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The microbial diversity associated with sulfide structures from three different hydrothermal vent fields was examined using phylogenetic analysis based on the small subunit ribosomal RNA gene (16S rDNA). Samples were collected from sites at 9°N, Guaymas Basin and the Juan de Fuca Ridge and analyzed with denaturing gradient gel electrophoresis (DGGE). In addition, clone libraries were constructed for two samples from Guaymas Basin. The *Bacteria* from Juan de Fuca Ridge all clustered within the ε-*Proteobacteria*, while the *Archaea* identified from all three sites were *Euryarchaeota*. Among the *Euryarchaeota* were sequences clustering within the *Methanococcales*, *Methanosarcinales*, *Methanobacteriales*, and *Methanomicrobiales*; the later two groups have not previously been identified as part of the microbial community at deep-sea hydrothermal vents. Two groups of sequences were identified that cluster with sequences previously only identified from hydrothermal systems in the western Pacific and the Mid-Atlantic Ridge; these groups may be endemic to deep-sea hydrothermal vents.

Microbial Diversity of Sulfide Structures from Hydrothermal Vent Sites at 9°N, Guaymas Basin and the Juan de Fuca Ridge

by

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Krista Longnecker, Author

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CONTRIBUTION OF AUTHORS

Dr. Anna-Louise Reysenbach was involved in the experimental design, execution of the research and analysis of the data for all of the manuscripts. Dr. Craig Cary and Dr. George W. Luther, III aided in the sample collection for the first two manuscripts. Dr. Margaret K. Tivey conducted the mineralogical analysis for the third manuscript and aided in the analysis of the data for that manuscript.

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1 Introduction

1.1 Background

Prior to the discovery of hydrothermal vents, their presence had been predicted based on global budgets of heat and metals, models of convective cooling of oceanic crust, anomalous heat flows at mid-ocean ridges, the presence of thermally altered basalt and metalliferous deep-sea sediments (Kennett 1982). However, scientists had not predicted the range of life forms that would be found at deep-sea hydrothermal vents. The first indications that biological life forms existed at deep-sea hydrothermal vents were still photographs taken during a cruise to the Galapagos Rift in 1976 (Lonsdale 1977). Later, the first submersible dives to hydrothermal vents near the Galapagos Rift in 1977 identified some of the larger invertebrates now classically associated with hydrothermal vents (Corliss et al. 1979). The unusual tube worms with their spectacular red plumes, the crabs crawling over the basalt, and the spaghetti worms swaying in deep-sea currents have

captured the imagination of much of the world thanks to television images, newspaper and magazine articles.

While the macrofauna may be visually stimulating to the rest of the world, the prokaryotes make life at hydrothermal vents interesting. This ecosystem is one based on chemolithotrophy rather than the photolithotrophy that prevails in most of the ecosystems on Earth. Chemolithotrophy is possible because the microorganisms are able to use the reduced chemical compounds in the hydrothermal fluid as an energy source. Not all microorganisms present at deep-sea hydrothermal vents are capable of this; however there are both free-living microorganisms and symbiotic *Bacteria* capable of chemolithotrophy and these organisms serve as the base of the food web at hydrothermal vents.

As in other ecosystems, the metabolic capacity of the microorganisms found at deep-sea hydrothermal vents is far from known. Prokaryotes that are involved in sulfur cycling at hydrothermal vents include the chemolithotrophic *Desulfurobacterium thermolithotrophum* (L'Haridon et al. 1998), the heterotrophic sulfur-reducing *Thermococcales* and the *Archaeoglobales* (Baross and Deming 1995). Two orders of methanogens are also found at hydrothermal vents: the *Methanococcales* which have been isolated from the East Pacific Rise (Jones et al. 1983), Guaymas Basin and 23°N on the Mid-Atlantic Ridge (Jeanthon et al. 1999), and *Methanopyrales* whose only described deep-sea isolate is from Guaymas Basin (Huber et al. 1989; Burggraf et al.

1991; Kurr et al. 1991). The list of species described from hydrothermal vents is short, but informative. The majority of species are involved in sulfur cycling, hydrogen oxidation, or methane production, thus linking the microbial community and the chemical environment at deep-sea hydrothermal vents.

In addition to cultures obtained from samples that were returned to the surface, there have been attempts to grow microorganisms in situ at several hydrothermal vent sites. Microbial CO_2 fixation has been measured from syringes pre-loaded with ^{14}C-labeled bicarbonate that were filled with hydrothermal fluid and left in place on the seafloor (Jannasch and Wirsen 1979; Jannasch and Mottl 1985; Wirsen et al. 1993). A chamber filled with solid media designed to select for aerobic thermophiles was deployed at Snakepit on the Mid-Atlantic Ridge (MAR) for three days. Following recovery of the chamber, enrichment cultures of *Bacillus*-like and *Thermotoga*-like microorganisms were obtained from pieces of the solid media (Marteinsson et al. 1997). Finally, a titanium chamber was deployed exposed to vent fluid for five days at a hydrothermal vent on the MAR (Reysenbach et al. 2000). After the chamber was returned to the surface, phylogenetic analysis based on 16S rDNA was used to analyze the microbial community that had grown on surfaces attached to the interior of the growth chamber (Reysenbach et al. 2000).

Phylogenetic analysis based on 16S rDNA relies on the conserved nature of the gene that encodes for the small subunit of the ribosome (the 16S

subunit). The gene is assumed to be conserved because all cells need to produce ribosomes in order to translate RNA into proteins. The establishment of large databases that are repositories of the sequences have made them readily available for comparisons among the three domains of life. Furthermore, the variable and conserved regions of the 16S rDNA have been delineated, so analysis of a community is possible using a variable region of the gene to identify the microorganisms present within a sample. For example, denaturing gradient gel electrophoresis (DGGE) relies on the polymerase chain reaction (PCR) to amplify a variable region of the 16S rRNA gene (Muyzer et al. 1993; Muyzer et al. 1996). One of the primers used in the PCR reaction has a GC-rich tail which is more resistant to denaturation. This PCR product is run on a gel with increasing amounts of denaturant in order to sort the PCR products at different locations in the gel based on differences in the gene's sequence. Sequence data can be obtained from the bands in the DGGE gel allowing identification of the organisms in the sample.

As in other ecosystems, the use of phylogenetic analysis based on 16S rDNA has expanded our view of the microbial community at hydrothermal vents beyond the diversity obtained in laboratory cultures. For example, the view of *Archaea* as extremophiles has been changing with the identification of archaeal sequences from non-extreme environments such as pelagic marine ecosystems (DeLong 1992; Fuhrman et al. 1992; DeLong et al. 1994; Massana et al. 1997; Massana et al. 1998; Massana et al. 2000), rivers and estuaries (Crump

and Baross 2000), and sediments (Vetriani et al. 1999). These environmental sequences cluster into two major groups that have yet to be obtained in laboratory cultures: the crenarchaeal Group I *Archaea* and the euryarchaeal Group II *Archaea*. Group I and Group II *Archaea* have also been identified at hydrothermal vents on the MAR (Reysenbach et al. 2000), Loihi Seamount (Moyer et al. 1998), and the western Pacific (Takai and Horikoshi 1999). While there have been attempts to subdivide the groups based on the samples' source, this has led to a confusing array of overlapping definitions that may or may not be metabolically or ecologically relevant. To avoid adding to the confusion, no new groups will be defined in this thesis and any sequences clustering with the Group II *Archaea* will only be identified as Group II *Archaea*. An exception will be made for the groups already defined at hydrothermal vents (Takai and Horikoshi 1999) and sequences from this research clustering within these hydrothermal groups will be identified following the existing nomenclature.

The use of 16S rDNA-based phylogenetic analysis has been used to identify the microbial community at several different deep-sea hydrothermal vents. The bacterial communities in microbial mats from Loihi Seamount and the Southern East Pacific Rise (SEPR) were primarily ε-*Proteobacteria* (Moyer et al. 1995; Longnecker and Reysenbach 2001). Conversely, the community from the in situ growth chamber was more diverse and included organisms that have been isolated from hydrothermal vents (*Thermococcales*, *Aquificales*, ε-

Proteobacteria, *D. thermolithotrophum*, and *Archaeoglobales*), and other groups that have not been cultured from deep-sea hydrothermal vents including the Bacteroides-Cytophaga-Flavobacterium group, β-*Proteobacteria*, and several novel lineages within the *Bacteria* (Reysenbach et al. 2000). The analysis of sulfide samples, water samples and sediments with a flux of hydrothermal fluid from sites in the western Pacific identified a broad array of sequences within the *Archaea*, most of which cluster into groups not previously identified from any other ecosystem (Takai and Horikoshi 1999).

1.2 Research objectives

While there have been sporadic attempts to examine the microbial communities at deep-sea hydrothermal vents using 16S rDNA-based phylogenetic analysis, there has been no systematic attempt to compare the communities at different sites within a single hydrothermal vent field. Nor has there been any attempt to examine the microbial community within a single sulfide structure in conjunction with its mineralogy. The primary goal of this thesis is to use phylogenetic analysis based on 16S rDNA to compare the archaeal and bacterial populations within a single flange and the archaeal populations at different sites within two different hydrothermal vent fields.

The first section of this thesis addresses the archaeal diversity of a sulfide chimney collected from 9°N on the East Pacific Rise (EPR). The chimney was divided into subsections in order to determine if differences in

the microbial populations could be correlated to differences in the temperature regime within the sulfide chimney. The results of the analysis indicated that different groups of *Archaea* do inhabit different regions of the sulfide chimney and their distribution supports hypotheses from models developed based on the energy available from microbial transformations of chemical compounds available in hydrothermal fluid (McCollom and Shock 1997).

The second section of the thesis examines the archaeal diversity of samples collected from Guaymas Basin. In conjunction with the DGGE analysis, the mineralogy of the chimney and flanges was visually determined in order to test the hypothesis that the archaeal diversity in the different sulfide structures would vary with the mineralogy. The samples from Guaymas Basin were analyzed with DGGE and clone libraries of almost full-length 16S rDNA were constructed for one chimney and one flange. The *Archaea* identified from Guaymas Basin include examples of *Archaea* that have only been identified at deep-sea hydrothermal vents and sequences that cluster within known orders of methanogens that have not previously been identified at deep-sea hydrothermal vents.

The third section of the thesis links more detailed mineralogy with the identification of *Bacteria* and *Archaea* present within a flange from the Juan de Fuca Ridge. The greatest number of DGGE bands for both *Bacteria* and *Archaea* were found within the section of the flange where the mineralogy indicated

the greatest temperature gradient. No *Archaea* and only two bacterial DGGE bands were identified from the hottest section of the flange which lay immediately above the pool of hydrothermal fluid.

The summary section of this thesis compares the patterns of microbial diversity observed in the sulfide structures collected from each of the three hydrothermal vent sites. While the samples were collected from different ridges and at sites with different chemical regimes, there are some broader conclusions that can be made about the microbial communities within the sulfide structures, some of which contrast with previously established views of hydrothermal vent microbiology.

2 Archaeal Diversity of a Sulfide Chimney from a Hydrothermal Vent at 9°N, East Pacific Rise

2.1 Abstract

The archaeal diversity associated with a sulfide chimney collected from 9°N on the East Pacific Rise (EPR) was determined using denaturing gradient gel electrophoresis (DGGE) of the V3 variable region of the 16S rRNA gene. The archaeal diversity appeared highest in the outer section of the chimney and lower in the inner and top sections. The diversity varied between the different sections of the chimney, although some sequences were obtained in multiple chimney sections. All of the sequences identified were euryarchaeotal with over half of the sequences clustering within the Group II *Archaea*. There were also sequences that clustered with the *Methanobacteriales* and *Methanomicrobiales*, two orders of methanogens that have not previously been identified at deep-sea hydrothermal vents.

2.2 Introduction

Within hydrothermal ecosystems, microorganisms occupy niches at the interface between the hot, reduced vent fluid and the cool, oxidized seawater (McCollom and Shock 1997). Sulfide chimneys form at hydrothermal vents at this interface as the minerals in the vent fluid precipitate when the

hydrothermal fluid comes in contact with seawater. Temperature and chemical gradients are created within the chimney which establish different microbial niches. The spatial distribution of microorganisms with different metabolic capacities has been modeled based on the energy available along the continuum of possible temperature and chemical conditions within a sulfide chimney (McCollom and Shock 1997). In addition to the models, microbial counts using epifluorescence microscopy have indicated that numbers of microorganisms decrease in the inner sections of a sulfide chimney (Chevaldonné and Godfroy 1997; Harmsen et al. 1997). Harmsen et al. also present data on the distribution of *Bacteria*, however no data are available on the identity or spatial distribution of *Archaea* within a sulfide chimney.

The identity of *Archaea* occupying different ecological niches at hydrothermal vents in the western Pacific was examined with molecular techniques based on phylogenetic analysis of the small subunit ribosomal rRNA gene (16S rDNA) (Takai and Horikoshi 1999). The samples analyzed were sections of sulfide chimneys, pore water from hydrothermal sediments, and high temperature vent fluid. Both *Euryarchaeota* and *Crenarchaeota* were identified. Furthermore, most of the sequences were not related to any cultured isolates nor to sequences identified from any other ecosystems (Takai and Horikoshi 1999).

Although *Archaea* were initially associated with extreme environments such as the hydrothermal vents in the western Pacific, they have also been identified in the cold waters of the open ocean and sediments that are distant from deep-sea hydrothermal vents (DeLong 1992; Fuhrman et al. 1992; DeLong et al. 1994; Massana et al. 1997; Massana et al. 1998; Vetriani et al. 1999; Crump and Baross 2000; Massana et al. 2000). There have been attempts to subdivide these sequences based on sample type; however, given the lack of metabolic data on the group, subdivisions based on sampling location or type seem premature. Fluorescently-labeled oligonucleotide probes have been used to describe the spatial distribution of the crenarchaeal Group I and the euryarchaeal Group II in marine ecosystems (Massana et al. 1997; Massana et al. 1998; Massana et al. 2000). The Group II *Archaea* dominate in the surface waters of the ocean, while the number of Group I *Archaea* is greater at depths below 200 m (Massana et al. 1997; Massana et al. 1998; Massana et al. 2000) and can reach 40% of the microbial community in the non-vent deep-sea at depths greater than 1000 m (Karner et al. 2001). Furthermore, both the Group I and Group II *Archaea* have been identified at deep-sea hydrothermal vents, though little information is available on their relative abundance since these studies relied on 16S rDNA clone libraries (Moyer et al. 1998; Takai and Horikoshi 1999; Reysenbach et al. 2000).

This study examines the diversity of *Archaea* located within a sulfide chimney collected from Io Vent at 9°N, EPR. The *Archaea* identified were all

Euryarchaeota and include new groups that have not been identified at deep-sea hydrothermal vents and other groups that may be endemic to hydrothermal vents.

2.3 Materials and methods

2.3.1 Sample collection and storage

The 9°N hydrothermal vent field is located on the East Pacific Rise (EPR) between the Clipperton and Siqueiros transform faults. This section of the EPR is a fast-spreading portion with a full spreading rate of 11 cm yr^{-1} (Haymon et al. 1991). Samples for molecular diversity analysis were collected during the Extreme 1 cruise to 9°N in May 1999.

The sulfide chimney was collected using the mechanical arm of the DSV *Alvin* and placed in a container on the exterior of the submersible. The lid of the container was closed during ascent to minimize contamination with seawater en route to the surface. After the sample was on board the ship, it was sectioned using a hacksaw with a carbide blade. Each section was homogenized in a mortar and pestle and divided into a series of tubes. The tubes were kept frozen at -80°C until the DNA extraction.

2.3.2 DNA extraction

A modified extraction protocol using cetyltrimethylammonium bromide (CTAB)(Ausubel et al. 1994) was used to extract DNA from the inner, outer and top sections of the beehive chimney from Io Vent. A 100 µl aliquot of the sample was centrifuged for 20 min, the supernatant removed, and the pellet resuspended in 500 µl of TE buffer (10 mM Tris-Cl and 1 mM EDTA). 600 µl of the extraction buffer (250 mM EDTA, 1.25% SDS, 8.3% Chelex-100, 0.66 mg ml^{-1} Proteinase K) was added along with additional TE to bring the volume in the centrifuge tube to 1.5 ml. The sample was rotated at 50°C for 3 h and then spun briefly to separate the Chelex-bound metals and cell lysate. The supernatant was transferred into two tubes and NaCl (0.8 M final concentration) and a CTAB/NaCl solution (1% CTAB and 0.7 M NaCl final concentration) was added to each tube. The sample was incubated at 65°C for 30 min. The solution was first extracted with chloroform:isoamyl alcohol (24:1) and then with phenol:choloroform:isoamyl alcohol (25:24:1). DNA was precipitated with an equal volume of 100% isopropanol overnight at 4°C. The sample was then spun at 4°C for one hour, washed with 70% ethanol and dried. The DNA was resuspended in 10 mM Tris.

2.3.3 Denaturing gradient gel electrophoresis (DGGE) analysis

PCR conditions were 5 µl of 10X Promega PCR buffer, 200 µmol dNTPs, 400 pmol of each primer, 1 U of Promega Taq, and 2.5 mM $MgCl_2$. 16S rDNA

was initially amplified with the universal primer 1492R (5'- GGT TAC CTT GTT ACG ACT T -3') and the archaeal specific primer 4F (5'- TCC GGT TGA TCC TGC CRG -3', where R=A or G). PCR conditions were initial denaturation at 94°C for 5 min, thirty-five cycles of 94°C, 30 s; 50°C, 30 s; 72°C, 90 s; and a final 10 min at 72°C. PCR product from this reaction was then used as a template for PCR with the archaeal specific primer 344F-GC (5'-ACG GGG CGC AGC AGG CGC GA-3') and 519RP (5'- GW ATT ACC GCG GCK GCT G-3', where W=A or T and K=G or T). The forward primer used to amplify the V3 variable region of the 16S rDNA also has a 40-nucleotide GC-rich section required for the analysis. PCR conditions for this reaction were a touchdown procedure with annealing temperatures starting at 71°C and decreasing to 61°C by 0.5°C increments every cycle for twenty cycles, and a final fifteen cycles at 61°C. The initial denaturation was 94°C, 5 min, the denaturation step for each cycle was 1 min, 94°C and the extension temperature at each cycle was 1 min, 72°C plus a final extension at 72°C for ten minutes (modified from (Casamayor et al. 2000). The negative control from the first reaction was also tested in the second reaction to confirm that amplification in the nested PCR was not a contaminant.

