37%) isolates were matched with the genus *Arthrobacter.* One
isolate, ym3fn1, matched most closely to the low "G+C", gram positive, Bacillus group. There were no gram negative culturable species detected from the YM3 sample. Figure 1 is constructed phylogeny of culturable isolates in YM3 based on 16S rDNA sequences. The phylogenetic tree displayed a better vision of microbial diversity, showing the major taxonomic groups that each strain fell into.

Comparison of fatty acid profile identification to that of 16S rDNA sequencing resulted that both systems identifying one Bacillus sp., eight Arthrobacter sp., and one Micrococcus sp. Two isolates of Microbacterium, ym3d3 and ym3ec3, as classified by 16S rDNA sequences, were grouped in Corynebacterium and Bacillus by MIDI analysis. Isolate ym3d8 was matched to Rhodococcus sp. by 16S rDNA sequence, and Bacillus marcerans by MIDI analysis. Two isolates of Streptomyces, two Nocardiodes and one Rhodococcus, based on 16S ribosomal DNA sequences, were unable to be classified by the MIDI system due to failure to match to the MIDI database. The unmatched isolates constituted 31% of the total isolates tested. Because the MIDI similarity coefficient for ym3d8 was only 0.257, it was below, although close to, the acceptance level of 0.30 for environmental isolates (2). Because isolates ym3f5 failed to demonstrate an acceptable match with Micrococcus.
or Bacillus (similarity coefficient of 0.125 and 0.09), it was not identified by the MIDI system.

Consistent with 16S ribosomal sequence results for YM3, there were no culturable isolates identified as gram negative bacteria by MIDI analysis.

Table 2 presents the classification results for 27 culturable isolates from the YM9 sample. "BLAST" search, using the 16S ribosomal DNA sequences, resulted in 24 of 27 culturable isolates from YM9 clustered into the Actinomycete group. They closely matched the genera Microbacterium, Agrococcus, Terrebacter, Dietzia, Nocardioides, Micrococcus, and Arthrohacter. Among them, Arthrohacter was again the dominant genus, accounting for 22.2% (six isolates out of 27) of the total isolates.

Of the 27 isolates previously grouped into the Actinomycetes, six isolates displayed questionable fatty acid profiles which could not be used for identification. Seven isolates did not match with any species in the fatty acid database. Therefore, 44.4% (12 out of 27) of the isolates were not adequately recognized by the MIDI system. Four isolates were found to match with Bacillus species by the MIDI method while 16S rDNA sequence data grouped them as Dietzia sp., Nocardioides sp., Microbacterium sp., and Agrococcus sp. Among the group of six Arthrohacter sp., which were confirmed by 16S rDNA sequences, four of them
matched *Arthrobacter oxydans* in the MIDI database, one matched with *Brevibacillus laterosporus*, and another showed a questionable MIDI data profile. Isolate ym9e8 was identified as *Arthrobacter sp.* by 16S rDNA, but it matched with *Micrococcus* by MIDI analysis with a low similarity coefficient of 0.124. ym9e2 was the opposite, it matched to *Micrococcus sp.* with 16S rDNA data, and *Arthrobacter sp.* in MIDI analysis. The remaining isolate did not grow on the standard MIDI growth medium, TSB (Tryptic Soy Broth), and therefore could not establish a fatty acid profile for identification.

There were three isolates that matched gram negative groups by 16S rDNA sequencing. One of them belonged to *Pseudomonas sp.*, and the other two were identified as *Acinetobacter sp.* The MIDI analysis identified these three gram negative species consistently with 16S ribosomal DNA sequence analysis. Unlike the YM3 sample, which contained only gram positive bacterial isolates, YM9 contained about 11% gram negative bacteria. Figure 2 is a phylogenetic tree of culturable isolates from YM9 based on 16S ribosomal DNA sequences, showing the diversity of culturable strains in this site. Again, majority of the isolates fell into *Arthrobacter* genus or its close relatives.

Figure 3 is a constructed phylogenetic tree from all isolates clustered in *Arthrobacter* and its close relatives.
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UMI
<table>
<thead>
<tr>
<th>Isolates</th>
<th>Classified by MIDI</th>
<th>Similarity coefficient</th>
<th>Classified by 16S rDNA</th>
<th>Sequence similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3FN1</td>
<td>Bacillus pantothenicus</td>
<td>0.191</td>
<td>Bacillus subtilis</td>
<td>96%</td>
</tr>
<tr>
<td>3A3</td>
<td>No Match</td>
<td></td>
<td>Rhodococcus sp.</td>
<td>97%</td>
</tr>
<tr>
<td>3D8</td>
<td>Bacillus macerans</td>
<td>0.257</td>
<td>Rhodococcus sp.</td>
<td>95%</td>
</tr>
<tr>
<td>3A4</td>
<td>No Match</td>
<td></td>
<td>Nocardioides sp.</td>
<td>93%</td>
</tr>
<tr>
<td>3D6</td>
<td>No Match</td>
<td></td>
<td>Nocardioides sp.</td>
<td>97%</td>
</tr>
<tr>
<td>3EC5</td>
<td>No Match</td>
<td></td>
<td>Streptomyces sp.</td>
<td>96%</td>
</tr>
<tr>
<td>3D7</td>
<td>No Match</td>
<td></td>
<td>Streptomyces griseus</td>
<td>93%</td>
</tr>
<tr>
<td>3D3</td>
<td>Corynebacterium</td>
<td>0.742</td>
<td>Microbacterium</td>
<td>96%</td>
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<tr>
<td></td>
<td>aquatic</td>
<td></td>
<td>laevaniformans</td>
<td></td>
</tr>
<tr>
<td>3EC3</td>
<td>Bacillus macerans</td>
<td>0.251</td>
<td>Microbacterium</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>Arthrobacter testaceum</td>
<td>0.218</td>
<td>laevaniformans</td>
<td></td>
</tr>
<tr>
<td>3E2</td>
<td>Arthrobacter oxydans</td>
<td>0.055</td>
<td>Arthro. globiformis</td>
<td>96%</td>
</tr>
<tr>
<td>3A2</td>
<td>Arthrobacter pascens</td>
<td>0.513</td>
<td>Arthrobacter sp.</td>
<td>92%</td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>Value</td>
<td>Comparison Species</td>
<td>%</td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------</td>
<td>-------</td>
<td>--------------------------</td>
<td>----</td>
</tr>
<tr>
<td>3F2</td>
<td>Arthrobacter pascens</td>
<td>0.371</td>
<td>Arthro. globiformis</td>
<td>91%</td>
</tr>
<tr>
<td>3D4</td>
<td>Brevibacterium sp.</td>
<td>0.542</td>
<td>Micrococcus luteus</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td>Arthrobacter sp.</td>
<td>0.414</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3E1</td>
<td>Arthrobacter nicotinae</td>
<td>0.578</td>
<td>Streptomyces sp.</td>
<td>98%</td>
</tr>
<tr>
<td>3D1</td>
<td>Arthrobacter  ilicis</td>
<td>0.456</td>
<td>Arthro. nicotianae</td>
<td>97%</td>
</tr>
<tr>
<td>3A1</td>
<td>Arthrobacter mysorens</td>
<td>0.745</td>
<td>Arthro.nicotianae</td>
<td>97%</td>
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<tr>
<td>3D5</td>
<td>Arthrobacter  ilicis</td>
<td>0.851</td>
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<td>97%</td>
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<tr>
<td>3F1</td>
<td>Arthrobacter  ilicis</td>
<td>0.867</td>
<td>Arthrobacter sp.</td>
<td>97%</td>
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<tr>
<td>3F5</td>
<td>Micrococcus lylae</td>
<td>0.125</td>
<td>Micrococcus luteus</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td>Bacillus brevis</td>
<td>0.090</td>
<td></td>
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Table 2. Tentative Identification of YM9 Culturable Isolates by MIDI System and 16S rDNA Sequences