PCR products were analyzed using denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1996). A 6% acrylamide gel was prepared in TAE (0.04M Tris, 0.02M glacial acetic acid, 1 mM EDTA). Within the acrylamide gel, a gradient of the denaturants increasing towards the

bottom of the gel was established. The denaturants were a combination of urea and formamide where 100% denaturant would be 40% (vol/vol) formamide and 7 M urea. The denaturing gradient used was from 20% urea/formamide to 60% urea/formamide, and the gel was run in a Bio-Rad DCode Universal Mutation Detection System at 60°C at 180 V for 2.5 h. The acrylamide gel was stained with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME) for 15 min and then viewed on an ultraviolet light box.

Bands from the DGGE were noted and removed by piercing the gel with a pipet tip. The tip was then placed in 10 mM Tris for 10 min at room temperature and 2 to 5 μl of this mixture was used as a template for PCR. The forward and reverse primers used correspond to the forward and reverse primers used in the previous PCR reaction, except that in this step the primers lack the GC-rich section required for DGGE. The PCR products were cleaned with the PCRpure SPIN kit (GeneMate, ISC BioExpress, Kaysville, UT) following the manufacturer's instructions. Both strands of the resulting PCR product were sequenced by cycle sequencing using fluorescent dideoxy terminators. The sequences were determined using an automated sequencer (ABI Prism 310 Genetic Analyzer) and were assembled with the AutoAssembler Program (Applied Biosystems, Inc.).

2.3.4 Phylogenetic analysis of the DGGE bands

Data from BLAST (basic logic alignment search tool) (Altschul et al. 1997) and the Ribosomal Database Project's (RDP) Similarity Matrix tool (Maidak et al. 2000) were used to identify sequences closely related to the sequences obtained from the DGGE gel. The closely related sequences and the 9°N sequences were aligned in ARB (Strunk and Ludwig 1995). Distance matrices of the ~180 nucleotides sequenced were constructed with PAUP (Swofford 1998) using the Jukes and Cantor algorithm (Jukes and Cantor 1969).

2.4 Results

The temperature at the orifice of the Io Vent chimney was estimated to be 150°C and was 48°C 3 cm above the orifice. DNA was extracted from each section of the beehive chimney from Io Vent. The DNA was extracted from the sulfide sample in low quantities, which necessitated the use of a nested PCR in order to get sufficient PCR product for DGGE analysis. When amplifying the samples with the DGGE primers, some of the samples would not amplify without the touchdown program. Therefore, for consistency, the touchdown PCR program was used for all of the samples. The banding pattern for samples that would amplify without the touchdown program was compared to their pattern if amplified with the touchdown program and the same pattern of DGGE bands was observed (Figure 2-1).

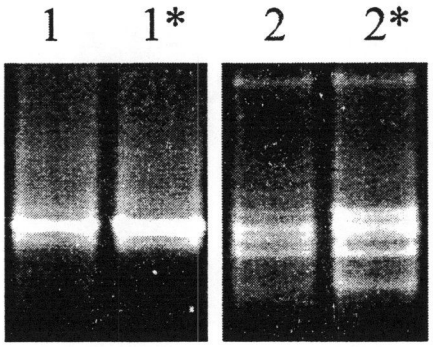

Figure 2-1. DGGE gel comparing samples amplified with a touchdown PCR program with those amplified with a non-touchdown PCR program. Two samples are shown and the samples marked with the * are the results of PCR reactions using the touchdown PCR program described in the text.

The results of the 16S rDNA-based phylogenetic analysis of the DGGE bands indicated that all of the *Archaea* were *Euryarchaeota* (Table 2-1). However, the pattern of DGGE bands indicated that the archaeal diversity was different for each section of the sulfide chimney (Figure 2-2). The outer section of the chimney had the highest number of DGGE bands with nine *Archaea*, whose sequences clustered within the Group II *Archaea*, the *Methanomicrobiales*, and the ANME-1 group. The inner section had fewer *Archaea* with sequences that clustered within the Group II *Archaea*, *Methanomicrobiales* and the *Methanobacteriales*. The top section had the fewest DGGE bands, with five *Archaea* whose sequences all clustered with the Group II *Archaea*.

Each section of the chimney also had multiple DGGE bands that clustered with the same species. For example, within the inner section of the chimney, two DGGE bands cluster with *Methanoculleus bourgense*. However one of the sequences is 18.4% distant from *Mcu. bourgense*, while the other is 28.5% distant. Multiple bands from the top section of the sulfide chimney clustered within the DHVE2 group and they ranged from 5% to 8.3% distant from pISA42.

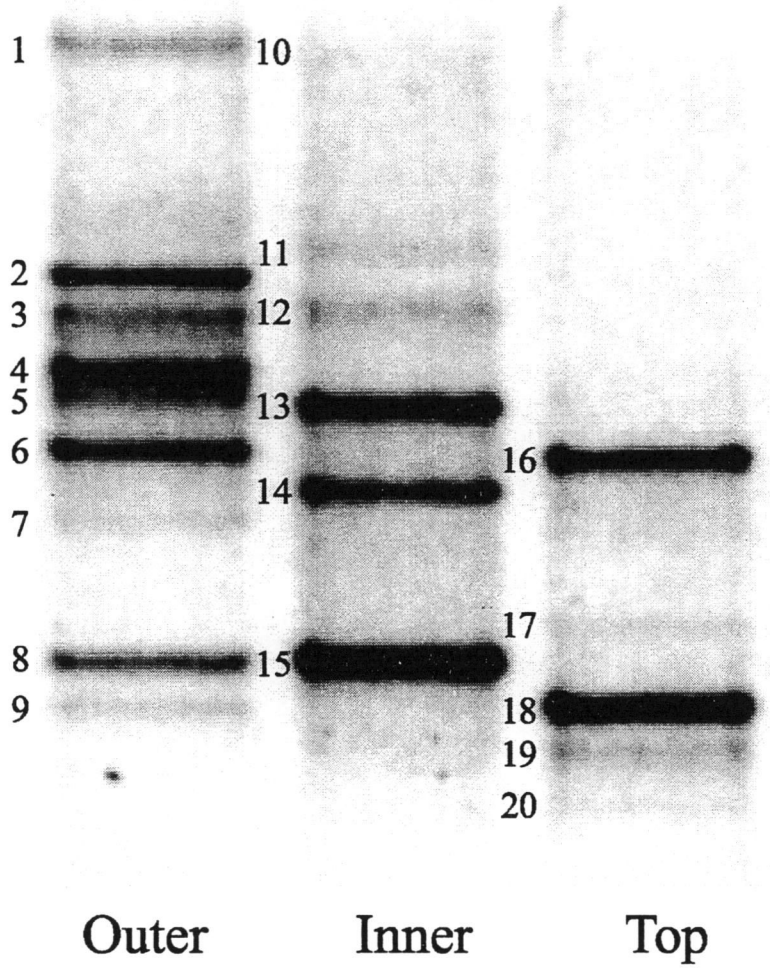

Figure 2-2. Negative image of the DGGE gel of the outer, inner and top sections of the sulfide chimney collected from Io Vent. The numbers adjacent to the bands identify which bands were removed from the DGGE gel and sequenced, and correspond to the band numbers shown in Table 2-1.

Table 2-1. Summary of bands identified from the beehive collected from Io Vent, 9°N and the results of the distance matrices conducted using the Jukes and Cantor model (Jukes and Cantor 1969). ANME-1 are sequences collected from marine sediments (Hinrichs et al. 1999), DHVE2 are sequences initially identified from hydrothermal vents in the western Pacific (Takai and Horikoshi 1999), and the WCHD sequences are clones from the methanogenic zone of a contaminated aquifer (Dojka et al. 1998).

Band #	**Section**	**Least distant species**	**Distance**	**Group**
1	Outer	WCHD3-02	19.9%	Group II *Archaea*
2	Outer	E31B1	21.2%	*Methanomicrobiales*
3	Outer	TA2e12	3.4%	ANME-1
4	Outer	WCHD3-33	21.4%	Group II *Archaea*
5	Outer	WCHD3-33	19.3%	Group II *Archaea*
6	Outer	WCHD3-33	19.5%	Group II *Archaea*
7	Outer	*Methanoculleus bourgense*	19.5%	*Methanomicrobiales*
8	Outer	pISA42	14.6%	DHVE2/Group II *Archaea*
9	Outer	VC2.1Arc13	9.8%	DHVE2/Group II *Archaea*
10	Inner	Same as Band 1		Group II *Archaea*
11	Inner	WCHD3-02	19.9%	Group II *Archaea*
12	Inner	WCHD3-33	19.0%	Group II *Archaea*
13	Inner	*Methanoculleus bourgense*	18.4%	*Methanomicrobiales*
14	Inner	*Methanoculleus bourgense*	28.5%	*Methanomicrobiales*
15	Inner	*Methanobacterium thermoautotrophicum*	30.4%	*Methanobacteriales*
16	Top	pMC2A10	17.0%	DHVE2/Group II *Archaea*
17	Top	pISA42	6.5%	DHVE2/Group II *Archaea*
18	Top	pISA42	8.3%	DHVE2/Group II *Archaea*
19	Top	pISA42	5.0%	DHVE2/Group II *Archaea*
20	Top	VC2.1Arc13	12.8%	DHVE2/Group II *Archaea*

2.5 Discussion

2.5.1 Archaeal diversity within the Io Vent sulfide chimney

All of the sequences clustered within the *Euryarchaeota*. There were sequences identified, such as those clustering with the DHVE2 group, that have previously been found in the western Pacific and the Mid-Atlantic Ridge (Takai and Horikoshi 1999; Reysenbach et al. 2000). There were other groups that have never been detected among the microorganisms from hydrothermal vents such as the *Methanobacteriales* and the *Methanomicrobiales*. Discovering *Archaea* at 9°N that previously were not known to be a component of the microbial community at deep-sea hydrothermal vents is not surprising given that early research at hydrothermal vents relied on laboratory cultures and only 1% of organisms can be obtained in laboratory cultures (Amann et al. 1995). Furthermore there have only been a few studies that relied on 16S rDNA sequences to identify the archaeal diversity at deep-sea hydrothermal vents (Moyer et al. 1994; Takai and Horikoshi 1999; Reysenbach et al. 2000).

Group II *Archaea* have been identified based on 16S rDNA at each of the hydrothermal vent sites previously studied (Moyer et al. 1994; Takai and Horikoshi 1999; Reysenbach et al. 2000) and more than half the sequences from 9°N cluster within the Group II *Archaea*. Different subgroups of the phylogenetically diverse Group II *Archaea* have been defined based on sequences obtained from hydrothermal vents in western Pacific (Takai and

Horikoshi 1999). Seven of the sequences from the DGGE gel cluster with the DHVE2 group defined by Takai and Horikoshi, while the remaining Group II *Archaea* cluster with sequences identified from a contaminated aquifer (Dojka et al. 1998). To our knowledge, the DHVE2 group has not been identified in any non-vent ecosystems and this, combined with its repeated identification at hydrothermal vents, may indicate the DHVE2 cluster within the Group II *Archaea* is endemic to deep-sea hydrothermal vents.

There are five orders of methanogens within the *Euryarchaeota* and the DGGE bands from 9°N included sequences that cluster within two of the orders: the *Methanomicrobiales* and the *Methanobacteriales*. Only the orders *Methanococcales* and *Methanopyrales* have previously been identified at deep-sea hydrothermal vents as 16S rDNA sequences (E. Corre, A.-L. Reysenbach, and D. Prieur, unpublished research) or as laboratory isolates (Jones et al. 1983; Huber et al. 1989; Burggraf et al. 1991; Kurr et al. 1991; Jeanthon et al. 1999). The *Methanomicrobiales* and *Methanobacteriales* have previously been identified in sediments, bioreactors, and digestors, although one of the *Methanobacteriales*, *Methanogenium tationis*, is from a moderately thermophilic (temperature between 37° and 40°C) solfataric pool in Chile (Whitman et al. 1992).

Although phylogenetic similarities do not necessarily imply metabolic similarities, all of the described *Methanomicrobiales* and *Methanobacteriales* are methanogens that use H_2 or formate as electron donors to reduce CO_2 into

methane (Whitman et al. 1992). The DGGE bands from 9°N are therefore also likely to be involved in methane production within the sulfide chimney from Io Vent. The methanogens most prominently associated with hydrothermal vents are the *Methanococcales*. This group was not identified with the methods used in this study. However, DNA was obtained from different sulfide chimneys using a different extraction method and/or amplified with a different reverse primer and these samples did include DGGE bands that were most closely related to *Methanococcus aeolicus*, a member of the *Methanococcales* that has not been fully described.

There is one DGGE band from the Io Vent chimney that clustered with the ANME-1 group (Hinrichs et al. 1999). This group has been implicated in methane-oxidation based on the depletion of ^{13}C in the lipids extracted from the sediments. The $\delta^{13}C$ values are indicative of the use of methane as a carbon and energy source rather than the use of CO_2 (Hinrichs et al. 1999). These organisms could be using the methane produced within the chimney by the *Methanobacteriales* or the *Methanomicrobiales* that were identified among the *Archaea* in the Io Vent sulfide chimney.

2.5.2 Spatial separation of *Archaea* in the Io Vent sulfide chimney

Energy is available at deep-sea vents at the interface between hydrothermal fluid and seawater; the amount of energy available to microorganisms has been modeled based on the mixing that occurs at this

interface within sulfide chimneys (McCollom and Shock 1997). Different amounts of energy are available from the geochemical reactions at different temperatures and a hypothetical distribution of microorganisms within a sulfide chimney was proposed based on the optimal temperature for each of the microbially-mediated geochemical reactions (McCollom and Shock 1997). At temperatures above 38°C methanogenesis, sulfate- and sulfur-reduction are energetically favorable, although sulfate-reduction may be limited because it requires sufficient diffusion of the sulfate from seawater through the chimney (McCollom and Shock 1997). The inner section of the Io Vent sulfide chimney contained three bands whose sequences clustered within known orders of methanogens. Their presence in the inner section of the chimney supports the model's hypothesis that methanogens will be present in the hotter sections of a sulfide chimney. The outer section of the chimney also contained methanogens, although only two of the nine bands from the outer section clustered with known methanogens. The initial chimney sections may have crossed the ecological niches and thus the presence of methanogens in the outer section of the chimney.

McCollom and Shock also postulated that the outer surfaces of a chimney would be inhabited by microorganisms capable of methane-oxidation; a reaction that is energetically favorable at temperatures less than 38°C (McCollom and Shock 1997). DGGE Band 3 was most closely related to the ANME-1 group of microorganisms and hypothetically this microorganism

can use methane as a carbon and energy source (Hinrichs et al. 1999). While anaerobic methane oxidation had been hypothesized as a metabolic process (Zehnder and Brock 1980), it has not been conclusively proven in laboratory cultures. Therefore methane-oxidation was considered as an aerobic process in the McCollom and Shock models. The presence of DGGE bands that cluster with anaerobic methane-oxidizers indicates the need to expand the models to include anaerobic methane oxidation as a process that may be occurring at deep-sea hydrothermal vents.

There was a decline in the number of DGGE bands in the inner and top section of the Io Vent chimney indicating a decline in the number of different *Archaea*. Counts with DNA stains and fluorescently-labeled oligonucleotide probes have indicated lower numbers of microorganisms are present in the interior sections of a sulfide chimney (Chevaldonné and Godfroy 1997; Harmsen et al. 1997). One hypothesis to explain the decrease in diversity in the interior of the Io Vent chimney is fewer niches are present in the hottest section of a sulfide chimney. Few niches result in a decline in the abundance of microorganisms as observed in previous studies (Chevaldonné and Godfroy 1997; Harmsen et al. 1997) and a decline in the number of different microorganisms as identified by DGGE in this study.

2.5.3 Identification of clusters of closely-related sequences within a chimney

Within the Io Vent sulfide chimney there were clusters of closely related 16S rDNA sequences related to WCHD3-33, pISA42 and *Mcu. bourgense*. Clusters of sequences from a single sample have been observed in other studies using 16S rDNA-based phylogenetic analysis (Giovannoni et al. 1990; Ferris et al. 1996; Ferris and Ward 1997; Moore et al. 1998). One hypothesis to explain this pattern is that each group is ecologically distinct and has adapted to a subset of the conditions experienced within a heterogeneous environment (Begon et al. 1996). Fluctuations in the geochemical regime have been observed at deep-sea hydrothermal vents. For example, the temperature of hydrothermal fluid at 9°N fluctuates due to cracking events and changes in subsurface plumbing (Von Damm et al. 1995; Fornari et al. 1998). Furthermore, as the parts of a chimney become sealed due to the precipitation of minerals, the path of hydrothermal fluid within the chimney will change (Tivey et al. 1999). These fluctuations in fluid flow would result in changes in the chemical conditions perceived by the microorganisms within the sulfide chimney. Perhaps the clusters of 16S rDNA sequences related to WCHD3-33, pISA42 and *Mcu. bourgense* are subpopulations that have developed in response to fluctuations in the flow and chemical composition of the hydrothermal vent fluid.

There are other possible explanations for the clusters of 16S rDNA sequences observed. There could be multiple copies of the gene present within a single organism. According to the Ribosomal RNA Operon Copy Number Database (rrndb), the *Euryarchaeota* have an average of 1.7 rRNA operons, although no data for the Group II *Archaea* or the *Methanomicrobiales* are available (Klappenbach et al. 2001). There could also be apparently multiple copies due to artifacts introduced during PCR (Qiu et al. 2001; Speksnijder et al. 2001). Without laboratory cultures of these sequences, distinguishing between the potential reasons for the gene clusters is not possible.

2.5.4 Conclusions

The archaeal diversity of the sulfide chimney sampled from 9°N on the EPR can be described by two distinct ecological patterns. The first pattern was the spatial separation of microorganisms within the sulfide chimney. The McCollom and Shock models based on the mixing of hydrothermal fluid and seawater hypothesized a distribution of microorganisms based on the temperature and geochemical regime within a chimney (McCollom and Shock 1997). The spatial distribution of some of the microorganisms within the Io Vent chimney support the results of the model, although hypotheses about the metabolic capacity of several of the microorganisms are impossible due to the lack of laboratory cultures. The second ecological pattern is multiple DGGE bands clustering with a single species that may be indicative of a community

that has evolved to allow different microorganisms to be active under different environmental conditions. Finally, the identification of the *Methanobacteriales* and the *Methanomicrobiales* represents the first identification of these orders of methanogens from deep-sea hydrothermal vents.

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2.6 Appendix

DNA was extracted from other 9°N sulfide samples and analyzed with DGGE. However the data are not presented in the body of the thesis because an attempt to reproduce the experiment was unsuccessful. DNA was extracted from sulfide chimney samples from M Vent and Q Vent, two sites located in the axial summit caldera north of Io Vent. The initial DGGE analysis of the M Vent and Q Vent sulfide samples indicated that all of the *Archaea* present were related to *Methanogenium* strains AK1 and ACE1_A. ACE1_A (GenBank accession number AY016504) and AK1 (S.C. Chong and D.R. Boone, unpublished results) were both isolated from cold ecosystems, an Antarctic marine ecosystem and permafrost in the Arctic respectively. However, AK1

was used as a positive control for some earlier experiments testing the efficiency of different extraction methods. Therefore the concern was raised that the DGGE bands in the M Vent and Q Vent sulfide samples were actually a contaminant. To address this concern, DNA was extracted from the M Vent and Q Vent sulfide samples a second time. The BLAST scores from the sequences obtained from the DGGE bands are given in Table 2-2. None of the DGGE bands are related to the *Methanogenium* strains and therefore the original data from Q Vent and M Vent were considered a contaminant and discarded.

Table 2-2. BLAST scores from sequences obtained from the second DNA extraction and DGGE gel of the M Vent and Q Vent sulfide chimneys.

DGGE Band	**Closest according to BLAST analysis**	**% identity**	**E score**
9-H.0212	pISA18	91%	1e-31
9-I.0212	pISA18	88%	0.004
9-J.0212	(no significant similarity)		
9-K.0212	pISA18	92%	9e-29
9-L.0212	pUWA9	94%	6e-12
9-M.0212	pISA42	80%	5e-10
9-N.0212	*Staphylothermus marinus*	84%	2e-14
9-O.0212	(no significant similarity)		
9-P.0212	pUWA9	84%	2e-6
9-Q.0212	(no significant similarity)		

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3 Archaeal Diversity in Sulfide Structures from Deep-Sea Hydrothermal Vents at Guaymas Basin

3.1 Abstract

The archaeal diversity associated with five active deep-sea hydrothermal vent sulfide structures from Guaymas Basin was determined using denaturing gradient gel electrophoresis (DGGE) of the V3 variable region of the small subunit ribosomal RNA gene (16S rDNA). For comparative purposes, a clone library of PCR-amplified 16S rDNA sequences (~1450 nucleotides) was constructed from DNA extracted from one flange and one chimney sample. In both analyses, the archaeal diversity was restricted to the *Euryarchaeota*. Two different reverse primers were used in the DGGE analysis and resulted in the identification of slightly different archaeal communities apparently due to PCR bias caused by mismatches between the primer and target sequences. If two flanges shared similar mineralogy, the diversity of *Archaea* identified was similar indicating a correlation between the minerals present and the *Archaea* present. Using DGGE, the diversity of *Archaea* in the chimney differed from the diversity in the flanges although some sequences were identified in the chimney and the flanges. However, there was no overlap in the clone libraries; the flange's clone library included

Methanococcales, *Thermococcales*, and the DHVE2 group initially identified at deep-sea hydrothermal vents in the western Pacific. The clone library for the chimney sample included *Methanosarcinales* and the DHVE7 group. The identification of *Methanosarcinales* is the first time this order of methanogens has been found within a deep-sea hydrothermal vent sample.

3.2 Introduction

The Guaymas Basin hydrothermal system is situated between Baja California and mainland Mexico. Due to its proximity to land, Guaymas Basin differs from most other hydrothermal systems because of the sediment layer that averages 100 m deep (Simoneit et al. 1979). Sediment accumulates on the sea floor at a rate exceeding 1 m per 1,000 years (Curray et al. 1979). Hydrocarbons present within the sediments at Guaymas are the result of thermal alteration of biological material, primarily autochthonous marine material and, to a lesser extent, allochthonous terrestrial runoff (Simoneit et al. 1979; Simoneit and Lonsdale 1982). The hydrothermal fluid at Guaymas Basin has a higher alkalinity, a higher ammonium concentration and a higher pH (~5.9) than hydrothermal fluid at vent sites that lack a sediment layer (Von Damm et al. 1985). Conversely, the concentration of metals such as Mn, Fe, Co, Cu and Zn is lower in the hydrothermal fluid because the higher pH levels cause the precipitation of metal sulfide minerals within the sediments before the fluid exits at the seafloor (Von Damm et al. 1985).

The organic compounds within hydrothermal fluid and sediments may be available to microorganisms as energy and carbon sources. The composition of hydrocarbons within sediments suggests microbial degradation of the hydrocarbons (Simoneit 1985; Bazylinski et al. 1988). Furthermore, the conversion of ^{14}C-labeled hydrocarbons into $^{14}CO_2$ has been demonstrated with sediment slurries from Guaymas and aerobic mesophilic microorganisms were isolated from these sediment slurries that were capable of utilizing hydrocarbons as their sole carbon and energy source (Bazylinski et al. 1989).

Archaea that have been isolated from Guaymas Basin include one of the most prevalent isolates from hydrothermal vents, namely the heterotrophic, sulfur-reducing *Thermococcales* (Canganella et al. 1998). Two orders of chemoautotrophic methanogens that can use the H_2 present in vent fluid to reduce CO_2 to methane have been isolated from deep-sea hydrothermal vents. Specifically, isolates obtained from Guaymas Basin include cultures of *Methanococcales* such as *Methanococcus jannaschii* (Jones et al. 1983; Jeanthon et al. 1999), *Mc. fervens* and *Mc. igneus* (Jeanthon et al. 1999), and the only *Methanopyrales* described from a deep-sea hydrothermal vent, *Methanopyrus kandleri* (Huber et al. 1989; Kurr et al. 1991).

This study assessed the archaeal diversity in flanges and a chimney collected from different sites at Guaymas Basin. *Archaea* were identified by sequencing 16S rDNA clone libraries and bands from denaturing gradient gel

electrophoresis (DGGE). Only *Euryarchaeota* were detected in both the clone libraries and from the DGGE bands. The sequences included *Archaea* previously identified at deep-sea hydrothermal vents and some sequences not previously reported from hydrothermal vent ecosystems.

3.3 Materials and methods

3.3.1 Sample collection and storage

Sulfide samples were collected during a cruise to Guaymas Basin (27° 0.8'N, 111° 25.4'W) in January 2000. At each site the maximum temperature of the hydrothermal fluid was noted prior to collecting the sulfide sample with the mechanical arm of the DSV *Alvin*. The samples were placed into separate sections of a container mounted on the basket of the submersible. The lid of the container was closed during ascent to minimize contamination with seawater during ascent. Once shipboard, the mineralogy of the samples was determined by visual inspection. The samples were then sectioned using a hacksaw with a carbide blade and homogenized in a mortar and pestle. The slurry was saved as aliquots at -80° C.

Six different samples (Table 3-1) were chosen for this study since they included at least one sample from every site visited in the axial rift valley known as the Southern Trough. One of the samples was a chimney structure, while the remaining five samples were flanges. Flanges differ from chimneys

in that they grow laterally out from the center of the structure and are therefore parallel to the seafloor; in contrast chimneys grow vertically up from the seafloor (Tivey 1995).

3.3.2 DNA extraction

DNA was extracted following a modified extraction protocol using cetyltrimethylammonium bromide (CTAB) (Ausubel et al. 1994). A 100 µl aliquot of the sample was centrifuged for 20 min, the supernatant removed, and the pellet resuspended in 500 µl of TE buffer (10 mM Tris-Cl and 1 mM EDTA). 600 µl of the extraction buffer (250 mM EDTA, 1.25% SDS, 8.3% Chelex-100) was added and the total volume brought to 1.5 ml with TE. The sample was mixed at 50°C for 1 h on a rotator. The Chelex-bound metals and cell lysate were removed by centrifugation. The supernatant was transferred into two tubes. NaCl (0.8 M final concentration) and a CTAB/NaCl solution (1% CTAB and 0.7 M NaCl final concentration) were added to each tube; the tubes were then incubated at 65°C for 30 min. The DNA was first extracted with an equal volume of chloroform:isoamyl alcohol (24:1) followed by an equal volume of phenol:choloroform:isoamyl alcohol (25:24:1). DNA was precipitated overnight at 4°C with an equal volume of 100% isopropanol. DNA was spun at 4°C for one hour, washed with 70% ethanol and dried. The DNA was suspended in 10 µl of 10 mM Tris.

Table 3-1. Summary of samples used for the analysis of the archaeal diversity of hydrothermal vents at Guaymas Basin.

Sample	Dive #	Site	Sample type	Maximum temperature	Mineralogy
G72	3519	Robin's Roost	Chimney	306° C	primarily sphalerite with some anhydrite, pyrrhotite, iron oxide, and calcite
G41	3518	K2	Flange/top	224° C	pyrrhotite and some pyrite
G203	3523	Kristin's Summit	Flange	299° C	primarily sphalerite and some pyrite and oxidized iron on the surface
G26	3517	Rebecca's Roost	Flange/top	170° C	Barite, some sphalerite and some pyrite
G98	3521	Rebecca's Roost	Flange/top	298.5° C	Soft, young flange, perhaps some barite, pyrrhotite
G106	3521	Rebecca's Roost	Flange/bottom	298.5° C	(same as above)

3.3.3 PCR conditions and denaturing gradient gel electrophoresis (DGGE)

DNA was amplified using PCR where the reaction conditions were 5 µl of 10X Promega PCR buffer, 200 µmol dNTPs, 400 pmol of each primer, 1 U of Promega Taq, and 2.5 mM $MgCl_2$. The region amplified for the DGGE analysis was the variable V3 region and all the samples used the same archaeal specific forward primer 344F-GC (5'-ACG GGG CGC AGC AGG CGC GA-3'). The primer also has a 40-nucleotide GC-rich section that is required for DGGE (Muyzer et al. 1993). Two different reverse primers were used in separate

reactions: 519R (5'- ATT ACC GCG GCT GCT GG -3') and 519RP (5'- GW ATT ACC GCG GCK GCT G-3', where W=A or T and K=G or T). Conditions for PCR were an initial denaturation at 94°C for 5 min, 35 cycles of 94°C, 30 s; 48°C, 30 s; 72°C, 30 s; and a final 10 min at 72°C.

One sample (G98) was initially amplified with the universal primer 1492R (5'- GGT TAC CTT GTT ACG ACT T -3') and the archaeal specific primer 4F (5'- TCC GGT TGA TCC TGC CRG -3', where R=A or G). PCR conditions were an initial denaturation at 94°C for 5 min, 40 cycles of 94°C, 30 s; 48°C, 30 s; 72°C, 30 s; and a final 15 min at 72°C. The PCR product was then used as a template for PCR with the primers used to amplify the V3 region as previously described.

PCR products were analyzed using denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1996). A 6% acrylamide gel was prepared in TAE buffer (0.04M Tris, 0.02M glacial acetic acid, 1 mM EDTA). A 20% to 60% urea/formamide denaturing gradient was produced using stock solutions of 40% (vol/vol) formamide and 7 M urea. The gel was run in a Bio-Rad DCode Universal Mutation Detection System at 60°C at 180 V for 2.5 h. The acrylamide gel was stained with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME) for 15 min and viewed on a UV light box.

The individual bands from the DGGE gel were removed by piercing the gel with a pipet tip. The tip was then placed in 20 µl of 10 mM Tris for 10 min at room temperature. 2 to 5 µl of this mixture was used as a template for PCR.

Conditions were as described above except the forward primer lacked the GC-rich section required for DGGE. The PCR products were cleaned with the PCRpure SPIN kit (GeneMate, ISC BioExpress, Kaysville, UT) following the manufacturer's instructions. Both strands of the PCR product were sequenced by cycle sequencing using fluorescent dideoxy terminators as previously described (Reysenbach et al. 2000). The sequences were resolved on an automated DNA sequencer (ABI Prism 310 Genetic Analyzer) and assembled with the AutoAssembler Program (Applied Biosystems, Inc.).

3.3.4 Calculation of Sorenson's index for the DGGE banding patterns to assess the similarity of the microbial diversity between samples

Sorenson's index is a numerical representation of the similarity between samples that has been used by others in analyzing DGGE data (Murray et al. 1998). This index is a qualitative determination of the similarity of microbial diversity between sites. Two samples were compared to determine what, if any, was the number of shared bands. Pairwise similarity values were calculated using Sorenson's index (Magurran 1988). $C_s = \frac{2j}{(a+b)}$

where j is the number of common bands shared between sample A and sample B, a is the total number of bands in sample A and b is the total number of bands in sample B. If C_s=1 then the band patterns are identical and all of the bands are shared between the two samples. If C_s=0 then no bands are shared.

3.3.5 Phylogenetic analysis of the DGGE bands

BLAST (basic logic alignment search tool) (Altschul et al. 1997) and the Ribosomal Database Project's (RDP) Similarity Matrix tool (Maidak et al. 2000) were used to identify sequences closely related to the sequences obtained from the DGGE gel. Sequences (~160 nucleotides) from the DGGE bands were aligned in ARB (Strunk and Ludwig 1995). Distance matrices of the entire region sequenced were constructed with PAUP 4.0b6 (Swofford 1998) using the Jukes and Cantor algorithm (Jukes and Cantor 1969).

3.3.6 Cloning of 16S rDNAs and restriction fragment length polymorphism (RFLP) analysis

The 16S rRNA genes from one chimney sample (G72) and one flange sample (G26) were amplified using PCR with the universal primer 1492R and the archaeal specific primer 4F. Conditions for PCR were an initial denaturation at 94°C for 5 min, 35 cycles of 94°C, 30 s; 50°C, 30 s; 72°C, 90 s; and a final 10 min at 72°C. The PCR products were purified with the Gelpure SPIN kit (GeneMate, ISC BioExpress, Kaysville, UT) following the manufacturer's instructions.

The PCR products were cloned into the pCR4 vector (Invitrogen, Carlsbad, CA). Plasmid DNA was extracted following the protocol of Sambrook et al. (Sambrook et al. 1989). Plasmid DNA was screened for inserts by PCR with the vector-specific M13 primers: M13F (5'-GTA AAA CGA CGG

CCA G-3') and M13R (5'-CAG GAA ACA GCT ATG AC-3'). The PCR-amplified inserts were digested with 1U of the restriction endonucleases *Msp*I and *Hin*PI following the manufacturer's instructions (New England Biolabs). The resulting products were separated by electrophoresis on a gel of 3.5% NuSieve 3:1 agarose (BioWhittaker Molecular Applications, Rockland, ME) with TBE buffer. The clones were separated into different phylotypes based on the RFLP banding patterns.

3.3.7 Sequencing and phylogenetic analysis of the clone libraries

Both 16S rDNA strands of one representative of each phylotype was fully sequenced by cycle sequencing as described previously (Reysenbach et al. 2000). Sequences were assembled using AutoAssembler (Applied Biosystems Inc.). The sequences were checked for the presence of chimeras using the RDP's CHIMERA_CHECK program (Maidak et al. 2000). The CHIMERA_CHECK analysis was run with and without the addition of the sequences obtained from this experiment. Potential chimeras were removed from further analysis.

Sequences were manually aligned in ARB (Strunk and Ludwig 1995). The alignment included close representatives that were identified based on data from BLAST (Altschul et al. 1997) and RDP's Similarity Matrix tool (Maidak et al. 2000). Approximately 1,450 nucleotides were sequenced and about 1,300 nucleotides in evolutionarily conserved regions were used for the

maximum likelihood analysis. Maximum likelihood trees were constructed using fastDNAml (Olsen et al. 1994). The optimal T value (T=1.4) was determined by comparing the likelihood score of trees constructed with a range of T values (T=1.0 to T=3.0) to a tree constructed with T=2.0. Once the optimal T value was determined, multiple maximum likelihood analyses were conducted with the sequence addition order jumbled to produce the optimum tree. Bootstrap data represent the percent scores from 100 replicates.

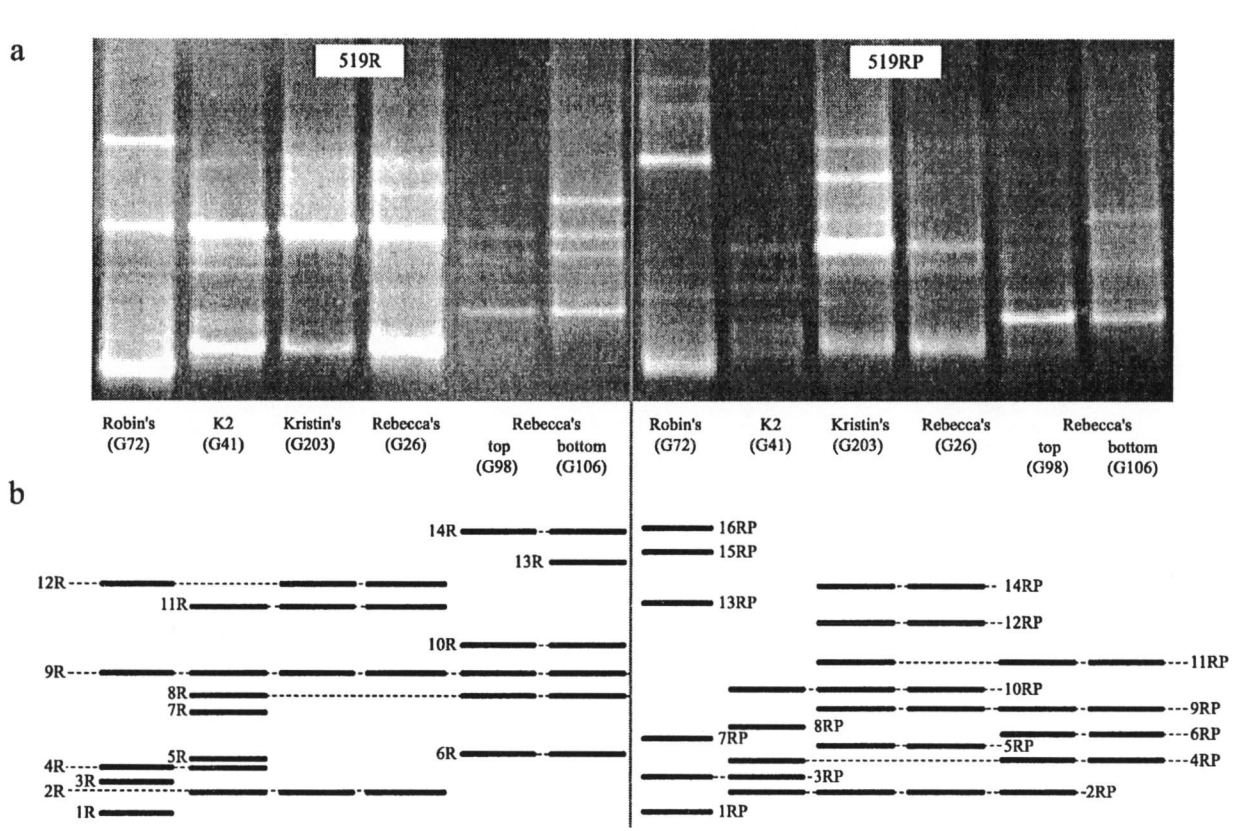

Figure 3-1. a. Image of denaturing gradient gel for the sulfide samples from Guaymas Basin. For lanes 1 through 6, 519R was used as the reverse primer and in lanes 7 through 12, 519RP was used as the reverse primer. The samples were loaded in the same order and the sites are marked in the figure. b. Schematic indicating the distribution of bands between the different sites and how the bands were numbered.