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Classification by MIDI system</th>
<th>Similarity coefficient</th>
<th>Classification by 16S rDNA</th>
<th>Sequence Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>9D1</td>
<td><em>Pseudomonas putida</em></td>
<td>0.097</td>
<td><em>Pseudomonas orientalis</em></td>
<td>97%</td>
</tr>
<tr>
<td>9CP4</td>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>0.246</td>
<td><em>Acinetobacter junii</em></td>
<td>97%</td>
</tr>
<tr>
<td>9AC1</td>
<td><em>Acinetobacter radioresistens</em></td>
<td>0.486</td>
<td><em>Acinetobacter junii</em></td>
<td>97%</td>
</tr>
<tr>
<td>9C6</td>
<td><em>Bacillus Macerans</em></td>
<td>0.516</td>
<td><em>Microbacterium sp.</em></td>
<td>96%</td>
</tr>
<tr>
<td>9B6</td>
<td>Flagged</td>
<td></td>
<td><em>M. imperiale</em></td>
<td>93%</td>
</tr>
<tr>
<td>9C2</td>
<td>No Match</td>
<td></td>
<td><em>Agrococcus jeensis</em></td>
<td>94%</td>
</tr>
<tr>
<td>9C1</td>
<td><em>Bacillus Macerans</em></td>
<td>0.346</td>
<td><em>Agrococcus jeensis</em></td>
<td>89%</td>
</tr>
<tr>
<td>9B2</td>
<td><em>Brevibacterium casei</em></td>
<td>0.250</td>
<td><em>Agrococcus jeensis</em></td>
<td>94%</td>
</tr>
<tr>
<td>9A6</td>
<td>No Match</td>
<td></td>
<td><em>Janthino limosus</em></td>
<td>95%</td>
</tr>
<tr>
<td>9B5</td>
<td>No Match</td>
<td></td>
<td><em>J. thuringensis</em></td>
<td>96%</td>
</tr>
<tr>
<td>9C3</td>
<td>No Match</td>
<td></td>
<td><em>Terre bacter sp.</em></td>
<td>97%</td>
</tr>
<tr>
<td>9B4</td>
<td>Flagged</td>
<td></td>
<td><em>J. thuringensis</em></td>
<td>94%</td>
</tr>
<tr>
<td>Sample</td>
<td>Identification</td>
<td>Similarity Score</td>
<td>Comment</td>
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<td>------------------</td>
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<tr>
<td>9E7</td>
<td>Flagged</td>
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<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9A7</td>
<td>Flagged</td>
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<tr>
<td>9AN3</td>
<td><em>Bacillus Macerans</em></td>
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<tr>
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<td><em>Gordona bronchialis</em></td>
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<td>9E4</td>
<td><em>Bacillus Macerans</em></td>
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</tr>
<tr>
<td>9C8</td>
<td>Flagged</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>9C5</td>
<td>No Growth</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>9B1</td>
<td><em>Arthrobacter oxydans</em></td>
<td>0.212</td>
<td>A. polychromogenes</td>
<td>96%</td>
</tr>
<tr>
<td>9C9</td>
<td><em>Arthrobacter oxydans</em></td>
<td>0.057</td>
<td>A. polychromogenes</td>
<td>98%</td>
</tr>
<tr>
<td>9A1</td>
<td><em>Arthrobacter oxydans</em></td>
<td>0.180</td>
<td>A. ramosus</td>
<td>95%</td>
</tr>
<tr>
<td>9E5</td>
<td>Flagged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9E8</td>
<td><em>Micrococcus luteus</em></td>
<td>0.124</td>
<td>A. oxydans</td>
<td>97%</td>
</tr>
<tr>
<td>9A5</td>
<td><em>Brevibacillus laterosporus</em></td>
<td>0.248</td>
<td>A. sp.</td>
<td>95%</td>
</tr>
<tr>
<td>9E2</td>
<td><em>Arthrobacter oxydans</em></td>
<td>0.216</td>
<td>Micrococcus luteus</td>
<td>96%</td>
</tr>
</tbody>
</table>
Figure 1. Classification of Culturable Isolates from Yucca Mountain YM3 by 16S rDNA Sequence
Figure 2. Classification of Culturable Isolates from Yucca Mountain YM9 by 16S rDNA Sequence

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Figure 3. Relatedness of Isolated Arthrobacter Strains in Yucca Mountain YM3 and YM9 Sites by 16S rDNA sequence
CHAPTER 3

MOLECULAR ANALYSIS OF MICROBIAL DIVERSITY
IN YUCCA MOUNTAIN

This chapter has been prepared for submission to Microbial Ecology and is presented in the style of that journal.
Abstract

Rock samples from two different subsurface geological sites in Yucca Mountain were selected for analysis of microbial diversity. YM3 is a highly fractured rock stratum, and YM9 is a relatively low permeability rock bed in which the high-level nuclear waste repository will be built. Analysis conducted for this study employed culture-independent molecular techniques. The process included direct extraction of total community DNA from the rock, PCR amplification of the 16S rDNA, and construction of a 16S rDNA clone library from the amplified fragments. Sequence determination of the 16S rDNA in the clone library showed a broad diversity in YM3. The bacterial strains ranged from High "G+C" gram positive species to α-, β-, and γ-proteobacteria of gram negative species. YM9 showed less diversity than YM3 which correlated with its geological characteristics. In YM9, only gram negative bacteria were detected, with 86.6% being Pseudomonas species. Diversities from total DNA extraction were also compared to the diversities derived from culturable isolates, which contained mostly the gram positive bacteria, Actinomycetes. The discrepancies between the diversity analysis of culturable isolates and the total DNA extraction are also discussed.
Introduction

Yucca Mountain is a proposed underground repository site for high-level nuclear waste. Characterization of the stability of this site for a long-term repository is critical. Assessment of the safety and health of the public by limiting or preventing nuclear wastes from reaching the accessible environments, such as surface or groundwater, is necessary. It has been reported that microorganisms have the ability to degrade construction materials (Diercks, 1991); initiate microbially influenced corrosion, which may in turn cause the degradation of waste containers (Jones, 1992; Lee, 1994; Pitonzo, 1996); alter the geochemistry of surrounding environments by metabolic activities; and influence the permeability of subsurface strata, and thereby, the fate of radioactive compounds (Hersman, 1997; Shaw, 1985). Therefore, an understanding of microbial diversity within this deep subsurface environment will be important in terms of predicting microbial activity and modeling the long term stability of a nuclear waste repository.

Microbial ecologists have long been constrained in their efforts to investigate the composition of natural microbial communities using traditional methods. Historically, classification of bacteria has been based on phenotypic traits, such as bacterial morphology, staining...
characteristics, utilization of various nutrients, types of metabolism, and the presence of specific enzymatic activities (Reeve, 1997; Smibert & Krieg, 1994). The characterization of those phenotypic traits relied mainly on bacterial isolates that were cultured in the lab. It is recognized that culture-dependent methods are biased, especially in the study of environmental samples. Since microorganisms can only be cultivated after their physiological niches are determined and duplicated in the lab, perhaps as little as 1% of the total microbial community has been characterized (Amann, et al., 1995; Haldeman, et al., 1997; Olsen and Bakken, 1987; Torsvik, et al., 1990; Ward, 1990). In investigations of subsurface microbiota, this has often been the case (Brockman, et al., 1992; Colwell, 1989; Haldeman, et al., submitted a; Marxsen, 1988; Vescio and Nierzwicki-Bauer, 1995). One notable exception was the subsurface sampling and analysis conducted at Savannah River Plant where nearly all of the microbiota detected were culturable (Fliermans, 1989).

Studies on characterization of volcanic tuff samples from Rainier Mesa, a structural analog to Yucca Mountain, showed that total biomass, based on phospholipid fatty acid analysis, exceeded culturable counts (Ringelberg, et al., 1996; Haldeman, et al., 1996). It is widely believed that a large proportion of the microbial community is often not detectable from environmental samples by use of

In the past few years, recombinant DNA methodologies and rapid nucleotide sequence determinations have changed the approach of microbial ecologists interested in phylogenetic studies. Of the macromolecules used for phylogenetic analysis, the 16S ribosomal RNA gene has proved to be the most useful for establishing evolutionary relationships, not only because of its high information content and conservative nature, but also its universal distribution (Lane, et al., 1985; Pace, et al., 1977). With development and improvement of DNA extraction methods, PCR amplification, and cloning technologies, it is now possible to evaluate genes of interest from natural bacterial populations for interpreting microbial phylogenies and diversity.

Several examples of discovery of previously uncultured microbes in natural environments have pushed the process of microbial identification toward nucleic acid sequencing. Studies of 16S ribosomal DNA amplified from natural populations of Sargasso Sea picoplankton indicated the presence of a novel uncultured microbial group, which appeared to be a significant component of this oligotrophic
bacterioplankton community (Giovannoni, et al., 1990). 16S ribosomal DNA sequence analysis of the 55°C cyanobacterial mat of Octopus Spring in Yellowstone National Park revealed numerous uncultured microorganisms in a natural community (Ward, et al., 1990). A recent phylogenetic survey of the microbiota in the arid land of the Southwestern United States performed in Kuske's lab (Kuske, 1997) found that most of the analyzed sequences fell into five novel clusters having no known cultured members.

Studies of microbial diversity within Yucca Mountain based on culturable isolates have been performed (Khalil, 1996; Kieft, et al., 1997; Wu 1998, Dissertation). The majority of the isolates recovered were found to be Arthrobacter species. None of the isolates was identified as gram negative bacteria in sample YM3, and only 11% were gram negative in sample YM9. From a geological point of view, YM9 is a rock bed with relatively low permeability, in which the high-level nuclear waste canisters would be placed, and YM3 is a highly fractured rock stratum. This type of fractured rock stratum was expected to contain relatively greater bacterial diversity due to the potential for water and nutrient flux. The analysis of 16S rDNA sequences from all culturable isolates at these sites showed that the diversity in YM9 was actually higher than in YM3. This raised the following questions: Were the dominant bacteria in this environment gram positive as seen
with the cultured isolates? How well do the culturable isolates represent the composition of actual microbial diversity in Yucca Mountain?

In this study, bacterial diversities at two sites in Yucca Mountain were examined using culture-independent methods. 16S rDNA was amplified from bulk DNA extracted directly from the rock. A clone library of 16S rDNA was constructed for each site, YM3 and YM9, to represent the composition of the native microbial community. Results were also compared with diversity from culturable isolates to estimate potential bias of culture-dependent analysis.

Materials and Methods

Sample Sites and Sample Collection:

Rock samples were collected according to the Yucca Mountain Characterization Project Plan (Hersman, 1994). Briefly, sampling faces within the ESF (Exploratory Studies Facility) were grossly prepared by mining support staff the evening before sample collection. An approximately 1 m² face was exposed in a vertical plane after at least 3.0 cm of rock was removed from the tunnel well. Immediately before sample collection, the rock face was cleaned by chipping off the recently exposed rock surface with sterile hand tools. Samples were chipped into sterile bags using fresh implements for each sampling position. Samples were
transported to the laboratory in coolers containing ice and sample analysis was initiated within 48 h. Rock samples for direct DNA extraction and analysis were preserved at -20°C until analyzed.

Rock samples from two locations were selected for this study. YM3, a highly fractured rock stratum which is 537 m away from the portal within the Tiva Canyon welded unit, and YM9, more than 2100 m from the portal and 300 m below the surface within Topapah spring welded unit, a relatively low permeability rock bed in which the waste canister would be placed (See Figure 2 in Chapter 1).

Direct DNA Extraction and Purification:

Extraction of DNA from 10 g rock samples was conducted by a modification of the method of Tsai and Olson (Tsai and Olson, 1991). Briefly, rock samples were ground using sterile mortars and pestles. Rock baked at 450°C for 4 h was used as a negative control. Samples were washed twice with 0.12 M sodium pyrophosphate solution by shaking at 200 rpm for 20 min, and then separated by centrifugation at 8000 rpm for 10 min before discarding the supernatant. Pellets were incubated with 5 ml of lysozyme solution (15 mg/ml lysozyme, 0.15 M NaCl, 0.1 M EDTA, pH 8.0) at 37°C for 3 h, with mixing every 30 min. After cooling on an ice bath for 5 min, 5 ml lysis buffer (0.1 M NaCl, 0.5 M Tris-HCl, pH 8, 10% SDS) was then added. Mixtures were
subjected to three cycles of freeze-thaw (ethanol-ice bath for 15 minutes followed by 65°C water bath for 20 min per cycle). After the final thaw, samples were placed on ice again. While on ice, 5 ml of saturated phenol (previously equilibrated with Tris-HCl, pH 8) was added. Samples were vortexed and phases were separated by centrifugation at 8,000 rpm for 10 min. The supernatant was collected in clean tubes and mixed with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). After centrifugation at 8,000 rpm for 10 min, the supernatant was collected again into a clean tube, mixed with an equal volume of chloroform-isoamyl alcohol mixture (24:1) and submitted to centrifugation at 8,000 rpm for another 10 min. The aqueous phase was collected and the DNA was precipitated with an equal volume of isopropyl alcohol. After being held at -20°C for at least 2 h, the mixture was centrifuged at 15,000 rpm for 10 min. The supernatant was discarded and pellet was washed with 70% ice-cold ethanol, and subjected to centrifugation again at 15,000 rpm for 10 min. The DNA pellets were dried and resuspended in 100 µl of sterile, nuclease-free water (Amresco, Ohio).

The extracted DNA was cleaned using a Spin-X centrifuge tube filter (Corning Costar Corporation, Cambridge, MA). The tube filter was pre-packed with Sephadex-G200 (Sigma Chemical Co.) previously saturated with TE (10 mM Tris, 1 mM EDTA). The crude extract was
loaded at the top of the Sephadex slant. DNA was collected by spinning the column at 3,000 rpm for 1 min.

16S rDNA Amplification and Cloning:

A pair of universal eubacterial primers, 16S-27f (5'-GAG AGT TTG ATC CTG GCT CAG-3'), 16S-1492r (5'-CTA CGG CTA CCT TGT TAC GA-3') (Bandi, et al., 1994), were used to amplify 16S rDNA (1,500 bp). One ml of cleaned extracted DNA was amplified for 35 cycles on a GeneAmp PCR System 2400 (Perkin Elmer). PCR cycling parameters included denaturing at 94°C for 30 sec, annealing at 52°C for 45 sec, and extension at 72°C for 1.5 min.

The PCR product was purified to free it of excess nucleotides and dimer primers, and was separated electrophoretically in a 1% TAE (Tris-Acetate-EDTA) agarose gel. The desired 1.5kb fragment was excised from the gel after staining with ethidium bromide (0.5 mg/ml) for 20 min. The amplified products were extracted from the gel slice using a Gene-Clean Kit (Bio101, Inc.) and resuspended in 25 ml of sterile nuclease-free water (Amresco Inc., Solon, Ohio).

About 10 ng of amplified 16S ribosomal RNA gene fragments was ligated into plasmid vector pCR™II and transformed into One Shot™ INVαF' competent cells using an Original TA Cloning Kit (Invitrogen Corp., San Diego, CA). White colonies with a 1.5 kb insert were scanned by PCR.
using universal vector primers M13f (5'-TGT AAA ACG ACG GCC AGT-3'), and M13r (5'-CAG GAA ACA GCT ATG ACC-3'). The colonies amplified with the 1.75 kb fragments were selected as positive clones for the construction of a 16S rDNA library. In this case, each clone represented an individual of the community.

**Analysis of the 16S rDNA Library by RFLP and Sequence Determination:**

The amplified 16S rDNA fragment in each clone was subjected to RFLP (Restriction Fragment Length Polymorphism) analysis with restriction endonucleases HaeIII and AvaII (Biolabs, New England). The digested fragments were separated electrophoretically in a 1% TAE agarose gel containing ethidium bromide (0.5 mg/ml). Each digestion pattern was compared and grouped. One to three clones from each RFLP pattern groups were subjected to sequencing. The sequencing templates amplified with M13f and M13r as described above were cleaned using a Gene-Clean Kit (Bio101, Inc.) before sequencing.

Sequencing was conducted using the DNA Sequencing Kit—Dye Terminator Cycle Sequencing Ready Reaction (Perkin Elmer, Applied Biosystems Division, Foster City, CA) with primer 16S-27f (5'-GAG AGT TTG ATC CTG GCT CAG-3'). The products of the cycle sequencing reaction were purified with Centri-Sep spin columns (Princeton Separations,
Adelphia, N.J.) to free them of extra florescent dye and primers. The sequencing was accomplished on an ABI 377 DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) using the 4.25% Acrylamide Burst-Pak Custom Gel (Owl Scientific, Inc., Woburn, MA).

Classification Using 16S rDNA Sequence Data:

Sequences from each clone were first analyzed for possible chimera formation using the CHECK_CHIMERA program in the Ribosomal Database Project (CHECK_CHIMERA Version 2.5, http://rdp.life.uiuc.edu/RDP/commands/chimera.html). Each sequence was then searched for the closest matching species in the database using the "BLAST" search program on line (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov; 1). The sequences were aligned along with representatives of published sequences included in the Ribosomal Database Project (Larsen, et al., 1993) and GenBank using the Clustal W program (Thompson, 1994). The phylogenies were constructed using PAUP (PAUP, Version 3.1.1, D. L. Swofford, Smithsonian Institution) by maximum-parsimony analysis. Bootstrap analyses were performed on 100 replicate data sets, with random-order sequence additions.
Results

Extracted DNA from the Rock Samples:

Since the number of bacteria in the rock sample was low, the extracted DNA was difficult to visualize and estimate by spectrophotometry or in agarose gels containing ethidium bromide (0.5 mg/ml). The success of extraction was detected by amplification of 16S ribosomal DNA from the total DNA pool with PCR. When PCR products were separated on 1% TAE agarose gels containing ethidium bromine, a bright band of 1.5kb which indicated the amplified 16S rDNA fragments was detected. Rock baked in a muffle oven was used as a negative control to insure that the extraction and amplification processes used were free of contamination.