3.3.8 Nucleotide sequence accession numbers

GenBank accession numbers for the full-length clones are AF356628-AF356648. Other sequences obtained from GenBank and used in the maximum likelihood analysis are as follows: *Methanolobus oregonensis* (U20152), *Methanomethylovorans hollandica* (AF120163), pISA16 (AB019758), pISA14 (AB019759), *Methanocorpusculum parvum* (M59147), *Methanoculleus thermophilicus* (M59129), *Halococcus morrhua* (X00662), *Haloferax volcanii* (K00421), VC2.1Arc13 (AF068820), pISA42 (AB019742), *Picrophilus oshimae* (X84901), *Thermoplasma acidophilum* (M38637), WCHD3-16 (AF050618), SB95-72 (U78206), *Methanothermus fervidus* (M59145), *Methanobacterium formicicum* (M36508), *Methanococcus aeolicus* (U39016), *Methanococcus jannaschii* (M59126), *Thermococcus celer* (M21529), VC2.1Arc16 (AF068821), *Pyrococcus furiosus* (U20163), *Archaeoglobus veneficus* (Y10011), *Ferroglobus placidus* (X99565), *Sulfolobus solfataricus* (X90478), and *Pyrodictium occultum* (M21087).

3.4 Results

3.4.1 Results from the DGGE analysis

The PCR products obtained using the 519R and 519RP primers for each of the samples were run on a single gel and the results are shown in Figure 3-1. A total of 14 bands were identified when the samples where amplified

with the 519R primer, while the 519RP primer identified a total of 16 bands. Furthermore, the pattern of DGGE bands obtained at each site varied with the different reverse primers.

Comparisons of the DGGE bands from the Kristin's Summit and Rebecca's Roost flanges using Sorenson's index indicated a high degree of similarity in microbial diversity between the two samples (Table 3-2, values of 1 and 0.92 for the 519R and 519RP primers, respectively). Both flanges also were hard flanges that were primarily sphalerite with traces of pyrite visible. The top and bottom sections of the flange sample from Rebecca's Roost also have a high Sorenson's index score (values of 0.91 and 0.89 for the 519R and 519RP primers, respectively), indicating a high degree of similarity in the archaeal community between the two sections. Both sections have a low degree of similarity with the flange sampled from Rebecca's Roost during a different dive (G26). The G26 flange sample was primarily barite with some pyrite and sphalerite, while the G98/G106 flange sample was a porous flange with smaller amounts of barite and pyrrhotite.

The differences between the two reverse primers became more apparent following the Sorenson's index calculations. When the 16S rDNA was amplified with the 519RP primer, the microbial diversity of the sulfide chimney collected from Robin's Roost was very different from that of the flanges since only one band was shared with one other site (K2). Conversely, when the 16S rDNA was amplified with the 519R primer, higher Sorenson's

index scores indicated a higher degree of similarity in the microbial diversity between the chimney and the flanges from Kristin's Summit and Rebecca's Roost (G26).

Table 3-2. Results of the Sorenson's index calculations for the sulfide samples collected from Guaymas Basin. The numbers in the upper right corner of the matrix indicate the number of bands shared between the two sites while the numbers in the lower left corner are the calculated Sorenson's index (see text for details).

519R and 344F-GC PCR products						
Sample	**Robin's Roost**	**K2**	**Kristin's Summit**	**Rebecca's Roost**	**Rebecca's Top**	**Rebecca's Bottom**
Robin's Roost		2	3	2	1	1
K2	0.33		3	3	1	1
Kristin's Summit	0.67	0.55		4	1	1
Rebecca's Roost	0.67	0.55	1.00		1	1
Rebecca's Roost Top	0.20	0.17	0.22	0.22		5
Rebecca's Roost Bottom	0.18	0.15	0.20	0.20	0.91	
519RP and 344F-GC PCR products						
Sample	**Robin's Roost**	**K2**	**Kristin's Summit**	**Rebecca's Roost**	**Rebecca's Top**	**Rebecca's Bottom**
Robin's Roost		1	0	0	0	0
K2	0.18		2	2	1	0
Kristin's Summit	0.00	0.33		6	3	2
Rebecca's Roost	0.00	0.36	0.92		2	1
Rebecca's Roost Top	0.00	0.20	0.50	0.36		4
Rebecca's Roost Bottom	0.00	0.00	0.36	0.20	0.89	

Approximately 160 nucleotides of sequence data from both strands of the 16S rDNA were assembled and aligned to other *Archaea*. Distance matrices

were constructed and a summary is given Table 3-3. To facilitate comparison of the archaeal sequences identified by site, Table 3-4 clusters the sequences identified by sulfide sample. All of the sequences identified were *Euryarchaetoa*. The majority of DGGE bands obtained with the 519R primer cluster within the *Methanococcales*. This group of bands is 3.1% to 36.7% distant from *Methanococcus aeolicus*, and is 16.7% to 41.8% distant from *Mc. jannaschii*.

The remaining sequences from the sulfide samples cluster into three groups. One DGGE band identified from the sulfide chimney (Band 16RP) is most closely related to *Methanoculleus bourgense*, a species within the *Methanomicrobiales*. Three bands (Band 3R, 1RP, and 13RP) were most closely related to a group of sequences associated with anaerobic methane-oxidation in marine sediments (Hinrichs et al. 1999). Finally the majority of the sequences obtained with the 519RP primer cluster within the Group II *Archaea* or with sequences previously identified at deep-sea hydrothermal vents. Sequences within groups identified from western Pacific hydrothermal vents include the sequences clustering with DHVE1, DHVE2, DHVE5 and DHVE7 (Takai and Horikoshi 1999).

Table 3-3. Summary of the distance matrices constructed with the sequences obtained from the DGGE bands from Guaymas Basin using the Jukes and Cantor correction (Jukes and Cantor 1969). The bands were amplified with two different reverse primers; those with the R suffix were amplified with 519R and those with the 519RP suffix were amplified with 519RP.

Group	**Least distant species**	**Band #**	**% distance**
Methanococcales	*Methanocaldococcus vulcanius*	2R	6.4%
	Methanococcus aeolicus	5R	17.8%
		7R	7.3%
		8R	8.3%
		9R	8.2%
		10R	34.0%
		11R	9.3%
		13R	22.8%
Group II *Archaea*/DHVE1	pMC2A24	6R	7.4%
Group II *Archaea*/DHVE2	pISA12	1R	36.8%
		4R	29.0%
Group II *Archaea*	ABS23	14R	13.2%
DHVE7	pISA14	12R	2.4%
ANME-1	BA1a2	3R	17.7%
Methanococcales	*Methanococcus aeolicus*	7RP	9.1%
		12RP	36.7%
		14RP	3.1%
Group II *Archaea*/DHVE1	pMC2A24	6RP	12.0%
		11RP	10.7%
Group II *Archaea*/DHVE2	pSSMCA108	3RP	33.2%
		10RP	31.3%
Group II *Archaea*/DHVE2	pISA42	4RP	35.7%
Group II *Archaea*/DHVE2	pMC2A10	8RP	21.1%
Group II *Archaea*	2C84	2RP	26.6%
		9RP	25.2%
Group II *Archaea*	WCHD3-02	5RP	21.7%
DHVE5	pISA1	15RP	37.0%
ANME-1	TA2e12	1RP	19.0%
		13RP	3.2%
Methanomicrobiales	*Methanoculleus bourgense*	16RP	18.9%

Table 3-4. Sequences obtained from Guaymas Basin and summarized by sulfide sample. The numbers in parentheses indicate number of DGGE bands that were identified.

519R primer			
Robin's Roost	**K2**	**Kristin's Summit**	**Rebecca's Roost**
Methanococcales	*Methanococcales* (6)	*Methanococcales* (3)	*Methanococcales* (3)
Group II *Archaea*: DHVE2 (2)	Group II *Archaea*: DHVE2	DHVE7	DHVE7
DHVE7			
ANME-1			

Rebecca's Roost top	**Rebecca's Roost bottom**
Methanococcales (4)	*Methanococcales* (5)
Group II *Archaea*: DHVE1	Group II *Archaea*: DHVE1
Group II *Archaea*	Group II *Archaea*

519RP primer			
Robin's Roost	**K2**	**Kristin's Summit**	**Rebecca's Roost**
Methanococcales	DHVE2 (4)	*Methanococcales* (2)	*Methanococcales* (2)
Methanomicrobiales	Group II *Archaea*	Group II *Archaea*: DHVE1	Group II *Archaea*: DHVE2
Group II *Archaea*: DHVE2		Group II *Archaea*: DHVE2	Group II *Archaea* (3)
DHVE5		Group II *Archaea* (3)	
ANME-1 (2)			

Rebecca's Roost top	**Rebecca's Roost bottom**
DHVE1 (2)	DHVE1 (2)
DHVE2	DHVE2
Group II *Archaea* (2)	Group II *Archaea*

3.4.2 Archaeal diversity obtained from the two clone libraries

Clone libraries were established for different sulfide structures, one chimney (G72) and one flange (G26), using 16S rDNA PCR product (~1490 nucleotides). Two separate clone libraries were constructed for sample G72.

The first clone library included eukaryotes; because we are interested in archaeal diversity these were eliminated from the second clone library by repeating the gel purification step and only cutting the band of shorter PCR product which corresponds to the archaeal 16S rDNA PCR product. Clones G72_C1, G72_C2, G72_C3, G72_C6 and G72_C12 are from the first clone library, while the remaining clones are from the second clone library. The two clone libraries identified the same groups of *Archaea*.

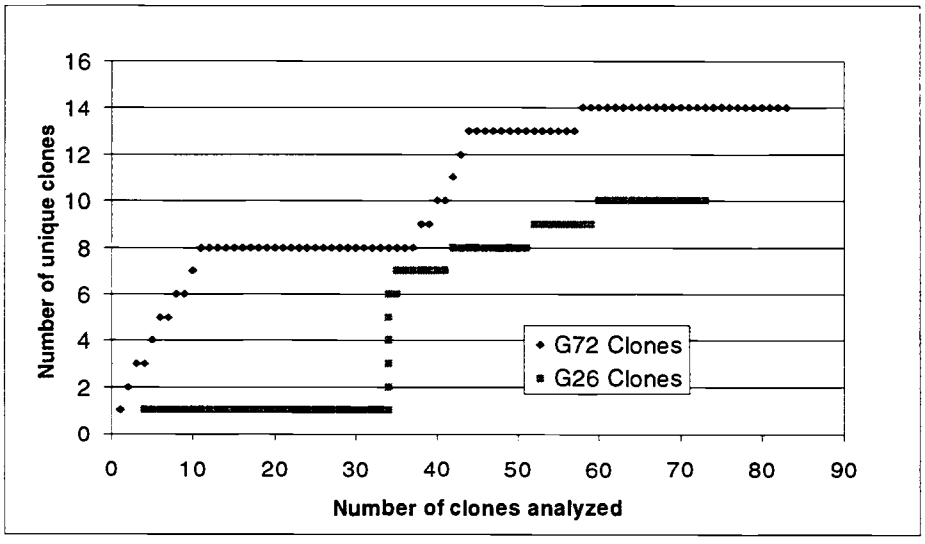

Figure 3-2. Collector's curve for the two Guaymas Basin clone libraries.

Clones were analyzed using RFLP analysis to identify unique phylotypes. A total of 82 clones were analyzed for the sulfide chimney sample (G72) and 14 different RFLP patterns were identified. For the flange sample

(G26), 41 clones were analyzed with 11 different RFLP patterns identified. In both cases, the collector's curve leveled off as further clones were analyzed indicating the number of clones sampled was a representative section of the community diversity (Figure 3-2).

A summary of the sequences identified from the two clones libraries and the distribution of the number of phylotypes is given in Table 3-5. Three sequences were not included in the analysis since they were identified as potential chimeras. In addition to the archaeal sequences, there were 3 eukaryotic DNA sequences identified from the sulfide chimney (G72); these sequences were over 70% of the sequences obtained from the first clone library. The percentages given in Table 3-5 do not include the phylotypes identified as eukaryotes. Most of the sequences from the flange clustered within the *Methanococcales*. The remaining sequences cluster within the *Thermococcales* and the DHVE2 group within the Group II *Archaea*. The majority of the sequences from the chimney clustered within the *Methanosarcinales* and 14.3% clustered within the closely related DHVE7 group.

Table 3-5. Summary of sequences obtained from the 16S rDNA clone libraries obtained for the G26 and G72 sulfide samples.

Sample	Group	Type sequence[1]	Other representatives[2]	Percent of clones[3]
Flange (G26)	*Methanococcales*			
	Mc. aeolicus	G26_C5	G26_C45	39.0%
			G26_C49	
	Mc. jannaschii	G26_C46	G26_C50	29.3%
			G26_C51	
			G26_C63	
			G26_C82	
	Thermococcales	G26_C48		26.8%
	DHVE2	G26_C73	G26_C56	4.9%
Chimney (G72)	*Methanosarcinales*	G72_C12	G72_C1	85.7%
		G72_C61	G72_C6	
	DHVE7	G72_C3	G72_C2	14.3%
		G72_C65	G72_C59	

[1]The type 16S rRNA sequence was used in the maximum likelihood and bootstrap analysis.

[2]Additional closely related sequences that were submitted to GenBank.

[3]The percentage of clones as identified by RFLP analysis was calculated considering each clone library separately.

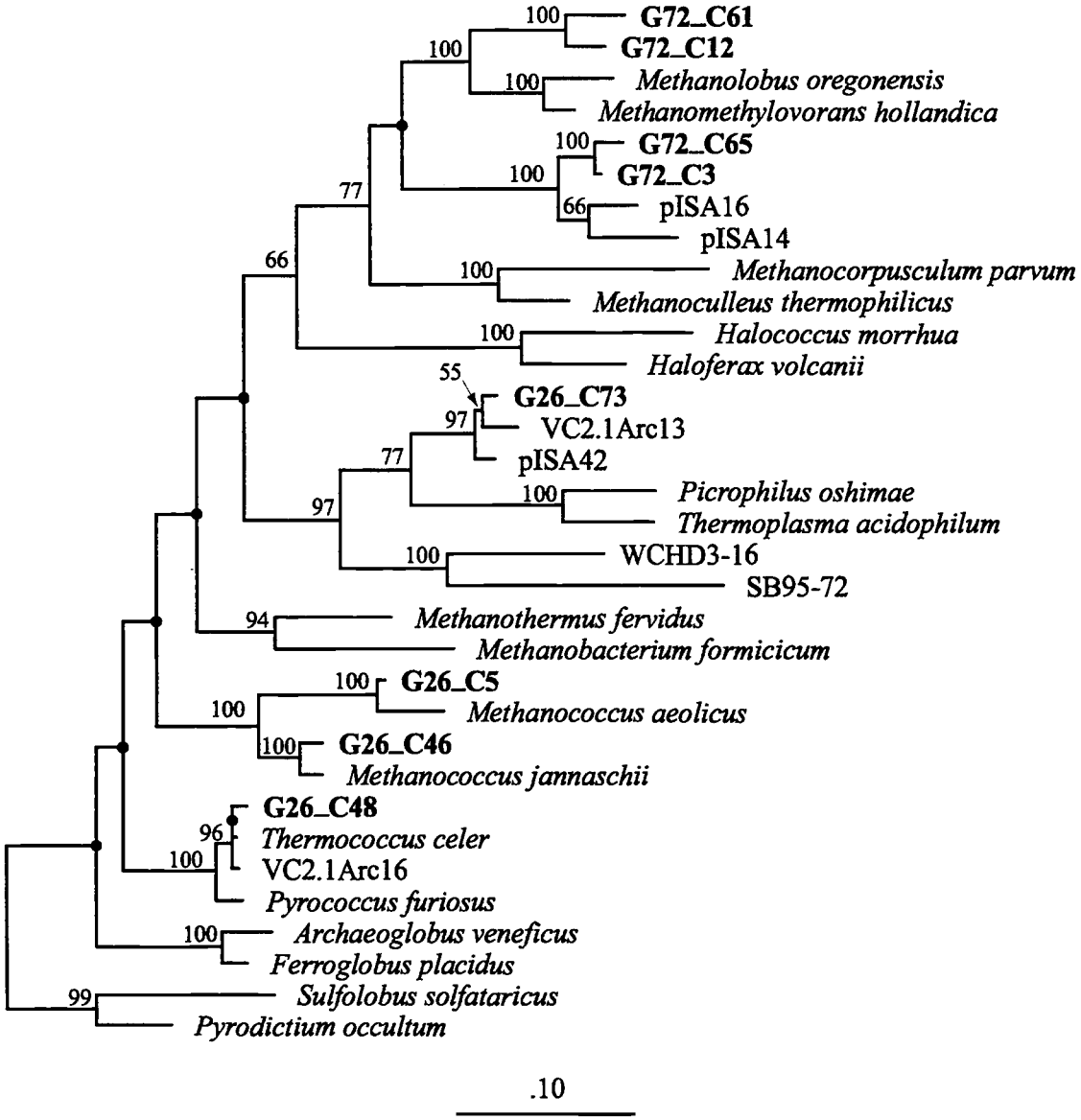

Figure 3-3. Phylogenetic analysis of the 16S rDNA sequences from the Guaymas Basin sulfide samples. The tree is the result of maximum likelihood analysis using *Sulfolobus solfataricus* and *Pyrodictium occultum* as the outgroup. The numbers at the nodes represent percent of bootstrap values obtained from 100 samplings. Scale bar represents 0.1 nucleotide substitutions per sequence position. Guaymas Basin sequences are marked in bold type.