Molecular Microbial Diversity in YM3 from Extracted Native DNA and Comparison with Diversity Derived from Culturable Isolates:

Sixty-five transformed colonies were collected from sample YM3. After amplification with M13f and M13r primers, 46 colonies containing an insert of 16S rDNA of 1.5 kb were selected to construct the clone library. Using restriction endonuclease AvaII and HaeIII for RFLP analysis of the amplified inserts produced diversified restriction
patterns. Sequence determination of representatives from each pattern group provided broad microbial diversity (Figure 1). There were four major taxa represented: the division of high "G+C" content gram positive bacteria, the α-proteobacteria, the β-proteobacteria, and the γ-proteobacteria within gram negative bacterial groups. Gram positive bacteria represented 43.5% of the populations in the clone library, and gram negative bacteria constituted the remainder (56.5%). When bacterial diversity, derived from native DNA extraction (Figure 1), was compared to that of the culturable isolates (Figure 1 in Chapter 2), there was a significant difference. The analysis of YM3 culturable isolates contained only gram positive bacteria, but native DNA analysis of the 16S rDNA showed that gram negative bacteria made up about 56.5% of the microbial community in that environment. Besides the species of β and γ-proteobacteria, native DNA analysis also recovered *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium* species in the α-proteobacteria group (11%), none of which have ever been cultured from this subsurface environment. *Arthrobacter sp.* were the major culturable isolates (Wu, 1998, Chapter 2), a finding that is consistent with the diversity from total native DNA analysis in *Arthrobacter* constituted about 37% of the total population.
Molecular Microbial Diversity in YM9 from Extracted Native DNA and Comparison with Diversity Derived from Culturable Isolates:

Eighty-four colonies containing a 1.5 kb 16S rDNA insert were collected in the 16S rDNA clone library for YM9. RFLP analysis, using restriction endonuclease AvaII and HaeIII, showed that 78% of the inserts fell in one restriction pattern group and the rest of the clones belonged to an additional four different pattern groups.

Six clones from the first major group and 1-3 clones from each of the other groups were subjected to insertion sequencing. When analyzing each sequence using CHECK_CHIMERA program, two clones, 9-1006 and 9-1009, were excluded from the analysis because of possible chimera formation. Molecular microbial diversity of YM9 is presented in Figure 2. Clone library only contained gram negative bacterial DNA from two major taxa: β-proteobacteria and γ-proteobacteria. Within β-proteobacteria, one clone was defined as Comamonas and seven others as Janthino, these constituting approximately 1.2% and 8.5% of the total, respectively. There were two major groups of γ-proteobacteria: Yersinia and Pseudomonas. While three clones were identified as Yersinia or its close relatives, 71 clones were classified as Pseudomonas.
species. *Pseudomonas* made up 86.6% of the total microbial population at this sampling site.

**Microbial Diversity Between Sample Site YM3 and YM9:**

Analysis using total DNA extracted directly from rock showed that microbial diversity in sample site YM3 was higher than in YM9. In YM3, bacteria recovered ranged from *Arthrobacter*, *Microbacterium* and other unidentified species of gram positive bacteria to α, β, and γ-proteobacteria, including nine major taxa of gram negative bacteria. In YM9, there were only gram negative bacteria recovered in the clone library, with 86.6% of the population classified as *Pseudomonas* species, while YM3 contained only 56.5% gram negative representatives.

Phylogenetic analysis of β and γ-proteobacteria strains in both YM3 and YM9 samples was presented in figure 3, showing the major portion of *Pseudomonas* species in YM9 was clustered with *Pseudomonas* species detected in YM3 sample (the cluster of 3-1047, 3-1002, 9-1030, 9-1003, and 9-1007). 3-1038 and 3-1006, along with 9-1076 were closely related to *Comamonas* species within β-Proteobacterium subdivision.
Discussion

Shifts in microbial community composition have been reported during handling and storage of volcanic tuff samples. These include the recovery of dormant or previously non-culturable microbial types (Fredrickson, et al., 1995; Haldeman, et al., 1994, 1995). These investigations hint at the importance of investigating non-culturable microbiota in natural environments, including the proposed Yucca Mountain repository in this study. It is recognized that addition of nutrient sources such as water, hydraulic fluid, diesel exhaust, etc., and perturbations associated with mining and construction will likely enhance shifts in composition of the microbial community. For example, in an investigation comparing microbiota associated with the tunnel invert (floor, an environment experiencing periodic perturbations) with those from tunnel walls at Rainier Mesa, microbial counts were several orders of magnitude greater in the perturbed environment, and higher numbers of anaerobic bacteria were recovered (Haldeman, et al., unpublished data). Thus, the importance of non-culturable bacteria needs to be assessed, and discerning their identity will provide insight into the potential of microbial activity within the repository.

In this study, molecular techniques were employed to perform a culture-independent survey of microbial diversity.
by analysis of 16S ribosomal RNA gene sequences present in
the DNA pool directly extracted from rock. While all of
the culturable isolates were identified as gram positive
bacteria, with *Arthrobacter* being the dominant species in
the YM3 sample, native DNA analysis of 16S ribosomal RNA
genes showed that *Arthrobacter* and other gram positive
species only constituted approximately 43.5% of the
population. Also, sequences from diverse species of gram
negative bacteria were recovered from the community DNA
extraction as well. Gram negative species made up the
other 56.5% of the total population in YM3, including
*Rhizobium, Comamonas, Pseudomonas* and six other major taxa.
In the YM9 sample, the same predominance of gram positive
culturable isolates, as high as 90%, was found. However,
the clone library of 16S ribosomal RNA gene sequences from
extracted native DNA contained all gram negative bacteria,
with 86.6% of the population in that environment being
*Pseudomonas* species.

One explanation for this discrepancy may be that even
though *Arthrobacter* and other gram positive bacteria were
most commonly cultured, gram negative bacteria, like
*Pseudomonas*, were more highly represented in the rock in
forms of dormant, viable but non-culturable, or dead cells.
It has been shown that in oligotrophic subsurface
environments, such as Yucca Mountain in this study, the
majority of the cells are under starvation stress because of the limitation of water and nutrients (Amy, 1997). When analyzed in the laboratory, plate counts from such environments are typically several orders of magnitude lower than direct counts (Giovannoni, 1990; Haldeman, et al., submitted; Kieft, 1997). Additionally, *Pseudomonas* species are well known to inhabit and populate soils. *Pseudomonas*, and other gram negative cells, have been shown to become viable but non-culturable under starvation conditions (Oliver, 1993). It is reasonable to suspect that the high proportion of *Pseudomonas* 16S rDNA presented in the DNA pool came from the large proportion of *Pseudomonas* species in the native microbial community at some time in the past.

Although the YM9 sample had 89% culturable gram positive cells, none were detected in the DNA pool. If the community was dominated by gram negative bacteria, the percentage of gram positive culturable species in YM9 may have been too low to be detected in a clone sample size of 82. An increase in the sample size of the clone library could possibly detect low numbers of gram positive bacteria in situ, including recovered culturable isolates.

A study on biomass of Yucca Mountain (Kieft, 1997) showed that dead cells accounted for as much biomass as living cells. Because of the extreme conditions of Yucca
Mountain, DNA of dead cells might be preserved better than in other environments (e.g., organically-rich and humid environments). Total DNA analysis might also include a portion of dead cells contributing preserved DNA. Diversity analysis from total DNA extraction might reflect the original colonization of the rock or represent a mixture of bacterial types from past and present.

Microbial phylogenies, estimated from native DNA directly extracted from rock, showed higher diversity in YM3 than in YM9. This was consistent with our hypothesis that microbial diversity is related to geology and geochemistry of the environment. YM3 is a highly fractured rock stratum in the subsurface where water and carbon are relatively abundant and available for microorganisms in this habitat. A more diversified microbial community was expected in this environment. On the other hand, YM9 is a subsurface environment with low permeability at the repository depth, bacteria adapted to the YM9 environment were likely those that had developed starvation survival strategies. In addition to that, the regional isolation of YM9 should also account for the lower diversity in this environment. In the analysis of culturable isolates, the estimated microbial diversity in YM3 and YM9 were totally opposite to the sequence analysis of directly extracted rock DNA (Wu, 1998, Chapter 2). Culturable isolates recovered were more diverse in YM9 than in YM3 (Figure 1
and Figure 2 in Chapter 2). Since culturable isolates usually represent only a small portion of the total microbial community, results from culturable isolates may be misleading regarding microbial diversity in situ. Diversity of the whole community is best represented by direct extraction of DNA followed by cloning and sequencing.

Analysis of 16S rDNA sequence in both clone library of YM3 and YM9 showed that the major group of *Pseudomonas* in the YM9 site was close related to *Pseudomonas* species detected in the YM3 site. Some culturable strains of *Arthrobacter* in both sites were also found to be clustered. This observation implied the broad distribution of some species in Yucca Mountain.

Another potential problem in comparing the extracted native DNA clone library to the culturable isolates is the high level of spatial heterogeneity found in subsurface samples (Amy et al., 1992; Haldeman and Amy, 1993). The natural spatial heterogeneity could account for much of the sample to sample variation. However, comparison of the culturable isolates to the extracted/cloned DNA within the same sample remains valid.

The concentration of DNA extracted from 10 g of rock was not observed visually and estimated by either spectrophotometry or ethidium bromine-containing agarose gel. The reason for this is that microbial abundance in
this oligotrophic environment is generally low, as confirmed by direct microscopic cell counts, plate counts, and phospholipid fatty acid concentration (Kieft, 1997). Since this biomass was approximately 1-100 thousand times lower than the surface samples, methods were employed to minimize dilution of extracted DNA (modified from Tsai's protocol, 1991). One challenge of direct extraction was to ensure that the lysozyme used to lyse the cells was DNA free. Because the concentration of cells in the rock samples was low, trace amounts of DNA in the lysozyme would confuse the results. The success of the extraction was confirmed by amplification of community 16S rDNA fragments using PCR.

PCR has become a popular tool for retrieval of the 16S rDNA from natural environments with the intent to define genera and species as representatives of the native microbial community. However, a potential hazard associated with PCR is the creation of recombinant or chimeric products (Brakenhoff, et al., 1991; Kopczynski, et al., 1994; Liesack, et al., 1991). All of the sequences in this study were analyzed for possible chimera formation using the CHECK_CHIMERA program in the Ribosomal Database Project. Two clones in YM9, 9-1006 and 9-1009 were eliminated from analysis because of the possible existence of chimeras. Caution needs to be taken since this program can not efficiently detect chimeras formed by two closely
related species. In those cases, secondary structure analysis is necessary to confirm the results (Liesack, 1991).

Another concern when explaining the discrepancy between sequence data from rock DNA extracts and culturable isolates is the series of methods that lead to the final sequence analysis. The procedure of lysis, extraction, purification, PCR amplification, cloning, and sequencing may all or, in some part, favor segments of certain species' DNA, e.g., Pseudomonas, at the expense of other bacterial types. An extensive experiment was performed to address this issue.

Reference


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endolithic habitats within the deep subsurface. Appl Environ Microbiol 58:3367-3373


count and activities of heterotrophic bacteria in subsurface samples. J Microbiol Meth 21:305-316


sequences for phylogenetic analysis. Proc Intl Acad Sci USA. 82:6955-6959


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Table 1. Tentative Identification of 16S rDNA Clones From Yucca Mountain YM3 Using BLUST Search

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Closest Matching Species Using BLUST Search</th>
<th>Classification</th>
<th>Sequence Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1013</td>
<td><em>Microbacterium arborescens</em></td>
<td>High “G+C” gram positive</td>
<td>95%</td>
</tr>
<tr>
<td>3-1027</td>
<td><em>Arthrobacter nicotianae</em></td>
<td>High “G+C” gram positive</td>
<td>92%</td>
</tr>
<tr>
<td>3-1058</td>
<td><em>Arthrobacter nicotianae</em></td>
<td>High “G+C” gram positive</td>
<td>93%</td>
</tr>
<tr>
<td>3-1040</td>
<td><em>Arthrobacter globiformis</em></td>
<td>High “G+C” gram positive</td>
<td>95%</td>
</tr>
<tr>
<td>3-1045</td>
<td><em>Arthrobacter liquefaciens</em></td>
<td>High “G+C” gram positive</td>
<td>86.9%</td>
</tr>
<tr>
<td></td>
<td><em>Frankia sp.</em></td>
<td>High “G+C” gram positive</td>
<td>86.6%</td>
</tr>
<tr>
<td>3-1007</td>
<td><em>Bradyrhizobium sp.</em></td>
<td>α-proteobacterium</td>
<td>98%</td>
</tr>
<tr>
<td>3-1008</td>
<td><em>Unidentified bacterium</em></td>
<td>α-proteobacterium</td>
<td>94%</td>
</tr>
<tr>
<td></td>
<td><em>Bradyrhizobium sp.</em></td>
<td>β-proteobacterium</td>
<td>94%</td>
</tr>
<tr>
<td>3-1011</td>
<td><em>Agrobacterium sp.</em></td>
<td>α-proteobacterium</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td><em>Agrobacterium tumefaciens</em></td>
<td>α-proteobacterium</td>
<td>94%</td>
</tr>
<tr>
<td>3-1001</td>
<td><em>Rhizobium sp.</em></td>
<td>α-proteobacterium</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td><em>Rhizobium loti</em></td>
<td>β-proteobacterium</td>
<td>96%</td>
</tr>
<tr>
<td>3-1017</td>
<td><em>Unidentified bacterium</em></td>
<td>β-proteobacterium</td>
<td>81%</td>
</tr>
<tr>
<td></td>
<td><em>Nevskia ramosa</em></td>
<td>β-proteobacterium</td>
<td>81%</td>
</tr>
<tr>
<td>3-1047</td>
<td><em>Pseudomonas anguilliseptica</em></td>
<td>β-proteobacterium</td>
<td>94%</td>
</tr>
<tr>
<td>3-1002</td>
<td><em>Pseudomonas aureofaciens</em></td>
<td>β-proteobacterium</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas putida</em></td>
<td>β-proteobacterium</td>
<td>96.6%</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th></th>
<th>Species</th>
<th>Class</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1020</td>
<td><em>Ideonella dechloratans</em></td>
<td>β-proteobacterium</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td><em>Ralstonia sp.</em></td>
<td></td>
<td>87%</td>
</tr>
<tr>
<td>3-1044</td>
<td><em>Beta proteobacterium</em></td>
<td>β-proteobacterium</td>
<td>97%</td>
</tr>
<tr>
<td>3-1038</td>
<td><em>Pseudomonas sp.</em></td>
<td>γ-proteobacterium</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td><em>Comamonas sp.</em></td>
<td></td>
<td>88%</td>
</tr>
<tr>
<td>3-1006</td>
<td><em>Pseudomonas testosteroni</em></td>
<td>γ-proteobacterium</td>
<td>99.5%</td>
</tr>
<tr>
<td></td>
<td><em>Comamonas sp.</em></td>
<td></td>
<td>93%</td>
</tr>
<tr>
<td>3-1039</td>
<td><em>Methylophilus methylotrophus</em></td>
<td>γ-proteobacterium</td>
<td>88%</td>
</tr>
</tbody>
</table>
Table 2. Classification of 16S rDNA Clone Library of YM3

<table>
<thead>
<tr>
<th>Classification</th>
<th># of Clones Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High G+C Gram Positive</td>
<td></td>
</tr>
<tr>
<td>Arthrobacter &amp; Relatives</td>
<td>17 (37%)</td>
</tr>
<tr>
<td>Microbacterium &amp; Relatives</td>
<td>2 (4.4%)</td>
</tr>
<tr>
<td>Other Unidentified</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>Gram Negative</td>
<td>26 (56.5%)</td>
</tr>
<tr>
<td>Proteobacterium α-subdivision</td>
<td></td>
</tr>
<tr>
<td>Rhizobium sp.</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>Bradyrhizobium sp.</td>
<td>2 (4.4%)</td>
</tr>
<tr>
<td>Agrobacterium sp.</td>
<td>2 (4.4%)</td>
</tr>
<tr>
<td>Proteobacterium β-subdivision</td>
<td></td>
</tr>
<tr>
<td>Comamonas &amp; Relatives</td>
<td>4 (8.7%)</td>
</tr>
<tr>
<td>Methylophiles &amp; Relatives</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>Rubrivivax &amp; Relatives</td>
<td>2 (4.4%)</td>
</tr>
<tr>
<td>Other unidentified</td>
<td>8 (17.4%)</td>
</tr>
<tr>
<td>Proteobacterium γ-subdivision</td>
<td></td>
</tr>
<tr>
<td>Nevakia &amp; Relatives</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>5 (10.9%)</td>
</tr>
</tbody>
</table>
Table 3. Tentative Identification of 16S rDNA Clones from YM9 Using BLUST Search

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Closest Matching Species Using BLUST Search</th>
<th>Classification</th>
<th>Sequence Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-1005</td>
<td><em>Yersinia sp.</em></td>
<td>γ-proteobacterium</td>
<td>93%</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-1059</td>
<td><em>Erwinia carotovora</em></td>
<td>γ-proteobacterium</td>
<td>88.5%</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
<td></td>
<td>88%</td>
</tr>
<tr>
<td>9-1003</td>
<td><em>Pseudomonas libaniensis</em></td>
<td>γ-proteobacterium</td>
<td>95%</td>
</tr>
<tr>
<td>9-1077</td>
<td><em>Pseudomonas libaniensis</em></td>
<td>γ-proteobacterium</td>
<td>92%</td>
</tr>
<tr>
<td>9-1090</td>
<td><em>Pseudomonas libaniensis</em></td>
<td>γ-proteobacterium</td>
<td>93%</td>
</tr>
<tr>
<td>9-1030</td>
<td><em>Pseudomonas viridiflava</em></td>
<td>γ-proteobacterium</td>
<td>95%</td>
</tr>
<tr>
<td>9-1076</td>
<td><em>Pseudomonas testosteroni</em></td>
<td>β-proteobacterium</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td><em>Comamonas sp.</em></td>
<td></td>
<td>87%</td>
</tr>
<tr>
<td>9-1087</td>
<td><em>Janthino lividum</em></td>
<td>β-proteobacterium</td>
<td>88%</td>
</tr>
<tr>
<td></td>
<td><em>Zoogloea sp.</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-1037</td>
<td><em>Janthino lividum</em></td>
<td>β-proteobacterium</td>
<td>94%</td>
</tr>
<tr>
<td>9-1057</td>
<td><em>Janthino lividum</em></td>
<td>β-proteobacterium</td>
<td>96%</td>
</tr>
<tr>
<td>9-1010</td>
<td><em>Janthino lividum</em></td>
<td>β-proteobacterium</td>
<td>92%</td>
</tr>
</tbody>
</table>
Table 4. Classification of 16S rDNA Clone Library Of Yucca Mountain YM9