At least one representative of each phylotype was included in the maximum likelihood trees produced and the results including the bootstrap analysis are given in Figure 3-3. The sequences from Guaymas Basin cluster into different groups whose position in the maximum likelihood trees is well supported by the bootstrap analysis.

3.5 Discussion

3.5.1 The archaeal community in sulfide samples from Guaymas Basin

The use of 16S rDNA-based methods on sulfide samples from Guaymas Basin has revealed *Archaea* not previously known to inhabit deep-sea hydrothermal vent ecosystems. The presence of *Methanomicrobiales* in the DGGE analysis and *Methanosarcinales* in the sulfide chimney's clone library represents the first identification of these groups at deep-sea hydrothermal vents (though *Methanomicrobiales* were also identified at 9°N and Juan de Fuca, see Chapters 2 and 4). Furthermore, the variation in the archaeal diversity between different sulfide samples emphasizes the need analyze samples with differing mineralogy before making larger conclusions about the microbial community present.

The use of DGGE allowed for comparisons between different sulfide samples based on the pattern of DGGE bands and the sequence data obtained from those bands. According to the distance matrices, sequence data from

multiple bands, either from the same sample or when comparing between samples, corresponded to the same *Archaea*. Such clusters have been noted before in studies using 16S rDNA sequence data (Giovannoni et al. 1990; Ferris et al. 1996; Ferris and Ward 1997). One hypothesis presented to explain these clusters is that heterogeneity in ecological conditions may lead to clusters of co-existing closely related organisms (Begon et al. 1996). Heterogeneity within a hydrothermal ecosystem may arise due to fluctuations in the flow of hydrothermal fluid causing variations in temperature (Karl et al. 1988; Chevaldonné et al. 1991; Fornari et al. 1998) and fluid chemistry (Von Damm et al. 1995; Butterfield et al. 1997). The microbial community within sulfide samples may be adapting to these fluctuations by allowing each subgroup to be active under different geochemical conditions.

3.5.2 *Archaea* in sulfide flanges from Guaymas Basin

Flange samples with similar mineralogy resulted in similar archaeal communities based on the Sorenson's index calculations. There was a high degree of similarity between the archaeal communities present in the flanges from Kristin's Summit and Rebecca's Roost (G26). Both the sphalerite and small amounts of pyrite present in both flanges results in similar ecological niches. The *Archaea* present within these two flanges were *Methanococcales*, Group II *Archaea*, and sequences clustering within the DHVE1, -2, and -7 groups.

Although sequences that are phylogenetically similar may not be metabolically similar, all of the organisms within the *Methanococcales* are capable of producing methane through the reduction of CO_2. *Methanococcales* have been cultured from hydrothermal vent sites (Baross and Deming 1995; Karl 1995; Jeanthon et al. 1999) and they were obtained during this cruise in laboratory cultures designed to enrich for microorganisms capable of hydrogen oxidation (Longnecker and Reysenbach, unpublished results). However, all these *Methanococcales* were related to *Mc. jannaschii* (Jones et al. 1983). In contrast, the DGGE bands were most closely related to *Methanococcus aeolicus*, a methanogen that has not previously been identified in any hydrothermal vents samples.

Group II *Archaea* were approximately 50% of the DGGE bands obtained in the Kristin's Summit and Rebecca's Roost flanges when the samples were amplified with the 519RP reverse primer. The Group II *Archaea* have previously been identified at Pacific and Atlantic deep-sea hydrothermal vents (Moyer et al. 1998; Takai and Horikoshi 1999; Reysenbach et al. 2000). The euryarchaeal Group II *Archaea* and the crenarchaeal Group I *Archaea* have also been identified in non-hydrothermal marine ecosystems (DeLong 1992; DeLong et al. 1994; Massana et al. 1997; Massana et al. 1998; Vetriani et al. 1999; Massana et al. 2000). In non-vent marine environments, the euryarchaeal Group II *Archaea* are more prevalent in the surface waters, while the crenarchaeal Group I *Archaea* increase at depths below the euphotic zone

(Massana et al. 1997; Massana et al. 1998). Furthermore, the crenarchaeal Group I *Archaea* are up to 39% of the microbial population (*Bacteria* and *Archaea*) at depths greater than 1000 m at a station in Hawaii (Karner et al. 2001). However no Group I *Archaea* were identified within the sulfide structures at Guaymas Basin indicating that the Group I *Archaea* may only be dominant in non-vent bathypelagic ecosystems.

The final sequences identified in both the Kristin's Summit and Rebecca's Roost flanges cluster with the DHVE7 group (Takai and Horikoshi 1999), although this group was only present when the samples were amplified with the 519R reverse primer. Maximum likelihood analysis indicates that the sequences within the DHVE7 group cluster with the ANME-1 group previously identified from marine sediments (Hinrichs et al. 1999). Based on the depletion of ^{13}C in the lipids extracted from the sediments, the ANME-1 group is hypothesized to be capable of anaerobic methane oxidation (Hinrichs et al. 1999). There are no laboratory cultures proven to be capable of anaerobic methane oxidation. However if these *Archaea* share the same metabolic capacity as the DHVE7 group they could be a sink for methane at deep-sea hydrothermal vents.

According to the Sorenson's index calculations, the K2 flange had low degrees of similarity when compared to the flanges from Kristin's Summit and Rebecca's Roost (G26). This low degree of similarity was based on the pattern of DGGE bands, yet the sequence data indicated the same types of sequences

were identified as in the other flanges. The mineralogy of the K2 flange indicated it was a hard flange of pyrite and pyrrhotite and thus comprised of different minerals than the other flanges. The differences in the DGGE banding pattern may be correlated to differences in the observed mineralogy if different *Archaea* inhabit different geochemical regimes within hydrothermal ecosystems.

The final flange was a soft, possibly newly formed, flange from Rebecca's Roost. The top and bottom sections of the flange had very high degrees of similarity indicating similar patterns of diversity in the *Archaea* present. The relatively uniform mineralogy of the flange may result in only one microbial niche that could be occupied by the microorganisms identified. Alternatively, the uniformity of the species between the top and bottom section may indicate that the microbial community had just settled into the newly formed sulfide structure. Microorganisms potentially could be transported from the subsurface to colonize a newly formed flange or be transported from other vents via water currents flowing along the seafloor. A model of larval transport at hydrothermal vents shows that the larvae are most often transported in near-bottom water currents (Kim and Mullineaux 1998) and, while not explicitly included in the model, microorganisms could be transported between vents in the same flow regime.

3.5.3 Archaeal community and mineralogy in the sulfide chimney

A different archaeal community was present in the chimney sample than in the flange samples from Guaymas Basin. *Methanomicrobiales*, *Methanosarcinales*, and sequences clustering with the ANME-1 and DHVE5 groups were only present in the sulfide chimney sample. Furthermore, where the distance matrices identified the same sequence types, they were represented by different DGGE bands in the chimney sample than in the flange samples. As in the flanges, mineralogy could be a component in defining the ecological niches within sulfide structures. The chimney from Robin's Roost and the flanges from Kristin's Summit and Rebecca's Roost were all comprised primarily of sphalerite. However while similar archaeal communities were identified within the two flanges, different *Archaea* were identified within the sulfide chimney sample. Therefore when comparing the microbial communities between chimneys and flanges, mineralogy seems less important than structural differences.

The identification of *Methanosarcinales* in the sulfide chimney is the first identification of this order of methanogens at deep-sea hydrothermal vents. *Methanosarcinales* are unique in their ability to use substrates beyond H_2 and CO_2 for methanogenesis since they also use acetate and methylated compounds such as methanol and methylamines (Jones et al. 1987). Previous attempts to culture *Methanosarcinales*-like organisms from deep-sea

hydrothermal vents have been unsuccessful (P. Rouvière and K.O. Stetter as cited in (Rouvière et al. 1992). Given the low percentage of prokaryotes that can be cultured in the laboratory (Amann et al. 1995), this negative result is not surprising. However their presence in the sulfide chimney from Guaymas Basin may be possible if the hydrocarbon-rich vent fluid at Guaymas Basin contains methylated compounds that the *Methanosarcinales* can use for methanogenesis. This could explain their presence at Guaymas Basin and absence at other deep-sea hydrothermal vent sites.

Methanomicrobiales were also identified within the sulfide chimney and absent from the Guaymas Basin flanges. To our knowledge, there are no laboratory cultures of *Methanomicrobiales* obtained from deep-sea hydrothermal vents, though isolates exist from non-vent systems such as sediments, bioreactors, and digestors (Whitman et al. 1992). The *Methanomicrobiales* were also identified from the 9°N chimney sample and the flange from Juan de Fuca Ridge (Chapters 2 and 4). Therefore the *Methanomicrobiales* can occupy both chimneys and flanges within hydrothermal systems, although they were only identified in a chimney at Guaymas Basin.

While the *Archaea* identified in the chimney differed from those in the flanges, the degree of difference varied depending on whether the analysis relied on DGGE or the clone libraries. The two clone libraries identified entirely different groups of *Archaea*. *Methanosarcinales* and the DHVE7 group

were identified in the chimney, while the flange included *Methanococcales*, *Thermococcales* and the DHVE2 group. Furthermore, *Thermococcales* and *Methanosarcinales* were abundant among the clones obtained, yet both groups were absent from the DGGE analysis. Thus while DGGE and clone libraries both indicate different archaeal communities are present in chimneys and flanges, the actual differences depend on the type of 16S rDNA-based analysis used.

3.5.4 Differences in the observed archaeal diversity obtained with the different reverse primers

The two reverse primers used in this study differed in the length of the primer and the presence of degeneracies. The 519R primer is a 17-mer and contains no degeneracies while the 519RP primer is a 19-mer and has two degeneracies. Two different primers were used because the 519R primer has three or more mismatches between the primer and crenarchaeal 16S rDNA sequences. Furthermore, in a test in the laboratory, the 519R primer was unable to amplify *Thermococcales* (data not shown) likely due to the two mismatches between the *Thermococcales* and the 519R primer. However the 519RP primer is not ideal for DGGE analysis since it is a degenerate primer and a single species could result in multiple bands if the same sequence is amplified with the different primer sequences.

In the analysis of the Guaymas Basin samples the 519R primer resulted in more DGGE bands that clustered within the *Methanococcales*, while the

519RP primer produced DGGE bands that primarily clustered within the Group II *Archaea*. The 519R primer only has one mismatch with the *Methanococcales* at the 5' end of the primer, while the 519RP primer has one to three internal mismatches with the *Methanococcales*. Yet this does not explain the absence of *Mc. jannaschii*-like sequences in the DGGE analysis since there are no mismatches between any *Mc. jannaschii* sequence and the 519R or 519RP primer. The prevalence of Group II *Archaea* amplified with the 519RP primer can be explained by the lack of mismatches between the primer and the Group II *Archaea*, conversely the 519R primer has one mismatch. The 519R primer has amplified Group II *Archaea* in other samples (Chapter 4). Differential amplification of DNA templates during PCR has previously been recognized as a problem (Reysenbach et al. 1992; Suzuki and Giovannoni 1996) and was seen in the Guaymas Basin samples since each of the reverse primers amplified different subsets of the archaeal community.

3.5.5 Conclusions

Using 16S rDNA-based tools to identify the archaeal community within sulfide structures from Guaymas Basin expanded the orders of methanogens known to inhabit deep-sea hydrothermal vents to include the *Methanosarcinales* and the *Methanobacteriales*. Additionally, *Archaea* were identified previously only found at deep-sea hydrothermal vents and therefore these *Archaea* may be endemic to hydrothermal ecosystems.

Examples in this group include the DHVE1, -2, and -5 sequences that have been previously identified in the western Pacific (Takai and Horikoshi 1999) and the Mid-Atlantic Ridge (Reysenbach et al. 2000). Other groups of sequences identified as endemic to hydrothermal vents include a group of ε-*Proteobacteria* found at hydrothermal vents in the Pacific and Atlantic (Reysenbach et al. 2000; Longnecker and Reysenbach 2001) and the *Methanopyrales* (Huber et al. 1989; Kurr et al. 1991). Finally, when considering a single type of sulfide structure, for example comparisons between flanges, variations in the archaeal community identified maybe correlated to variations in mineralogy. However when comparing between chimneys and flanges, the differences between the two types of sulfide structures seems more important than similarities in mineralogy.

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4 Microbiology and Mineralogy of a Flange Collected from a Deep-Sea Hydrothermal Vent on the Juan de Fuca Ridge

4.1 Abstract

The correlation between the mineralogy and microbial diversity of a flange collected from the Juan de Fuca Ridge was examined using phylogenetic analysis based on the small subunit ribosomal RNA gene (16S rDNA). DNA was extracted from each section of the flange and 16S rDNA was amplified with primers modified for denaturing gradient gel electrophoresis (DGGE). Analysis of the DGGE results indicated that the section of the flange with the largest number of DGGE bands coincided with the section that had the largest change in mineralogy. The hottest section of the flange contained no archaeal DGGE bands and the lowest number of bacterial DGGE bands. Although several different groups of *Bacteria* have been identified from other hydrothermal vents through the use of molecular tools and laboratory cultures, all of the *Bacteria* within the Cantilever flange were related to ε-*Proteobacteria* that have been identified from other deep-sea hydrothermal vent fields. The *Archaea* from the flange were all *Euryarchaeota*, most of which clustered within the *Methanomicrobiales*. Methanogens have been previously described from hydrothermal vents, however the

identification of *Methanomicrobiales* expands the breadth of methanogens present in hydrothermal ecosystems.

4.2 Introduction

The interaction between microorganisms and their surrounding minerals can result in small-scale and large-scale changes to the ecosystem. Microorganisms can catalyze the transformation of minerals in their vicinity by directly changing the oxidation state of a mineral or by causing a chemical reaction to occur with their byproducts. For example, microbial oxidation of pyrite leads to the formation of water with lower pH values (Edwards et al. 1998; Edwards et al. 2000; Edwards et al. 2001). Additionally, sulfate-reducing microorganisms within biofilms have been shown to produce sulfide that can precipitate with zinc to form spherical aggregates of sphalerite (Labrenz et al. 2000).

Within deep-sea hydrothermal vent chimneys, microorganisms have been hypothesized to be responsible for pitting that created 1 μm long rod shapes on the external surface of a chimney collected from a deep-sea hydrothermal vent in the southeastern Pacific Ocean (Verati et al. 1999). Geochemical analyses of the sections lead to the hypothesis that microorganisms were leaching minerals from the sulfide's external surface via a multi-phase process that solubilized the iron and sulfur minerals within the chimney (Verati et al. 1999). While the authors model the processes that occur

on the surface of the chimney and propose microorganisms that may be involved, the actual identity of the microorganisms is unknown (Verati et al. 1999).

Based on the metabolism of laboratory cultures, microorganisms living in deep-sea hydrothermal vent systems can be involved in the cycling of sulfur, methane, hydrogen, and iron (Karl 1995). Phylogenetic analysis based on 16S rDNA of samples from deep-sea hydrothermal vents has also, not surprisingly, identified organisms with as yet unknown metabolic capabilities. Relevant to the present study are sequences that cluster with the uncultured Group II *Archaea* that have been identified from Pacific (Moyer et al. 1998; Takai and Horikoshi 1999) and Atlantic (Reysenbach et al. 2000) hydrothermal vents. Previous studies using 16S rDNA-based analysis have focused on hydrothermal fluid, sulfide chimneys, and microbial mats. The diversity of microorganisms present within flanges and their potential role in the altering the mineralogy of the flanges is largely unknown.

A flange is a ledge in a sulfide-sulfate-silicate structure that has grown perpendicular to the main body of a sulfide chimney, so that the resulting flange is roughly parallel to the sea floor (Delaney et al. 1992; Tivey et al. 1999). Flanges range in size from centimeters to up to 5 m along the border between the flange and the body of the sulfide chimney. The formation of a flange has been hypothesized to occur when the internal pressure of a sulfide chimney increases and forces the flow of hydrothermal fluid laterally out of

the sulfide chimney. This increase in internal pressure is due either to increased mineral precipitation which blocks the flow of fluid or because of increased fluid drag resulting from the lengthening of fluid channels within the sulfide chimney (Delaney et al. 1992). Hydrothermal fluid is trapped beneath the flange in a concave area of the flange. The fluid then either rises vertically through the flange and cools until it exits the top of the flange or is pushed out of the flange at the lip of the flange, the section furthest from the body of the sulfide chimney (Delaney et al. 1992). Growth of the flange by precipitation of minerals occurs during the interaction of hydrothermal fluid and seawater at the outer lip of the flange (Tivey et al. 1999).

Flanges are not found at all deep-sea hydrothermal vent fields. The presence of flanges at the Endeavour vent field has been attributed to the higher pH (pH 4.2-4.5, measured at 25°C) due to increased ammonia concentrations within the vent fluid (Tivey et al. 1999). The increased pH of the vent fluid leads to amorphous silica deposition within the flange, this has been hypothesized to be critical in strengthening the flange so it can grow laterally from the sulfide chimney (Tivey et al. 1999). Silica lends increased stability to the flange since it is stronger than the anhydrite it replaces and is more resistant to dissolution by low temperature fluids than anhydrite (Tivey and Delaney 1986; Delaney et al. 1992).

This study examines the microbial diversity within a flange collected from the Endeavour section of the Juan de Fuca Ridge. Molecular

phylogenetic analysis based on 16S rDNA was used to identify the members of the microbial community and compare the diversity present in different sections of the flange. Both *Archaea* and *Bacteria* were present with the majority of the *Archaea* closely related to methanogens, while the *Bacteria* all clustered within the ε-*Proteobacteria*.

4.3 Materials and Methods

4.3.1 Sample collection and storage

The flange was collected on October 9, 1999 during DSRV *Alvin* dive #3480. The flange was removed from the Cantilever site (47°57'N, 129°6'W) on the Main Endeavour Field on the Juan de Fuca Ridge. The flange was examined to determine the different mineralogical regimes and then sectioned as indicated in Figure 4-1 for molecular and mineralogical samples. The samples for molecular analysis were homogenized in a mortar and pestle and divided into a series of tubes. They were then frozen on the ship at -80°C and kept frozen until the DNA extractions.

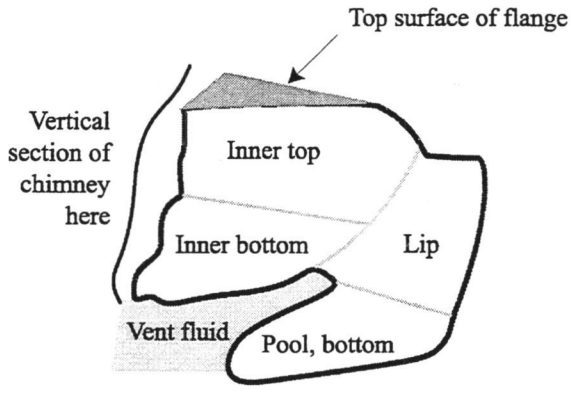

Figure 4-1. Schematic of the flange from the Cantilever site on the Juan de Fuca Ridge. After the flange was returned to the ship, it was cut in half. The diagram shows the sections as divided for mineralogy. The half used for analysis of the microbial community was a mirror image of what is shown in the figure.

4.3.2 Extraction of DNA and amplification of 16S rDNA with PCR

DNA was extracted following a modified extraction protocol using cetyltrimethylammonium bromide (CTAB) (Ausubel et al. 1994). A 50 µl aliquot of the sample was centrifuged for 20 min, the supernatant removed, and the pellet resuspended in 400 µl of TE buffer (10 mM Tris-Cl and 1 mM EDTA). 300 µl of extraction buffer (250 mM EDTA, 1.25% SDS, 8.3% Chelex-100, 0.66 mg ml^{-1} Proteinase K) was added. The sample was incubated at 50°C for 3 h and then spun briefly to separate the Chelex-bound metals and cell lysate. The supernatant was transferred to a new tube and 5M NaCl (0.8 M final concentration) and a CTAB/NaCl solution (1% CTAB and 0.7 M NaCl

final concentration) was added. The sample was incubated at 65°C for 30 min. The DNA was first extracted with chloroform:isoamyl alcohol (24:1) and then with phenol:choloroform:isoamyl alcohol (25:24:1). DNA was precipitated with an equal volume of 100% isopropanol overnight at 4°C.

PCR conditions were 5 µL of 10X Promega PCR buffer, 200 µmol dNTPs, 400 pmol of each primer, and 1 U of Promega Taq. PCR conditions for *Archaea* required an additional 1 mM $MgCl_2$ in addition to the 1.5 mM $MgCl_2$ already present in the buffer. *Archaea* were initially amplified with the universal primer 1492R (5'- GGT TAC CTT GTT ACG ACT T -3') and the archaeal specific primer 4F (5'- TCC GGT TGA TCC TGC CRG -3', where R=A or G). PCR conditions were initial denaturation at 94°C for 5 min, forty cycles of 94°C, 30 s; 48°C, 30 s; 72°C, 30 s; and a final 15 min at 72°C. PCR product from this reaction was then used as a template for PCR with the archaeal specific primer 344F-GC (5'-ACG GGG CGC AGC AGG CGC GA-3') and 519R (5'- ATT ACC GCG GCT GCT GG -3'). PCR conditions for this reaction were initial denaturation at 94°C for 5 min, thirty-five cycles of 94°C, 30 s; 48°C, 30 s; 72°C, 30 s; and a final 10 min at 72°C. The negative control from the first reaction was also tested in the second reaction to confirm that amplification in the nested PCR was not a contaminant. *Bacteria* were amplified with the universal primer 519R and the bacterial specific primer 338F-GC (5'-TCC TAC GGG AGG CAG CAG-3'). PCR conditions for this reaction were initial denaturation at 94°C for 5 min, thirty cycles of 94°C, 30 s; 50°C, 30 s; 72°C, 30 s;

and a final 10 min at 72°C. The forward primers used to amplify this V3 variable region of the *Archaea* and *Bacteria* have a 40-nucleotide GC-rich section required for the analysis.

4.3.3 Analysis of PCR products and sequencing

PCR products were analyzed using denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1996). A 6% acrylamide gel was prepared in TAE (0.04 M Tris, 0.02 M glacial acetic acid, 1 mM EDTA). Within the acrylamide gel, a gradient of increasing denaturant towards the end of the gel was established. The denaturants were a combination of urea and formamide where 100% denaturant would be 40% (vol/vol) formamide and 7 M urea. The denaturing gradient used was from 20% urea/formamide to 60% urea/formamide and the gel was run in a Bio-Rad DCode Universal Mutation Detection System at 60°C at 180 V for 2.5 h. The acrylamide gel was stained with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME) for 15 minutes then viewed on an ultraviolet light box.

Bands from the DGGE were noted and removed by piercing the gel with a pipet tip. The tip was then placed in 10 mM Tris for 10 minutes at room temperature. 2 to 5 µl of this mixture was used as a template for PCR with the forward and reverse primers that correspond to the forward and reverse primers used in the previous PCR reaction, except that in this step the primers lack the GC-rich tail required for DGGE. The PCR products were cleaned with

the PCRpure SPIN kit (GeneMate, ISC BioExpress, Kaysville, UT) following the manufacturer's instructions. Both strands of the resulting PCR product were sequenced by cycle sequencing using fluorescent dideoxy terminators. The sequences were determined on an automated sequencer (ABI Prism 310 Genetic Analyzer). Sequences were assembled with the AutoAssembler Program (Applied Biosystems, Inc.).

4.3.4 Phylogenetic analysis of the DGGE bands

Data from BLAST (basic logic alignment search tool) (Altschul et al. 1997) and the Ribosomal Database Project's (RDP) Similarity Matrix tool (Maidak et al. 2000) were used to identify sequences closely related to the sequences obtained from the DGGE gel. Sequences were aligned in ARB (Strunk and Ludwig 1995). Maximum likelihood trees were constructed with approximately 1,400 nucleotides in conserved regions using fastDNAml (Olsen et al. 1994). Sequences amplified from the DGGE gel were added to the maximum likelihood trees using the interactive parsimony option in ARB using a filter so only the sequence data encompassed by the DGGE bands would be used in the parsimony analysis. Distance matrices only considering the stretch of sequence data obtained from the DGGE gel were created with PAUP (Swofford 1998). Several different models were tested and since each gave approximately the same results (data not shown), the results from the Jukes and Cantor model (Jukes and Cantor 1969) are presented.

Sequences obtained from GenBank and included in the phylogenetic analysis were, for the *Archaea*: *Methanofollis liminatans* (AF095271), *Methanocorpusculum parvum* (M59147), *Methanoculleus thermophilicus* (M591290), *Methanomicrobium mobile* (M59142), *Methanosaeta thermoacetophila* (M59141), *Methanolobus vulcani* (U20155), WCHD3-16 (AF050618), SB95-72 (U78206), *Ferriplasma acidophilum* (AJ224936), *Thermoplasma acidophilum* (M38637), *Haloferax volcanii* (K00421), *Halobacterium halobium* (X03407), *Methanobacterium formicicum* (M36508), *Ferroglobus placidus* (X99565), *Archaeoglobus veneficus* (Y10011), *Methanococcus jannaschii* (M59126), *Methanococcus infernus* (AF025822), *Thermococcus celer* (M21529), *Pyrococcus furiosus* (U20163), *Pyrodictium occultum* (M21087), *Sulfolobus solfataricus* (D26490), *Thermofilum pendens* (X14835). For the *Bacteria*, the following sequences were included in the analysis: *Thiomicrospira denitrificans* (L40808), PVB_73 (U15106), PVB_55 (U15105), VC2.1Bac32 (AF068806), symbiont of *Alvinella pompejana* (L35520), symbiont of *Rimicaris exoculata* (U29081), VC2.1Bac4 (AF068786), VC2.1Bac31 (AF068805), *Bacteroides ureolyticus* (L04321), *Campylobacter jejuni* (Z29326), *Wolinella succinogenes* (M88159), *Helicobacter pullorum* (L36143), VC2.1Bac7 (AF068788), S17sBac19 (AF299125), *Desulfovibrio desulfuricans* (M34133), *Escherichia coli* (U70214).

4.4 Results

4.4.1 Temperature and mineralogy data

The results of the mineralogy analysis (M.K. Tivey, manuscript in preparation) indicate that the inner bottom section of the flange was the hottest and most reducing section of the flange. The minerals present were pyrrhotite, pyrite, chalcopyrite, sphalerite, and wurzite in the reducing zones. The inner top section contained pyrite and marcasite, indicative of a less reducing and colder region. The pool section of the flange had the largest temperature gradient, which extended from 2°C at the bottom to 332°C at the top.

4.4.2 Distribution of *Archaea* and *Bacteria* within the Cantilever flange

The DGGE analysis of the Cantilever flange identified both *Bacteria* and *Archaea* (Figure 4-2). *Bacteria* were identified from all four sections of the flange, while *Archaea* were only identified from the pool, inner top and lip sections of the flange. The hottest section of the flange, the inner bottom, included no *Archaea*. A higher volume of the extracted DNA was required for the bacterial PCR for the inner bottom section than for the other parts of the flange (data not shown), suggesting lower concentrations of DNA in the inner bottom section.

The number of DGGE bands varied between sections of the flange. The pool section of the flange had the highest number of bacterial and archaeal DGGE bands (Figure 4-3). The lip and the inner top section of the flange had approximately the same number of DGGE bands, while the hottest section of the flange (the inner bottom) had the lowest number of bacterial DGGE bands and no *Archaea*. The results from the DGGE analysis do not allow determination of the biomass or abundance of *Bacteria* and *Archaea* within the flange.

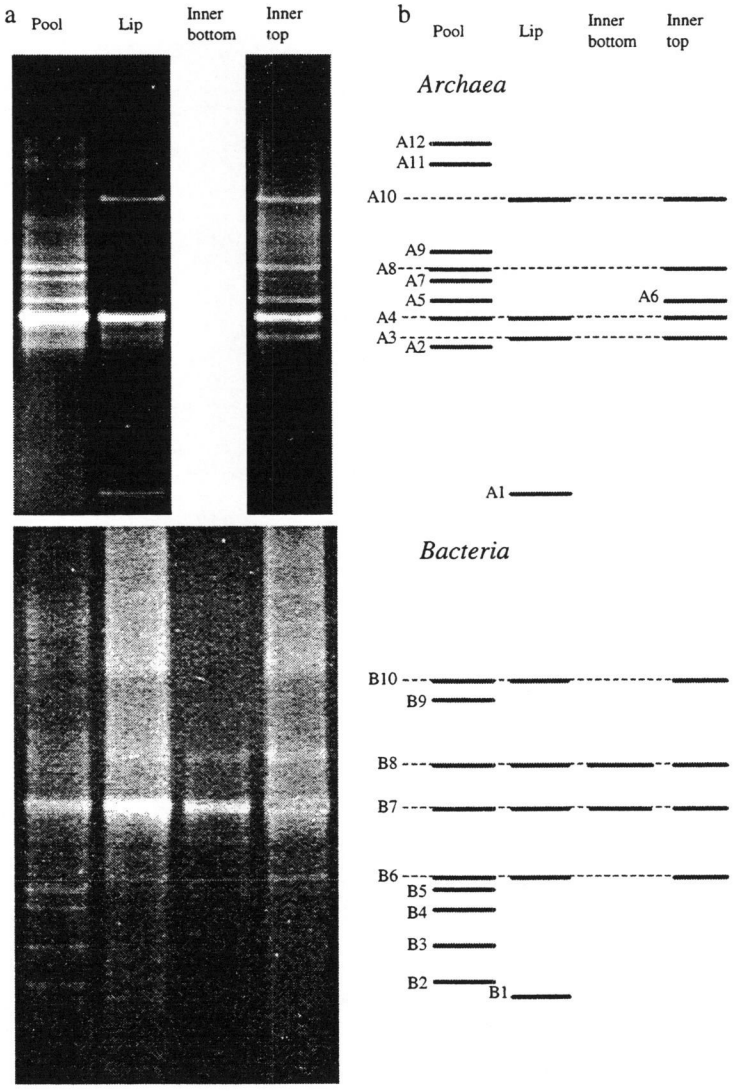

Figure 4-2. a. Image of denaturing gradient gel electrophoresis gel (DGGE) of the sections of the flange from the Cantilever flange. The upper panel is the data from *Archaea* and the lower panel is the data from the *Bacteria*. No *Archaea* were obtained from the inner bottom section of the flange. b. A schematic indicating which bands were identified from the DGGE gel.

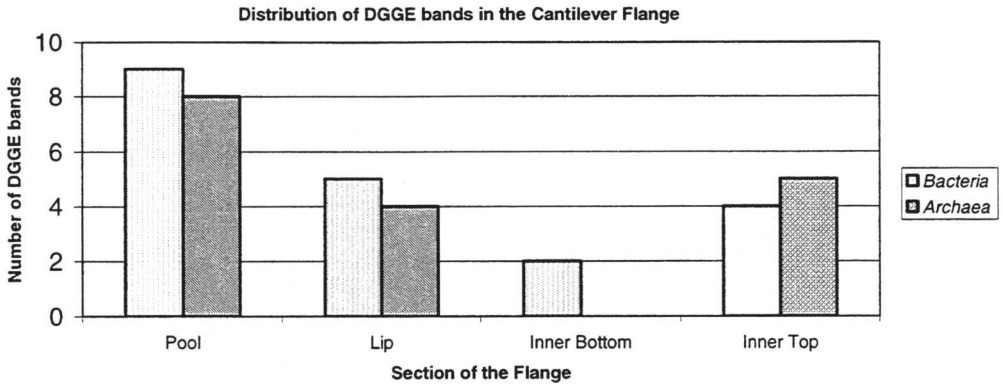

Figure 4-3. Number of DGGE bands within each section of the Cantilever flange. A total of ten different *Bacteria* and twelve different *Archaea* were observed.

4.4.3 Identification of *Archaea* in the flange

All of the *Archaea* identified from the Cantilever flange were *Euryarchaeota*. Table 4-1 is a summary of the archaeal bands identified from the Cantilever flange and the results of the distance matrices constructed using the Jukes and Cantor algorithm. One band (JdF-A4) was present in all three sections of the flange that contained *Archaea* and is most closely related to Clone Rot12 which was identified from anoxic sediments in the Rotsee (Switzerland) (Zepp Falz et al. 1999). Based on the distance matrices, most of the DGGE bands (10 out of 12) are related to Clone Rot12, which is within the *Methanoculleus bourgense* subgroup of the *Methanomicrobiales*. The remaining two bands (JdF-A1 and JdF-A10) are most closely related to ABS23, a sequence

identified from a consortium inhabiting the roots of rice plants that clusters within euryarchaeal Group II (Großkopf et al. 1998).

Table 4-1. Summary of archaeal bands from the DGGE analysis of the Cantilever flange collected from the Juan de Fuca Ridge. The least distant species was determined by distance matrices conducted using the Jukes and Cantor model. Band numbers correspond to the numbers in Figure 4-2.

Band Number	**Least distant species**	**% distance**	**Present in which sections of the flange**
JdF-A1	ABS23	29.3%	Lip
JdF-A2	Clone Rot12	17.3%	Pool
JdF-A3	Clone Rot12	17.3%	Lip, inner top
JdF-A4	Clone Rot12	26.5%	Pool, lip, inner top
JdF-A5	Clone Rot12	23.6%	Pool
JdF-A6	Clone Rot12	17.5%	Inner top
JdF-A7	Clone Rot12	17.7%	Pool
JdF-A8	Clone Rot12	16.4%	Pool, inner top
JdF-A9	Clone Rot12	18.4%	Pool
JdF-A10	ABS23	13.2%	Lip, inner top
JdF-A11	Clone Rot12	16.4%	Pool
JdF-A12	Clone Rot12	16.4%	Pool

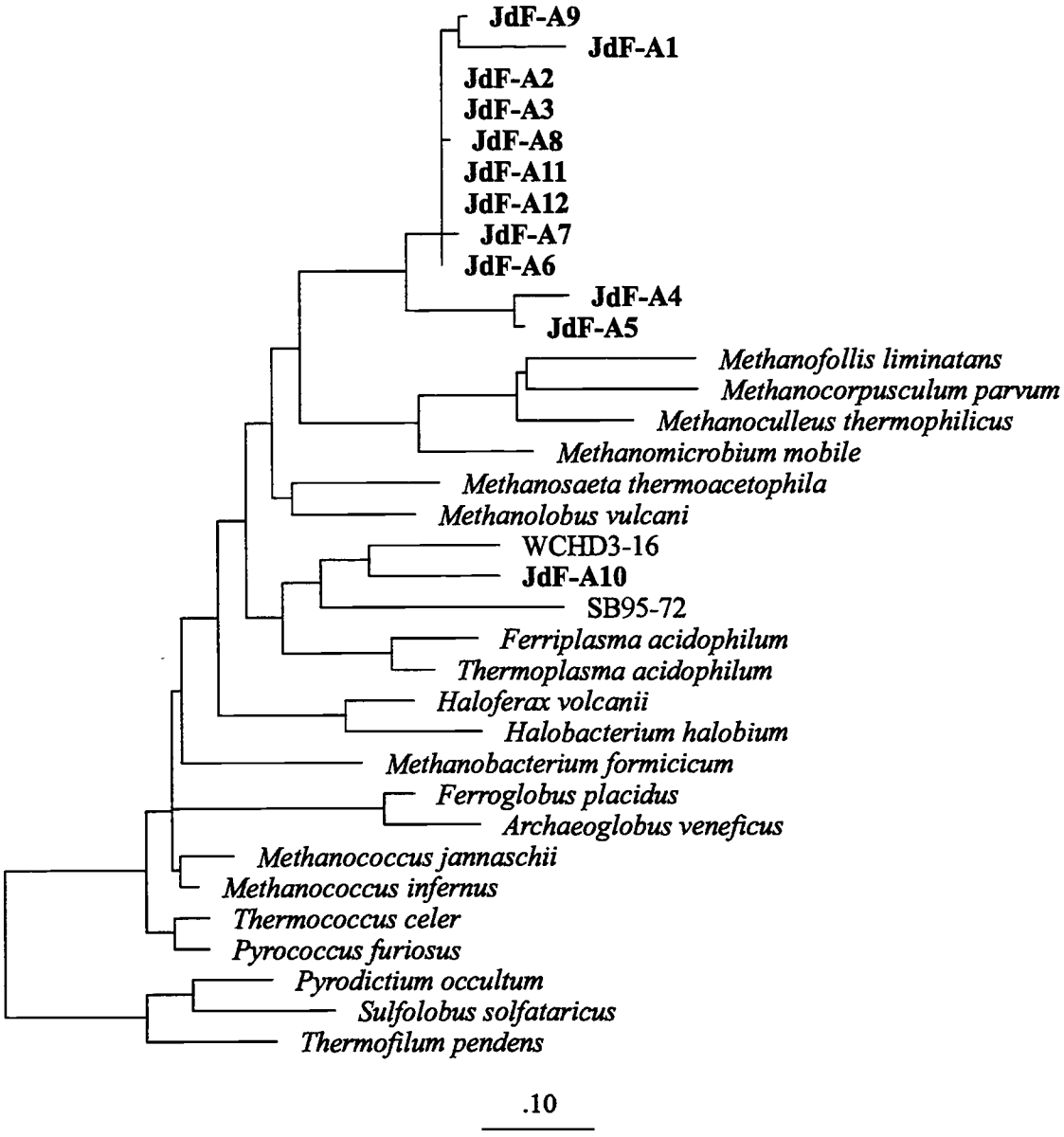

Figure 4-4. Phylogenetic analysis of the *Archaea* sequenced from the DGGE bands from the Cantilever flange. Band numbers correspond to the numbers in Figure 4-2. The tree was constructed using maximum likelihood analysis for the full length sequences. Once the branch points were confirmed by bootstrap analysis, the shorter DGGE bands were added using the parsimony criterion and are indicated in bold in the figure. The scale bar represents 0.10 nucleotide substitutions per sequence position for the full length sequences.