<table>
<thead>
<tr>
<th>Gram Negatives</th>
<th># of Clones Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteobacterium, β-subdivision</strong></td>
<td></td>
</tr>
<tr>
<td>Comamonas &amp; Relatives</td>
<td>1 (1.2%)</td>
</tr>
<tr>
<td>Janthino &amp; Relatives</td>
<td>7 (8.5%)</td>
</tr>
<tr>
<td><strong>Proteobacterium, γ-subdivision</strong></td>
<td>74 (90.2%)</td>
</tr>
<tr>
<td><em>Yersinia, E.coli</em> and Relatives</td>
<td>3 (3.7%)</td>
</tr>
<tr>
<td><em>Pseudomonas &amp; Relatives</em></td>
<td>71 (86.6%)</td>
</tr>
</tbody>
</table>
Figure 1. Microbial Diversity of 16S rDNA from Native DNA Extraction in YM3

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Figure 2. Microbial Diversity of 16S rDNA from Native DNA Extraction in YM9
Figure 3. Relatedness of Microbial Diversity of 16S rDNA Detected in Native DNA Extraction in YM3 and YM9
CHAPTER 4

TECHNICAL BIAS ANALYSIS

DURING DETERMINATION OF BACTERIAL PHYLOGENY

FROM WHOLE COMMUNITY DNA EXTRACTIONS

This chapter has been prepared for submission to the Journal of Microbiological Methods and is presented in the style of that journal.
Summary

Analysis of microbial diversity using 16S ribosomal RNA gene sequences extracted from environmental samples has gained wide acceptance by microbiologists. Because the process is independent of culturing, it has been proven to be a powerful tool for detecting novel populations in microbial communities. However, the process contains a series of steps that may cause bias in the recovery of DNA fragments from a given environmental sample. Therefore, quantification of the components of the microbial community using 16S ribosomal DNA remains problematic. To estimate the potential bias of the process of molecular analysis as a whole, 10 bacterial strains, which are commonly found in soil habitats, were seeded into sterile rock in equal amounts. A 16S ribosomal DNA (rDNA) clone library was constructed after direct cell lysis, total DNA extraction, polymerase chain reaction (PCR) amplification of 16S rDNA with a pair of universal bacterial primers, and cloning of the amplified fragments. The proportion of each strain in the final clone library was estimated by the Restriction Fragment Length Polymorphism (RFLP) technique. The process showed no significant difference between gram positive and gram negative bacterial groups. However, certain species within each group were favored at the expense of others. *Pseudomonas*, represented by three species in the sample, was underestimated.
Introduction

Viable plate counts and/or most-probable-number techniques have been, and frequently still are, used for quantification of active bacteria in various environmental samples. Microbial ecologists realize that culturable bacteria represent only a small portion of the whole community, because laboratory culture conditions only select for certain organisms. It is now accepted that the majority of cells in the environment are viable but do not form visible colonies on plates (Amann, et al., 1995; Olsen and Bakken, 1987; Torsvik, et al., 1990).

Developments in molecular technology have changed the face of microbial ecology. With the advance of PCR and nucleotide sequencing, it is now possible to amplify a specific gene, particularly microbial 16S rDNA genes, from mixed DNA directly extracted from the environment. Phylogenetic analysis of environmental microorganisms using genetic molecular markers can then be used independently of the culturing step.

The power of a molecular marker used for phylogenetic inference is widely accepted (Olsen, et al., 1986; Woese, 1987). However, it is impossible to quantify the number of species represented in the environment using genetic molecules amplified by PCR from the total extracted DNA (Amann, et al., 1995; Farrelly, et al., 1995). There are several steps in analysis, which are open to error or bias,
including cell lysis, DNA extraction and purification processes, PCR amplification, choice of primers and cloning vectors and methods. While errors or bias related to methodology may be overcome or minimized with improved techniques, the cellular and genomic properties of bacteria may cause significant bias during analysis. First, the genome size and gene copy number (rRNA gene number), which vary from species to species, are two parameters that will affect the actual population ratio in the PCR products from the community (Cole, et al., 1994; Farrelly, et al., 1995). Second, lysis conditions of different cells, which are partially controlled by cell membrane contents, and physiological stage of the cells are other variables of microbes that may contribute to bias in the molecular analytical process (Silve and Batt, 1995).

Studies of microbial diversity at Yucca Mountain have shown a significant discrepancy between the analysis of culturable isolates and the clone library of 16S rDNA from a mixed population nucleic acid extraction (Wu, 1998). While Arthrobacter and other gram positive cells were the most dominant culturable isolates, sequences in the 16S rDNA clone library from native microbial DNA demonstrated that Pseudomonas and other gram negative Proteobacteria were the most abundant taxa. In the YM9 sample, no gram positive bacterial sequences were detected and Pseudomonas sp. accounted for about 86.6% of the total populations by
native DNA analysis. One explanation for this discrepancy may be that even though Arthrobacter were most commonly cultured, Pseudomonas species were more highly represented in the rock in forms of dormant, viable but non-culturable (VBNC), or dead cells. Another possibility is that technical bias existed in the series of methods that lead to the final sequence analysis, including lysis, DNA extraction, purification, PCR amplification, cloning, and cycle sequencing. The protocols may all, or in some part, favor segments of Pseudomonas or other Proteobacteria DNA at the expense of other bacterial types.

To address these possible technical biases, 10 bacterial strains were selected and seeded onto ground sterile rock in equal amounts. The seeded rock was then subjected to the same process of diversity analysis along with YM3 and YM9 rock samples. The proportion of each strain in the clone library was determined by RFLP and used to estimate the potential bias of the entire process.

**Materials and Methods**

Ten ATCC bacterial strains commonly found in soil were selected for this study (Table 1). Each strain was first cultured in 100 ml of R2B, a low nutrient medium designed for culturing environmental microorganisms. Cultures were incubated in an EnvironShaker (LAB LINE Instruments, Inc., Illinois) at 25°C (approximately 3 d) until reaching late
stationary phase. Three ml of each culture was fixed with 3 ml of 4% filter sterilized formaldehyde (1:1 vol/vol). Cultures were stored after mixing equal volumes of culture with 20% sterile glycerol in cryogenic vials (Nalge Company, New York) and frozen at -70°C until use.

Fixed cultures were used to perform the acridine orange staining and enumeration by fluorescent microscopy (Optiphot, Nikon, Inc.). Then, one vial of each frozen culture was thawed and approximately $1 \times 10^6$ cells of each strain were seeded into 10 g of sterile rock, which had been previously baked at $400^\circ C$ for 6 h.

The seeded rock sample was then subjected to the same protocol as described previously for DNA extraction (Wu, 1998a), 16S rDNA amplification using PCR (GeneAmp PCR System 2000, Perkin Elmer), then amplified fragments were ligated into the plasmid vector pCR™II and transformed into One Shot™ INV®F' competent cells using an Original TA Cloning Kit (Invitrogen Corp., San Diego, CA). Each fragment in the clone library was analyzed by RFLP (Restriction Fragments Length Polymorphism). Clones were first amplified with vector primers M13f (5'-TGT AAA ACG ACG GCC AGT-3') and M13r (5'-CAG GAA ACA GCT ATG ACC-3'). The clones containing a 1.75 kb amplified product were selected as positive clones and subjected to PCR again using universal eubacterial 16S rDNA primers, 16S-27f (5'-GAG AGT TTG ATC CTG GCT CAG-3') and 16S-1492r (5'-CTA CGG
CTA CCT TGT TAC GA-3'). Amplified fragments from each clone were digested with restriction endonuclease AvaII and separated on a 1% TAE agarose gel containing ethidium bromide (0.5 μg/ml). Each clone was then identified by comparing its AvaII endonuclease digestion pattern to the patterns of the ten original isolates. The experiment was repeated twice and the colonies were pooled. The proportion of each strain recovered by the whole process was then used to estimate the potential bias of the analysis.

Results and Discussion

The 16S rDNA of each pure strain was amplified and subjected to AvaII digestion. When digested fragments were separated on an agarose gel, there were nine distinct restriction patterns that later served as pattern standards. *Micrococcus luteus* and *Arthrobacter globiformis* could not be separated by the AvaII single digestion. For the purpose of this study, further recognition for these two species was not necessary. They were grouped as high "G+C" content gram positive bacteria.