The placement of the DGGE sequences within maximum likelihood trees resulted in trees with the archaeal DGGE sequences present as a coherent group, minus the one DGGE band that is only present in the lip and inner top sections of the flange (JdF-A10) which clusters within the Group II *Archaea*. Depending on the choice of full-length sequences, the large group only clustered with the *Methanomicrobiales* as shown in Figure 4-4, or was in a cluster equidistant from the *Methanomicrobiales* and the *Methanosarcinales*. Given that the distance matrices support the placement of the DGGE bands within the *Methanomicrobiales* and not the *Methanosarcinales*, we feel that the evidence is stronger that the sequences from the Cantilever flange are in fact *Methanomicrobiales*.

4.4.4 *Bacteria* identified from the Cantilever flange

Bacteria were amplified from all sections of the flange. All of the bands sequenced from the DGGE gel are related to ε-*Proteobacteria* (Table 4-2). Two bands (JdF-B7 and JdF-B8) were present in all four sections of the flange. According to the distance matrices, JdF-B7 is most closely related to VC2.1Bac32, an ε-*Proteobacteria* sequence obtained from an in situ growth chamber deployed on the Mid-Atlantic Ridge (Reysenbach et al. 2000). JdF-B8 was also present in all sections of the flange; it is most closely related to the epibiont of the vent shrimp *Rimicaris exoculata*.

Table 4-2. Summary of *Bacteria* identified from Cantilever flange. The least distant species was determined by distance matrices conducted using the Jukes and Cantor model. Band numbers correspond to numbering in Figure 4-2.

Band #	**Least distant species**	**% distance**	**Sections of flange with this band**
JdF-B1	VC2.1Bac31, -4	1.2%	Lip
JdF-B2	VC2.1Bac31, -4	0.7%	Pool
JdF-B3	VC2.1Bac31, -4	12.7%	Pool
JdF-B4	VC2.1Bac31, -4	1.3%	Pool
JdF-B5	VC2.1Bac31, -4	1.3%	Pool
JdF-B6	VC2.1Bac31, -4	2.7%	Pool, lip, inner top
JdF-B7	VC2.1Bac32, PV*B*_55	2.7%	Pool, lip, inner bottom, inner top
JdF-B8	Symbiont of *Rimcaris exoculata*	4.1%	Pool, lip, inner bottom, inner top
JdF-B9	VC2.1Bac31, -4	3.4%	pool
JdF-B10	VC2.1Bac31, -4	1.3%	Pool, lip, inner top

*In the region used for the DGGE analysis the following pairs are 100% similar: VC2.1Bac31 and VC2.1Bac4; VC2.1Bac32 and PV*B*_55.

Several different bands that were primarily present in the pool section of the flange are most closely related to VC2.1Bac4 and VC2.1Bac31, sequences identified from an in situ growth chamber deployed on the Mid-Atlantic Ridge (Reysenbach et al. 2000). A distance matrix for this group indicates that the bands differ by a maximum of 12% over the 180 nucleotides that were sequenced.

The results of the phylogenetic analysis agree with the distance matrices and are shown in Figure 4-5. The bacterial sequences cluster into

three groups: one with *Thiomicrospira* sp., one with the epibionts of *Rimicaris exoculata* and *Alvinella pompejana*, and the final group with VC2.1Bac4 and VC2.1Bac31.

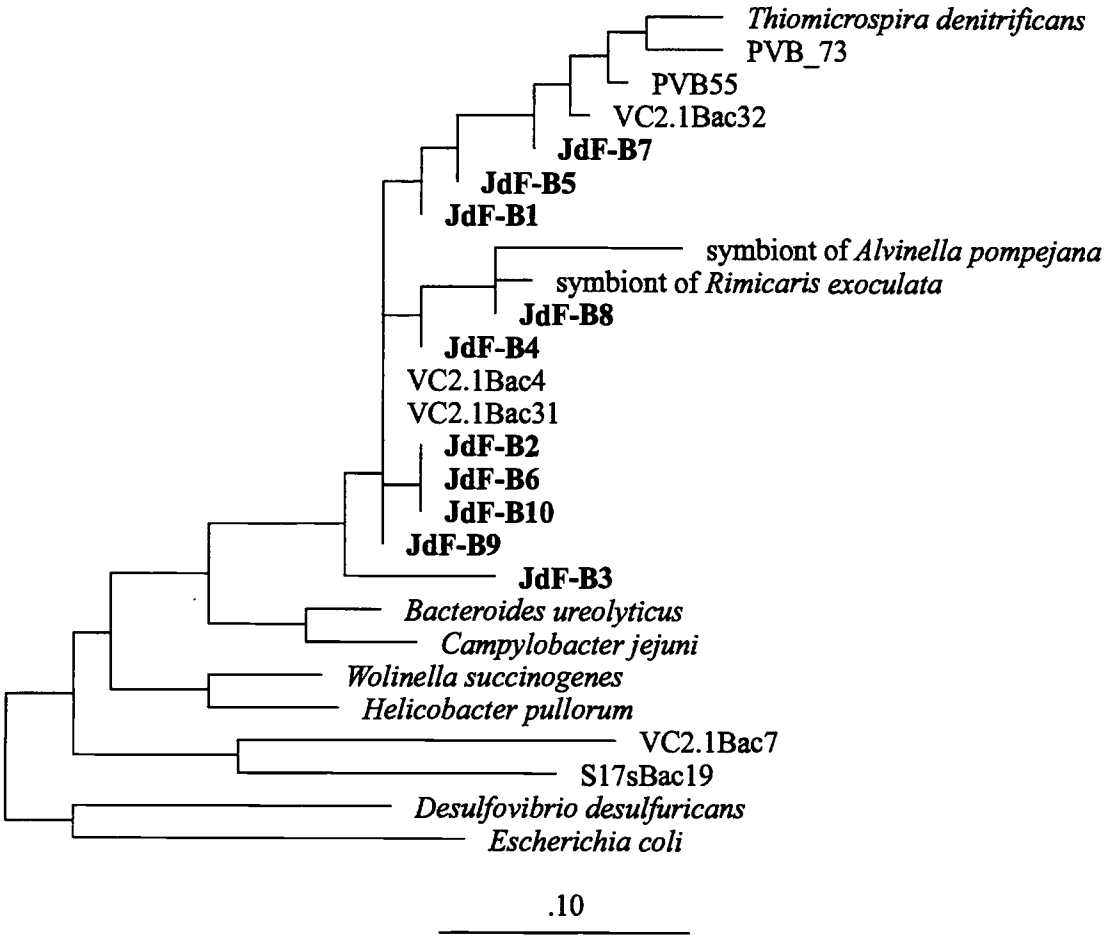

Figure 4-5. Phylogenetic analysis of the bacterial sequences obtained from the DGGE bands from the Cantilever flange. Band numbers correspond to the numbers in Figure 4-2. The tree was constructed using maximum likelihood of full-length 16S rDNA sequences. Once the branch points were confirmed by bootstrap analysis, the shorter DGGE bands were added using parsimony as the criterion. The scale bar, which indicates 0.1 nucleotide changes per position, is only applicable for the full length sequences.

4.5 Discussion

4.5.1 Spatial distribution of microorganisms within a flange

The highest number of DGGE bands from the Cantilever flange were found in the pool section of the flange which, according to the mineralogy, has the largest temperature range. The *Bacteria* and *Archaea* identified from the pool section may in fact be distributed at different points within the pool section depending on the optimal temperature range required for growth. Thermal gradients are also observed at terrestrial hot springs and changes in the microbial population have been correlated to the cooling of the high temperature fluid as it moves further from the spring's source (Ferris et al. 1996; Ferris and Ward 1997). Conversely, the low number of DGGE bands in the inner bottom section of the flange highlights the low diversity of microorganisms capable of surviving in the hottest section of the flange.

The abundance and distribution of *Archaea* and *Bacteria* has previously been examined in a flange collected from Endeavour Ridge on the Juan de Fuca Ridge using the distribution and abundance of different lipids (Hedrick et al. 1992). Based on the concentration of ether lipids, *Archaea* were absent from the top, and therefore coolest, section of the flange. The highest concentration of *Archaea* occurred just below the top section of the flange and

decreased towards the bottom, and therefore hottest, section of the flange (Hedrick et al. 1992). The concentration of polar lipid fatty acids indicated that *Bacteria* were most abundant in the upper layers of the flange (Hedrick et al. 1992). Therefore *Bacteria* and *Archaea* both decreased in abundance in the hotter sections of the flange.

Since the DGGE data are not quantitative, direct comparisons between the lipid analysis and the DGGE data from the Cantilever flange are not possible. However, there may be a correlation between the decrease in biomass observed by Hedrick et al. and the low number of DGGE bands in the section of the Cantilever flange located immediately above the hydrothermal fluid. In other words, the decline in the number of different microorganisms identified by the DGGE analysis may be correlated to a decrease in biomass in the hotter sections of a flange.

4.5.2 Archaeal diversity in the Cantilever flange

Methanogens have previously been recognized as important components of the microbial community present at deep-sea hydrothermal vents. Thermophilic methanogens such as *Methanococcales* and *Methanopyrus kandleri* have been cultured from deep-sea hydrothermal vents (Jones et al. 1983; Huber et al. 1989; Kurr et al. 1991). Furthermore, *Methanococcales* have been identified by 16S rDNA-based phylogenetic analysis on a sample from an in situ growth chamber deployed at Snake Pit on the Mid-Atlantic Ridge (E.

Corre, A.-L. Reysenbach, and D. Prieur, unpublished results) and from a black smoker chimney from the Myojin Knoll (Takai and Horikoshi 1999).

The identification of sequences clustering within the *Methanomicrobiales* represents the first identification of this order from deep-sea hydrothermal vents thereby expanding the breadth of methanogens known to inhabit hydrothermal vents. *Methanomicrobiales* that have been cultured all reduce CO_2 using H_2 or formate as an electron donor (Whitman et al. 1992). They have been previously identified from a diverse array of habitats including: marine and lake sediments, swamps, bioreactors, and sewage sludge (Whitman et al. 1992). One isolate (*Methanofollis tationis*) is from a moderately thermophilic solfataric pool in Chile (Zellner et al. 1999).

Given the metabolic similarities between the other *Methanobacteriales* identified, the *Archaea* from the Cantilever flange are likely autotrophs that produce methane using the H_2 in the vent fluid. Vent fluid collected from the Endeavour section of the Juan de Fuca Ridge has been shown to have high concentrations of methane with a very low $^{13}C/^{12}C$ ratio (Lilley et al. 1993). While the source of the methane is most likely thermal decomposition of organic matter, methane-producing microorganisms could also be the source of the methane (Lilley et al. 1993). The former hypothesis can not be addressed by this study, however the identification of sequences related to known methanogens would support the hypothesis that some of the methane in the vent fluid may be due to microbial methane production.

One sequence (JdF-A10) clusters within the loosely described euryarchaeal Group II *Archaea*. As yet, there are no cultured organisms within this group, however sequences have been obtained from a variety of aquatic ecosystems including coastal waters off the eastern and western United States (DeLong 1992; Fuhrman et al. 1992; Fuhrman et al. 1993) and Antarctica (DeLong et al. 1994), aquifers (Dojka et al. 1998), marine sediments (Vetriani et al. 1998; Vetriani et al. 1999) and hydrothermal vents (Takai and Horikoshi 1999; Reysenbach et al. 2000). While marine planktonic *Archaea* (grouped as both euryarchaeal Group II *Archaea* and crenarchaeal Group I *Archaea*) have been shown to take up amino acids (Ouverney and Fuhrman 2000), no other metabolic data about these groups are available.

4.5.3 Diversity of *Bacteria* identified from the Cantilever flange

Although there are at least 40 divisions of *Bacteria* that have been identified from clone libraries of microbial communities (Hugenholtz et al. 1998), all of the *Bacteria* identified from the Cantilever flange clustered within the *Proteobacteria*. *Proteobacteria* (α, β, and γ) have been identified from a variety of marine and coastal ecosystems (DeLong et al. 1993; Suzuki et al. 1997; Cottrell and Kirchman 2000). However, ε-*Proteobacteria* have thus far been restricted in marine ecosystems to deep-sea hydrothermal vents (Moyer et al. 1995; Polz and Cavanaugh 1995; Cary et al. 1997; Reysenbach et al. 2000) and shallow coastal marine water (Taylor et al. 1999).

The ε-*Proteobacteria* identified from Cantilever are all closely related to ε-*Proteobacteria* that have been identified from other deep-sea hydrothermal vent ecosystems. Most of the DGGE bands were most closely related to VC2.1Bac4, a sequence identified from an in situ growth chamber deployed on the Mid-Atlantic Ridge (MAR) (Reysenbach et al. 2000). While the majority of the bacterial clones (40% according to restriction fragment length polymorphism analysis) identified from the in situ growth chamber were ε-*Proteobacteria*, only 9% of the ε-*Proteobacteria* were related to VC2.1Bac4. ε-*Proteobacteria* were also the majority of bacterial clones identified from microbial mats collected at hydrothermal vents at Loihi Seamount (Moyer et al. 1995) The dominant ε-*Proteobacteria* from Loihi clustered within the Thiovulum group, and DGGE bands JdF-B7 and JdF-B9 also clustered within this group. The expansion of the distribution of ε-*Proteobacteria* to the Juan de Fuca Ridge indicates that ε-*Proteobacteria* may be widely distributed among deep-sea hydrothermal vents and points to the necessity for further research on the role of these organisms within hydrothermal ecosystems.

The final type of ε-*Proteobacteria* identified from the Cantilever flange clustered with ectosymbiont of the vent shrimp, *Rimicaris exoculata*. The *R. exoculata* symbiont was the only phylotype present on the shrimps' carapace and extremities and this same phylotype was 51% of the community associated with sulfide samples collected from Snake Pit on the MAR (Polz and Cavanaugh 1995). There are no *R. exoculata* known to be present at the

Juan de Fuca hydrothermal vents (T. Shank, personal communication). Therefore, the cluster of sequences related to the vent shrimp epibiont may indicate that these sequences are broadly distributed beyond the MAR in the free-living microbial community present at deep-sea hydrothermal vents.

The results from the Juan de Fuca Ridge flange sample are also notable for the species that were not observed within the flange. DGGE is capable of detecting organisms that are greater than 1% of the total cells present in the community (Muyzer et al. 1993; Casamayor et al. 2000). Based on the number of species that are described in the literature, it would seem that *Thermococcales* and *Methanococcales* would be prevalent throughout deep-sea hydrothermal ecosystems. However neither group was identified from within the flange. *Thermococcales* have been cultured from an event plume sampled following an eruption on the North Gorda Ridge (Summit and Baross 1998). Based on optimum growth temperatures that exceeded the temperatures of the event plumes, the hypothesis is that these *Thermococcales* originated in the subsurface and were carried in the hydrothermal vent fluid to the seafloor during the eruption (Summit and Baross 1998). However, *Thermococcales* cultured from sulfide chimneys from the Endeavour section of the Juan de Fuca Ridge are phylogenetically distinct from *Thermococcales* isolated from diffuse hydrothermal fluid collected in the same region (Summit and Baross 2001). If *Thermococcales* are primarily located in the subsurface or are only

present in low numbers in the sulfide structures, they may not be identified by DGGE analysis.

There were also species of *Bacteria* notably absent from the flange sample. Sulfide chimneys from the MAR are populated by the autotrophic sulfur-reducing *Desulfurobacterium thermolithotrophum* (L'Haridon et al. 1998) that are up to 40% of the community within the sulfide chimneys (Harmsen et al. 1997). These organisms could be absent from the DGGE analysis of the flange sample because 1) they were present in such low numbers, 2) because they are only found in the chimney section of the sulfide edifices or 3) they are not present at Pacific hydrothermal vents.

Understanding the microbial diversity present within a hydrothermal flange is an important step towards understanding the potential role microorganisms may play in altering the mineralogy of the flange. This study represents a first step towards combining the microbiology of a flange with the mineralogy. Future research will examine the impact of microorganisms on the mineralogy within a flange.