Although all ten types of seeded bacteria were detected with RFLP analysis, the distribution of each type of recovered clone was not even. The protocol appears to be biased for or against certain species. Figure 1 shows the number of clones that were recovered for each seeded
species. A $\chi^2$ test showed that there was no significant difference between species in the gram positive group (including *Micrococcus luteus*, *Arthrobacter globiformis*, and *Bacillus subtilis*) and the gram negative group (including *Alcaligenis faecalis*, *Comamonas testasteroni*, *Xanthomonas maltophilia*, *Acinetobacter calcoaceticus*, *Pseudomonas aeruginosa*, *Pseudomonas diminuta*, and *Pseudomonas putida*) ($\alpha=0.01$).

All three species of the genus *Pseudomonas* were recovered in the clone library. When the recovery of *Pseudomonas* species was compared to the remaining four species of gram negative bacteria, there was a significant difference between the *Pseudomonas* sp. and the other gram negative bacteria. The *Pseudomonas* sp. was not favoured by the process.

Within the gram positive bacteria, the low "G+C" species, *Bacillus subtilis*, made up about 80% of the gram positive species recovered. This was approximately 47% over the original 33% at the time of seeding. Therefore, the high "G+C" group, which included *Micrococcus luteus* and *Arthrobacter globiformis*, was not favoured by this process.

Even though the ratio of the recovered seeded species was biased to certain individual bacteria, the experiment
showed no significant difference between gram positive and gram negative groups. Therefore, using the techniques of this study, a relatively close picture of the community with respect to groups designated by gram reaction will be obtained.

Analysis using 16S rDNA sequences from the total extracted DNA in rock sample YM9, showed that about 86.8% of the sequences in the clone library consisted of *Pseudomonas* (Wu, 1998). Because the genus *Pseudomonas* is not favoured by the process according to the seeded bacteria experiments, it is possible that the actual percentage of *Pseudomonas in situ* may be even higher than estimated. One possible explanation is that over very long periods of nutrient deprivation, *Pseudomonas sp.* may have become viable-but-non-culturable (VBNC) cells (Oliver, 1993). Even though the gram positive bacteria, such as *Arthrobacter*, were highly represented among the culturable isolates, *Pseudomonas* was the most prevalent taxa in this specific subsurface environment. *Arthrobacter*, along with some other gram positive bacteria, appeared to have superior ability to remain culturable over long periods of time.

In an attempt to grow the isolates used in the seeding experiment under conditions similar to the natural soil environment, the cells were cultivated in a low nutrient
medium, R2A (Difco, Laboratories, Detroit). It has been observed that the proliferation of cells in low nutrient and rich nutrient media result in drastically different cell shapes and sizes. When incubated in a rich medium such as TSB (tryptic Soy Broth), the cells displayed enlarged, elongated and filamentous shapes. In low-nutrient medium, R2A, cells were obviously miniaturized and displayed either coccibacilli or small rod shapes (observation only, data not shown). These observations correlate with the dwarf cells observed in other oligotrophic subsurface environments (Kieft, 1996). In environments where nutrient and water are limited, the majority of cells enter a special stage as the strategy for starvation. Nearly total metabolic arrest may occur in some cell types (Amy, 1997), cell shapes are miniaturized (Kieft, 1996, Matin, 1989), and macromolecular quantities and cellular density are changed (Amy, 1983). Studies have shown that cellular and biochemical alterations happen during starvation (Amy, 1983, Kieft, 1994), and the cell membrane transport mechanisms may be enhanced in low-nutrient environments (Geesey and Morita, 1979). Specific cellular changes of various species in low nutrient environments may indeed alter the lysis condition for each type of cell in the community. These cellular alterations may contribute significantly to differential extraction of DNA from the environmental samples. There is also evidence
for differential macromolecular degradation for starved cells. In one case, ribosomal RNA was preferentially degraded, which will in turn alter the total copy number of 16S rDNA (Matin, 1989).

The genome size and gene copy number as a result of PCR amplification of 16S rRNA genes from a mixture of bacterial species has been examined (Farrelly, et al., 1995) and shown to correlate with gene amplification from a mixture of community DNA. In studies of environmental samples, where genome size and gene copy number are not known for all the species, quantification of populations in microbial communities is not possible from analysis of 16S rDNA clone libraries.

In the seeded rock study, all 10 species were recovered using the techniques described. Both gram positive and gram negative groups were found even though individual species were represented in varying quantities. The methods of DNA extraction and gene amplification are powerful enough to detect novel species in the environment and estimate the relative abundance of major groups.

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Table 1. 10 ATCC Seeding Strains

<table>
<thead>
<tr>
<th>ATCC #</th>
<th>Bacteria strain</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>381</td>
<td>Micrococcus luteus</td>
<td>High &quot;G+C&quot;, Gram Positive</td>
</tr>
<tr>
<td>B776</td>
<td>Arthrobacter globiformis</td>
<td>High &quot;G+C&quot;, Gram Positive</td>
</tr>
<tr>
<td>6051</td>
<td>Bacillus subtilis</td>
<td>Low &quot;G+C&quot;, Gram Positive</td>
</tr>
<tr>
<td>10145</td>
<td>Pseudomonas aeruginosa</td>
<td>Gamma Proteobacterium,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gram Negative</td>
</tr>
<tr>
<td>11568</td>
<td>Pseudomonas diminuta</td>
<td>Gamma Proteobacterium,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gram Negative</td>
</tr>
<tr>
<td>12633</td>
<td>Pseudomonas putida</td>
<td>Gamma Proteobacterium,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gram Negative</td>
</tr>
<tr>
<td>23055</td>
<td>Acinetobacter calcoaceticus</td>
<td>Gamma Proteobacterium,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gram Negative</td>
</tr>
<tr>
<td>13637</td>
<td>Xanthomona maltophilia</td>
<td>Gamma Proteobacterium,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gram Negative</td>
</tr>
<tr>
<td>11996</td>
<td>Comamonas testasteroni</td>
<td>Beta Proteobacterium,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gram Negative</td>
</tr>
<tr>
<td>8750</td>
<td>Alcaligenis faecalis</td>
<td>Beta Proteobacterium,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gram Negative</td>
</tr>
</tbody>
</table>
Figure 1. Proportion of 10 Seeded Bacteria Recovered
CHAPTER 5

GENERAL DISCUSSION

Microbiological research of the subsurface at Yucca Mountain has included anaerobic microbiology (Clarkson et al., 1996), autotrophic and heterotrophic diversity (Khalil et al., 1996), distribution and limiting nutrients of heterotrophic microbiota (Haldeman et al., 1996; Kieft et al., 1997), in situ characterization of microbiota by lipid analysis (Ringelberg et al., 1996), microbially influenced corrosion (Pitonzo et al., 1996), and other possible impacts that microbiota may have on man-made materials associated with repository construction (Horn et al., 1996; Horn and Meike, 1995). Since most of these researches were based on laboratory studies of culturable bacteria isolated from the rock, it is possible that estimation of microbial diversity is incomplete, because only a small fraction of the microbial community can be cultured from natural environments (Brockman, et al., 1992; Colwell, 1989; Haldeman, et al., submitted a; Marxsen, 1988; Vescio and Nierzwicki-Bauer, 1995). This may be especially true of
the subsurface, where cells have survived starvation and age extremes over geological time.

The research presented in this dissertation is the only analysis of the subsurface microbial community in Yucca Mountain using total DNA extracted from the tuffs. Molecular techniques were applied in this study to identify the culturable isolates obtained from subsurface tuff deep within Yucca Mountain, and to describe the microbial diversity in situ in the absence of culture-based methods. The topics covered in this chapter include: 1) identification of culturable isolates by 16S rDNA sequences; 2) the microbial community represented by total extracted DNA; 3) microbial ecology; and 4) technical concerns.

Identification of Culturable Isolates by 16S rDNA Sequences

Using 16S rDNA sequencing techniques to identify culturable isolates from Yucca Mountain has proven to be a fast and accurate approach. About 400 bp were obtained for each isolate, and comparisons of sequences to data bases of previously described microbes can place the isolates to the genus level. The results from 16S rDNA sequencing were compared to MIDI analysis (Microbial Identification System, Co.), an independent phenotypic identification system, in this study. Even though, the use of the MIDI system has proven to be a more time/cost efficient method of
determining the identity of strains and their relatedness (Haldeman, 1994), and results from MIDI analysis were consistent with the 16S ribosomal DNA sequencing when interpreting the major groups of isolates. However, the MIDI system failed to detect some of the isolates from Yucca Mountain. This is mainly due to the limitation of the MIDI data base. Since the MIDI system was developed primarily for identification of clinically related isolates, it is necessary to establish an environmental microbial database to fulfill the functions desired in this study. Although some attempts to do so have been made, the variety of lipids composing a bacterium are not as great as the potential combinations available with sequencing nucleic acids, and therefore, nucleic acid sequencing will always provide greater discrimination when a large variety of genera are involved.

A standard procedure required by MIDI analysis is incubation of each isolate on tryptic soy agar plates for 24 h prior to fatty acid extraction. This stabilizes the conditions under which membrane lipids are formed, such as temperature, nutrient composition, and different growth stages (Malmcrona-Friberg, et al., 1986; Oliver and Stringer, 1984). In this study, some isolates failed identification by the MIDI system due to the lack of initial growth on standard trpticase soy agar (TSA). Because of the oligotrophic characteristics of the Yucca
Mountain environment, culturable isolates were originally recovered on a low nutrient medium, R2A. When inoculated in TSA medium for the MIDI analysis, the rich nutrients may have suppressed the growth of some truly oligotrophic microorganisms. For efficient application of the MIDI system to environmental microbiology, especially subsurface microbial research, a new MIDI library is needed that uses low-nutrient media as its standardized growth regime.

Selection of bacterial types for this study was based on the assumption that colonies of the same morphotype represent the same species or biotype. Much of the research in the deep subsurface has relied on selection and characterization of representative colonies with distinct colony morphotypes (Haldeman and Amy, 1993), however, it has been shown that genetic diversity within physiologically similar bacteria can be extensive (Jimenez, et al., 1990). Studies based on colony morphology would certainly lose some of the potential diversity, yet represent a reasonable first step to investigation of isolates through rDNA sequencing.

**Microbial Community Represented by Total Extracted DNA**

By analyzing 16S rDNA sequences taken from a total DNA extraction of rock, a clone library was generated that eliminated the bias of cultivation and thus better represents the range of microbial diversity in Yucca
Mountain. Using this approach, several bacterial families were recovered which were not obtained by culturing techniques. DNA analysis detected 56% of the microbial community of sample YM3 as gram negative species, and 100% of sample YM9 as gram negative bacteria, a significant shift from the composition of culturable isolates. In the YM3 sample, all of the culturable isolates were identified as gram positive species, with the majority classified as Arthrobacter sp. or other high "G+C" gram positive species.

Similar results were obtained for the YM9 sample, except that 10% of the isolates were identified as gram negative bacteria, which included Pseudomonas and Acinetobacter groups. The other 90% of the isolates belonged to the high "G+C" gram positive bacteria, and again, Arthrobacter was the major representative.

Since Pseudomonas sp. and some other gram negative bacteria are very common inhabitants that exist in nearly all kinds of environmental samples, it was a surprise that culturable isolates from two study sites at Yucca Mountain, YM3 and YM9, contained none or only 10% of gram negative bacteria, respectively. Results showed that while commonly represented by DNA sequences, Pseudomonas and other gram negative bacteria were underestimated by culturable isolates.
It is reasonable to suspect that even though *Pseudomonas* sp. are highly represented in the tuffs, they might be dormant, viable-but-none-culturable (VBNC), or even exist as dead cells. It has been shown that in oligotrophic subsurface environments, such as Yucca Mountain in this study, the majority of cells were under starvation stress because of the remote feature of the sites and the limitation of water and other nutrients (Amy, 1997 and Kieft, et al., 1997). *Pseudomonas* and some other gram negative species have been shown to become VBNC under starvation conditions (Oliver, 1993). The cells that were VBNC could not be recovered in laboratory media although, after a resuscitation process they could regain some of the original the culturability, as demonstrated by Pitonzo, et al (Pitonzo, et al., 1998). Shift in microbial composition after disturbance, perturbation, or handling and storage of the samples from Rainier Mesa, a similar environment to Yucca Mountain, further suggests the existence of dormant and VBNC cells in this type of environment (Fredrickson, et al., 1995; Haldeman, et al., 1994, 1995;). Such findings have also confirmed observations from direct microscopic counts that plate counts only represent a small portion of the natural diversity.

Dead cells in Yucca Mountain tuffs were nearly as numerous as living cells (Kieft, et al., 1997), as indicated by the concentration of diglyceride fatty acid
(0.2-2.3 pmol g⁻¹), a measure of dead cell biomass, and phospholipid fatty acid (0.1-3.7 pmol g⁻¹), a measure of live cell biomass, extracted directly from the tuffs. Therefore, it is reasonable to suspect that undetected gram negative cells might be primarily dead cells. Detection and analysis of the microbial community from the rock-extracted DNA pool would not be able to separate live cells from dead cells contributing preserved DNA. Due to the low permeability of the rock, and low water and nutrient content, DNA of dead cells might very well be preserved better than in other environments, e.g., organically rich and humid environments. Therefore, a total DNA diversity analysis might reflect a mixed diversity of the past and the present. This is a hazard in using a culture-independent method to interpret the diversity in an extreme environment like the subsurface in Yucca Mountain. Finding a way to identify the live and active populations in environments will still be a challenge to microbiologists of the future.

Bacteria recovered as culturable isolates from Yucca Mountain, such as Arthrobacter and Actinomycetes groups, were represented in the clone libraries in small numbers or were not detected at all. This phenomenon was also detected by other researchers regarding bacterial diversity of various environments (Stackbrandt, et al., Kuske, et al, 1997). The proportion of culturable isolates in
environmental samples is often too low, and the resulting size of the clone library from oligotrophic environments is not big enough to detect all members of the culturable group. Increasing the size of the clone library, if possible, may increase the chance of detecting more culturable representatives in the DNA pool.

Since microbial abundance is near the detectable limit (Kieft, et al., 1997), DNA extraction from 10 g of rock was not sufficient to be directly quantified using standard spectrophotometer or ethidium bromine stain. Sufficient copies of 16S rDNA were prepared by amplification using PCR. There is a potential hazard of creating recombination or chimeric products (Kopczyński, et al., 1994; Brakenhoff, et al., 1991; Liesack, et al., 1991). Even though all of sequences were analyzed using the CHECK_CHIMERA program in the Ribosomal Database Project, the chimeras formed by two closely related species can not be efficiently detected with this program. A secondary structure analysis might be necessary in those cases.

Microbial Ecology

Selection of a placement site for high-level nuclear waste canisters is largely based on the geological and geochemical characteristics of the volcanic tuff in Yucca Mountain. The YM9 sample was obtained from the repository level. Because it is located in a rock bed composed of
unsaturated tuff with relative low permeability, lower microbial diversity was expected than at the YM3 site, which came from a highly fractured rock stratum. Analysis of microbial/DNA diversity resulting from direct extraction of rock material confirmed this hypothesis. In the YM3 sample, both gram positive and gram negative bacteria were recovered. The gram negative bacteria included α-subdivision proteobacteria (such as Rhizobium sp., Brandyrhizobium sp., and Agrobacterium sp.), β-subdivision proteobacteria (such as Comamonas sp., Alcaligenes sp.), and γ-subdivision proteobacteria (including most species of Pseudomonas). Also, 43.5% of the DNA molecules were identified as coming from high "G+C" gram positive bacteria, these being indicative of the major portion of culturable isolates: Arthrobacter sp. In the YM9 sample, only Gram negative bacteria were detected, with 90% being from of γ-subdivision proteobacteria and the other 10% from the β-subdivision proteobacteria. On the other hand, analysis from the culturable isolates demonstrated the reverse situation in which YM9 was higher in microbial diversity than YM3. Therefore, this study, like so many others, demonstrates the questionable reliability of diversity estimations based on culturable isolates. Although this appears to be the case, caution must be taken
when using material from ancient environments since much of
the biomass, and therefore its remaining DNA, may be dead.
If so, it is not indicative of the current microbial
community, only that of long ago. In fact, it may be most
indicative of the original colonization and rain recharge
influences.

While diversity between two study sites, YM3 and YM9,
were vary, there were certain species highly related
evolutionarily, represented both by culturable isolates and
DNA extraction. The relatedness of species between two
geologically different sites may imply the broad existence
of these strains.

Technical Concerns

Even though the molecular techniques used did not show
significant preference when interpreting the major
bacterial diversity into gram positive and gram negative
bacteria, there are certainly biases within each group.
When tested with 10 known common soil bacteria types,
seeded in the sterile rock, three species of Pseudomonas
were recovered in the final clone library, but their
percentage was significantly lower than that of other
species. This result implies that the actual number of
Pseudomonas sp. in Yucca Mountain might be even higher than
detected.
The Significance of this Project

The detection of genetic diversity within a natural environment can be considered the very first step towards the understanding of the role that bacteria play in an ecosystem. Sequence analysis of 16S rDNA fragments directly extracted from Yucca Mountain eliminated bias associated with culturing steps and presented a more complete picture of microbial diversity in situ. Even though the total number of microbes in Yucca Mountain, an oligotrophic environment, was estimated to be low, the influence of interactions between the indigenous microbial community and radioactive wastes may have significant impact on alteration of radionuclide transportation in the long term. Additionally, microbes may play a key role in microbially influenced corrosion of repository structures including radioactive material containers. The research presented in this dissertation will facilitate the identification of potential factors that may limit microbial growth and activity in Yucca Mountain. The identity of major populations of microorganisms in Yucca Mountain will also be important for development of strategies for the storage of high-level nuclear waste and the maintenance of the repository integrity.
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