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5 Summary

5.1 Summary of findings at the three different sites

Several patterns emerge when the data presented in the three previous sections are viewed without the distinctions made based on location and other research goals. The presence of *Methanomicrobiales* at all three sites studied and *Methanobacteriales* at 9°N expands the diversity of methanogens known to be present at deep-sea hydrothermal vents. Conversely, the lack of *Methanococcus jannaschii* in the DGGE analysis at any of the sites is interesting given the prevalence of this species in laboratory cultures from deep-sea hydrothermal vents and the presence of sequences related to *Mc. jannaschii* in the clone library.

The group of organisms that are potentially involved in anaerobic methane oxidation (the ANME-1 group) expands the types of microorganisms that are involved in methane cycling at deep-sea hydrothermal vents. The methanogens and methane-oxidizers are postulated to be separated within sulfide chimneys because of the temperature dependence of the different metabolic reactions (McCollom and Shock 1997) and this is supported by the presence of the ANME-1 group only in the outer section of the Io Vent chimney. The ANME-1 group was also seen in the sulfide chimney from Robin's Roost at Guaymas Basin, but was not observed in any of the flange

samples analyzed. Maximum likelihood trees indicate that the ANME-1 group and DHVE7 group are contained within the same cluster and this raises the question as to whether or not the DHVE7 group is also getting its carbon and energy from methane. Aerobic methane-oxidizers are known at deep-sea hydrothermal vents and their habitats include hydrothermal plumes (de Angelis et al. 1993), the exterior surfaces of some vent invertebrates (de Angelis et al. 1991) and as symbionts within bathymodiolid mussels (Cavanaugh et al. 1987; Cavanaugh et al. 1992).

The prevalence of the euryarchaeal Group II *Archaea* was interesting given that previous studies of this group have indicated they are relatively more abundant in the surface waters of marine ecosystems while the crenarchaeal Group I *Archaea* increase proportionately at depths below the euphotic zone (Massana et al. 1997; Massana et al. 1998; Massana et al. 2000). The results from 9°N, Guaymas Basin and the Juan de Fuca Ridge indicate that the Group II *Archaea* are a component of the sequences identified in vent ecosystems as was also seen at Loihi Seamount, the MAR, and the western Pacific (Moyer et al. 1998; Takai and Sako 1999; Reysenbach et al. 2000).

Several lines of evidence from this study support the hypothesis that there are species endemic to deep-sea hydrothermal vents. To our knowledge, the sequence groups identified from samples collected at hydrothermal vents in the western Pacific (Takai and Sako 1999) have only been expanded by the addition of sequences from the MAR (Reysenbach et al. 2000). The DGGE

bands from Guaymas Basin and 9°N clustered with three of the groups defined by Takai and Horikoshi and these groups may be examples of *Archaea* only found at deep-sea hydrothermal vents. All of the *Bacteria* identified from the Cantilever flange were ε-*Proteobacteria* and they were most closely related to ε-*Proteobacteria* previously identified from deep-sea hydrothermal vents. The question of endemism is a difficult one to address since studies that rely on phylogenetic analysis based on 16S rDNA often find novel division level groups. However repeatedly finding these groups of sequences from different hydrothermal vent sites and no other locations supports the notion that these groups may indeed be unique to hydrothermal vents.

Data from each of the three sites support the theory that heterogeneity in ecological conditions may lead to clusters of co-existing closely related organisms (Begon et al. 1996). Different clusters of microorganisms occurred at each of the vent sites studied. Microorganisms at hydrothermal vents may have adapted to fluctuations in the flow of hydrothermal fluid if different subgroups are only active for a subset of the observed environmental conditions. Clusters of closely related microorganisms have also been observed within thermally-heated microbial mats and theoretically allow the community to live in a fluctuating environment (Ferris et al. 1996; Ferris and Ward 1997). This niche specialization has also been observed in *Prochlorococcus* sp. that maintain an adaptation to different light regimes even after being

cultured in identical laboratory conditions for several years (Moore et al. 1998).

5.2 Using DGGE to analyze unknown communities

DGGE is an excellent method to gain an understanding of the microbial diversity of a large number of samples without the need to establish large clone libraries for each sample. However, in the samples from 9°N and Guaymas Basin there were specific bands which actually contained multiple *Archaea* within a single band. This was evident in the electropherograms since places in the DNA sequence were a mix of different bases.

The major obstacle with the DGGE analysis was the different microorganisms identified by the two reverse primers. The 519R primer amplified sequences primarily related to *Mc. aeolicus*. While the 519RP primer was also able to amplify *Mc. aeolicus*, it identified an array of Group II *Archaea* that were usually not amplified with the 519R primer. This difference was most apparent in the data from Guaymas Basin, but also occurred in the data from 9°N. The data presented here highlight the impact the choice of primers can have on the apparent microbial diversity identified with DGGE. As new species are identified within the *Archaea* perhaps the best action plan is to reexamine the primers to ascertain that microorganisms likely to be identified have no more than one mismatch with the primer.

However, checking for mismatches between the primer and the sequences will not eliminate the problem of PCR bias. Even if the 519R and 519RP primers were able to amplify the same organisms, the brightness of the bands did vary. Other researchers have tried to quantify the abundance of a group based on the intensity of the DGGE band, however the outcome from the two different reverse primers points to the difficulties in obtaining reliable data correlating the brightness of a DGGE band with its abundance.

5.3 Future work

Different microorganisms seem to inhabit chimneys with different mineralogy. However further research is needed to confirm this conclusion since there were no chimney/flange pairs collected, nor was the mineralogy exhaustively studied at all the hydrothermal vent sites. In addition, future research should address the link between microorganisms and their ability to alter the mineralogy of a structure. Microorganisms are known to be actively or passively involved in geochemical transformations and this activity within sulfide flanges has not been addressed.

Since DGGE analysis is not quantitative, the relative abundance of the different groups of microorganisms identified is unknown. The use of fluorescent in situ hybridization (FISH) has been successful in other sulfide chimney samples (Harmsen et al. 1997; Harmsen et al. 1997), although there can be some difficulties with autofluorescence which requires special

treatment of the samples. Given that the *Methanomicrobiales* and *Methanobacteriales* have never been identified from deep-sea hydrothermal vents, the use of group-specific probes to target those sequences would give more information about their abundance and location within the sulfide structures. Furthermore, the location and abundance of the Group II *Archaea* would be interesting since they are apparently recurrent members of the microbial community at hydrothermal vents.

In addition to determining the diversity of microorganisms present in these samples, an increased understanding of their metabolic capacity would aid in determining the role of each group in the geochemical cycles at deep-sea hydrothermal vents. While obtaining laboratory cultures of each different group would be ideal, it is unrealistic due to the time and effort involved in obtaining pure cultures. Furthermore actually obtaining, in the laboratory, a representative diversity of the microorganisms present in the field is not a given since the isolates obtained can be a small fraction of the diversity observed in the field (Ward et al. 1998). In the absence of laboratory cultures, obtaining some information about the metabolic capacity of specific groups is possible by combining FISH with microautoradiography (Lee et al. 1999; Ouverney and Fuhrman 1999; Cottrell and Kirchman 2000). Since a variety of substrates can be radioactively-labeled, some basic questions can be answered that were raised in the course of this research. Are the Group II *Archaea* autotrophs or heterotrophs? Do the metabolic substrates for the methanogens

identified from Guaymas Basin extend beyond H_2, CO_2 and formate to include methylated compounds that are found within the vent fluid from Guaymas? Do the group of sequences that cluster with the putative methane-oxidizers actually use methane as a carbon source? The answers from these questions could be used to help direct attempts to obtain laboratory cultures of the new groups of microorganisms identified at Guaymas Basin, Juan de Fuca, and 9°N, EPR.

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APPENDIX

Appendix

A1. Development and testing of extraction methods

The extraction method used in this thesis was chosen after a series of other extraction methods were tried, and a subset of those methods tested, in order to identify the best method for extracting DNA from sulfide samples. The method was chosen based on two criteria. The method had to be able to extract DNA from which the 16S rRNA gene could be amplified using PCR. Second, the method could obtain the greatest microbial diversity using the number of DGGE bands present as an indicator of diversity.

Methods that were tried and eliminated due to their inability to extract any DNA were: the proprietary IsoQuik method (ORCA Research, Inc., Bothell, WA), a modified sucrose lysis method (Giovannoni et al. 1990) with additional sample lysis using a microwave (C. D. Takacs, personal communication), and a method that involved the use of silica beads from Qiagen (Valencia, CA). Pre-treatment of the sulfide sample with oxalic acid (Lovley and Phillips 1988) followed by a modification of the method described in the thesis was also eliminated.

Once several successful extraction methods were identified, their ability to identify the microbial community present was compared. A single sulfide sample from 9°N was thawed and divided into a series of tubes, all of which

were refrozen at –80°C. The tubes were then individually thawed and the DNA extracted using the following three extraction methods.

The first method was a variation of the method described in the thesis. This method, called Chelex/CTAB, involved an initial incubation step for two hours at 42°C. Final precipitation of the DNA was either at –80°C for 20 minutes, or overnight at –20°C. Aside from these changes, the method is as described in the body of the thesis.

The second method was a published method given in full in Appendix A.2 (Vetriani et al. 1998), that involves an initial lysis step with multiple enzymes (lysozyme, pronase, mutanolysin, RNAse). An additional lysis step involving Tris, EDTA, SDS and Proteinase K was also incorporated into the method. This method was tried as published with six extractions involving organic compounds (twice with phenol, twice with phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform:isoamyl alcohol (24:1)) and also tried with only one phenol and one phenol:chloroform:isoamyl alcohol (25:24:1) extraction.

The final method tested was initially developed for ferns (Dempster et al. 1999) and was tried at the suggestion of our collaborators at the University of Delaware. The full protocol for this method is in Appendix A.3. Cell lysis occurred in a buffer with high salt concentrations, 2-mercaptoethanol, CTAB and polyvinylpyrrolidone (PVP). This method was tested both as published with the RNAse incubation and without the RNAse incubation.

Following extraction of the DNA with each of the three methods, the V3 variable region of the 16S rRNA gene was amplified with the primers designed for DGGE as described in the thesis. The results of the DGGE gel are shown in Figure A-1. Each of the extraction methods only resulted in a single DGGE band. However since the diversity in the sample was unknown, further analysis was required.

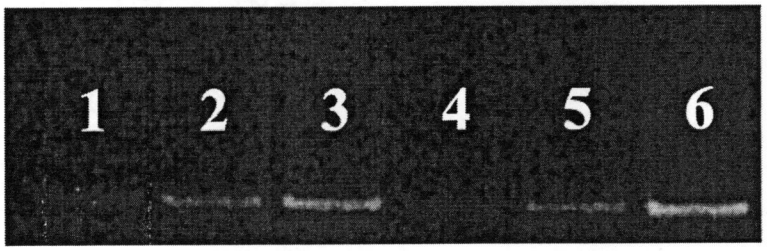

Figure A-1. DGGE gel run to examine the efficiency of the different extraction methods' ability to amplify the archaeal community in the sulfide sample from 9°N, EPR. Samples were extracted with the following methods: Lane 1 (Chelex/CTAB method with a 20 min precipitation at –80°C), Lane 2 (Multi-enzyme method with six extractions with organic solvents), Lane 3 (Chelex/CTAB method with an overnight precipitation at –20°C), Lane 4 (Multi-enzyme method with two extractions with organic solvents), Lane 5 (Fern extraction method with one precipitation step), Lane 6 (Fern extraction method with two precipitation steps).

Using a different sulfide sample from 9°N, the three extraction methods described above were used to extract DNA from three mixtures: 1) a control containing cultures of *E. coli* and AK-1, a psychrophilic methanogen, 2) only the sulfide sample, and 3) a combination of the sulfide sample and the control cells. Following extraction, the variable V3 region of the 16S rRNA gene was

amplified with the DGGE primers. Since the Chelex/CTAB method was unable to extract DNA from the sulfide sample alone, it was not included on the DGGE gel shown in Figure A-2.

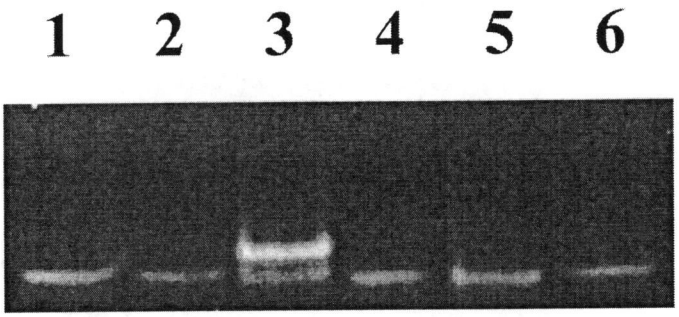

Figure A-2. DGGE gel comparing the fern extraction method with the multi-enzyme extraction method. Samples were loaded onto the gel as follows. Lane 1 (Fern extraction method, controls only), Lane 2 (Fern extraction method, sulfide and controls), Lane 3 (Fern extraction method, sulfide only), Lane 4 (Multi-enzyme extraction method, controls only), Lane 5 (Multi-enzyme extraction method, sulfide and controls), Lane 6 (Multi-enzyme extraction method, sulfide only).

Since the fern extraction method resulted in more DGGE bands for the sulfide sample (two bands compared to the one band for all of the other methods), it was tried as an extraction method for several Guaymas Basin sulfide samples. However, no DNA was obtained from the Guaymas Basin sulfide samples using the fern extraction method. Further discussions in the lab led to the alterations in the Chelex/CTAB extraction protocol. Specifically

the initial incubation temperature was increased to 50°C and the precipitation step changed to an overnight precipitation at 4°C.

The four sections of the sulfide flange from Cantilever were then extracted using the newly-modified Chelex/CTAB method. *Bacteria* were amplified by PCR direct from the DNA extraction from all four sections of the flange. *Archaea* could be amplified from the inner top section of the flange if a nested PCR was used (as described in Chapter 4). Subsequently the UltraClean Soil DNA kit from MolBio Laboratories was also tested on the flange samples from Cantilever. No *Bacteria* could be amplified from the DNA obtained using this kit, however *Archaea* could be amplified from the inner top section of the flange direct from the DNA extraction. The DGGE gel from these first two extractions from Cantilever is shown in Figure A-3. Although the kit allowed amplification of the *Archaea* in the inner top section of the flange without the use of nested PCR, its inability to extract *Bacteria* that could be amplified by PCR led to its elimination as an extraction method.

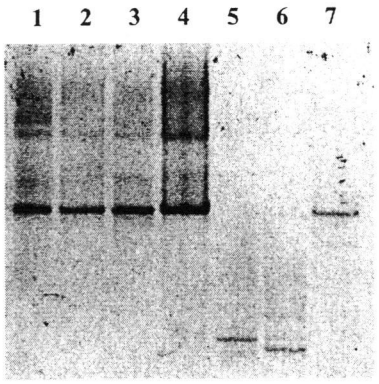

Figure A-3. DGGE gel following the first two extractions from the Cantilever flange. Samples were loaded on the gel in the order listed in Table A-1.

Table A-1. Summary of the first two extractions from the Cantilever flange. Number refers to the lane number in the DGGE gel in Figure A-3.

Number	Domain	Location in the flange	Extraction protocol	Comments
1	*Bacteria*	Pool	Chelex/CTAB	
2	*Bacteria*	Lip	Chelex/CTAB	
3	*Bacteria*	Inner Bottom	Chelex/CTAB	
4	*Bacteria*	Inner Top	Chelex/CTAB	
5	*Archaea*	Inner Top	UltraClean Soil DNA kit	PCR using 519R as the reverse primer
6	*Archaea*	Inner Top	Chelex/CTAB	Nested PCR using 519R as the reverse primer
7	*Archaea*	Inner Top	Chelex/CTAB	Nested PCR using 519RP as the reverse primer

Finally the Chelex/CTAB protocol was modified to include an initial 3 h incubation at 50°C and this is the method seen in the DGGE gels printed in Chapter 4 of the thesis. The three hour incubation was deemed the best method since it resulted in a greater microbial diversity based on the number of DGGE bands present in each section of the flange.

A.2 Fern extraction method (Dempster et al. 1999)

Suspend sample in 500 µl of extraction buffer (100 mM Tris-Cl (pH=8.0), 1.4 M NaCl, 20 mM EDTA, 0.4% (vol/vol) 2-mercaptoethanol, 2% (wt/vol) CTAB, 1% PVP (mol. wt. 360 000)) that has been heated to 55°C. Incubate the sample for 15 minutes at 55°C. Add 500 µl of chloroform:isoamyl alcohol (24:1) and shake on vortex for 20 minutes. Spin for 15 minutes and then transfer the aqueous layer to a fresh tube. Precipitate the DNA with an equal volume of 100% isopropanol and 0.5 volume 5M NaCl at –70°C for one hour. Spin for 10 minutes and wash with 100% ethanol. Resuspend the pellet in 100 µl TE, add 1 µl RNAse (1 mg/ml) and incubate at 37°C for 30 minutes. Precipitate the DNA with 2 volumes freezer-cold 100% ethanol and 0.1 volume 3M sodium acetate at –70°C for one hour. Spin for 30 minutes at 4°C, wash with freezer-cold 80% ethanol and dry pellet.

The method was tried as published above and with a single precipitation step that involved precipitating the DNA with 2 volumes

freezer-cold 100% ethanol and 0.1 volume 3M sodium acetate at –70°C for one hour.

A.3 Multi-enzyme method (Vetriani et al. 1998)

Suspend sample in 300 µl TE and add the following enzymes: 200 µl lysozyme (50 mg/ml), 20 µl pronase (10mg/ml), 8 µl mutanolysin (5000 U/ml), 4 µl RNAse (10 mg/ml). Incubate at 37° C for one hour. Add 250 µl of extraction buffer (20 mM Tris-Cl (pH=8.0), 100 mM EDTA (pH=8.0), 100 µL/mL proteinase K, 1% SDS). Incubate for one hour at 37°C and 30 minutes at 55°C. Extract twice with phenol, twice with phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform:isoamyl alcohol (24:1). Precipitate the DNA with 0.1 volume 3M sodium acetate and 2 volumes of freezer-cold 100% ethanol on ice for one hour. Spin 30 minutes at 4°C, wash with 70% ethanol, and dry the pellet.

The method was tried as published with all six extraction steps and with only two extraction steps: once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1).