BACTERIAL COMMUNITY STRUCTURE AND PHYLOGENETIC DIVERSITY OF HYDROTHERMAL VENTS AT AXIAL VOLCANO, JUAN DE FUCA RIDGE

by

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Accepted in Partial Completion
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MASTER'S THESIS

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ABSTRACT

The community structure and phylogenetic diversity of bacterial communities were determined from four hydrothermal vent sites at Axial Volcano, Juan de Fuca Ridge. Genomic DNA was extracted from sampled microbial communities and amplified using SSU rDNA primers specific to the domain *Bacteria*. PCR products were then used to generate clone libraries from each sample. ARDRA was performed on individual SSU rDNA clones to determine the dominant operational taxonomic units (OTUs). Fifteen dominant bacterial OTUs were identified from the 249 clones screened. Overall, organismal diversity in all samples examined was relatively high as determined by rarefaction. Phylogenetic analyses were performed to determine the genetic relatedness of the representative phylotypes. Three of the sample sites were located in the caldera's recent 1998 lava flow (Marker 33 Vent, Snow Blower and Easy Vent), while the other site (North Rift) was on an old lava flow north of the caldera. Marker 33 Vent microbial mats were dominated by members of the ϵ-*Proteobacteria* (17.2%) and δ-*Proteobacteria* (8.6%). The floc-ejecta collected from the Snow Blower Vent contained representatives from the ϵ-*Proteobacteria* (25.1%) and Green Non-Sulfur *Bacteria* (12.5%), possibly originating from the deep subsurface. The Easy Vent bacterial mat community was dominated by members of the ϵ-*Proteobacteria* (14.3%) and γ-*Proteobacteria* (5.4%). The distal microbial mat from North Rift was dominated by members of the Flexibacter-Cytophaga-Bacteroides division (43%), possibly representing additional trophic level interactions. The organismal and phylogenetic diversity of the ϵ-*Proteobacteria* found at the new lava vent sites was exceptional, indicating the ecology of these potentially sulfur-oxidizing microorganisms plays a significant role at hydrothermal vents especially after recent eruptive events.

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INTRODUCTION

Axial Volcano (Figure 1) lies in the center of the Juan de Fuca Ridge which is located about 300 km from the coasts of British Columbia, Washington, and Oregon and rises ~1,000 meters above the normal ridge axis to the north and south (Davis, 2000). The summit of the volcano is a large rectangular caldera 8 km long and 4 km wide running from northwest to southeast (Figure 2). Intense seismic activity recently occurred on January 25, 1998, lasted for twelve days, and included over 8,000 earthquakes detected by the U.S. Navy's Sound Surveillance System, a real-time hydroacoustic monitoring system (SOSUS; Dziak and Fox , 1999). An integrated response study began in August 1998 to examine the physical, chemical, and biological characteristics of Axial Volcano (Feely *et al.*, 1999) as part of the New Millennium Observatory (NeMO; Embley and Baker, 1999) project. The primary goal of the project has been to investigate the effect of dike intrusions and eruptions on the microbiology, geochemistry, and macrobiology of the vents at Axial Volcano (Embley and Baker, 1999).

Axial Volcano is located on a spreading center where divergent tectonic plates of the Earth spread apart allowing the rise of magma toward the surface of the seafloor. This in turn causes convective heat flow by drawing the seawater into the oceanic crust where it is heated, transformed chemically by the interaction with the basalt, and then rises due to increased buoyancy toward the surface creating a unique ecosystem on the seafloor which supports other micro- and macro-fauna (Jannasch, 1995; Jannasch and Mottl, 1985; Karl, 1987; McCollom and Shock, 1997). Since the discovery of hydrothermal vents in the late 1970s (Corliss *et al.*, 1979; Grassle *et al.*, 1979; Lonsdale, 1977), these vent fields have been characterized as either warm vent fields or hot vent fields, with flow rates of 0.5 to 2 cm per

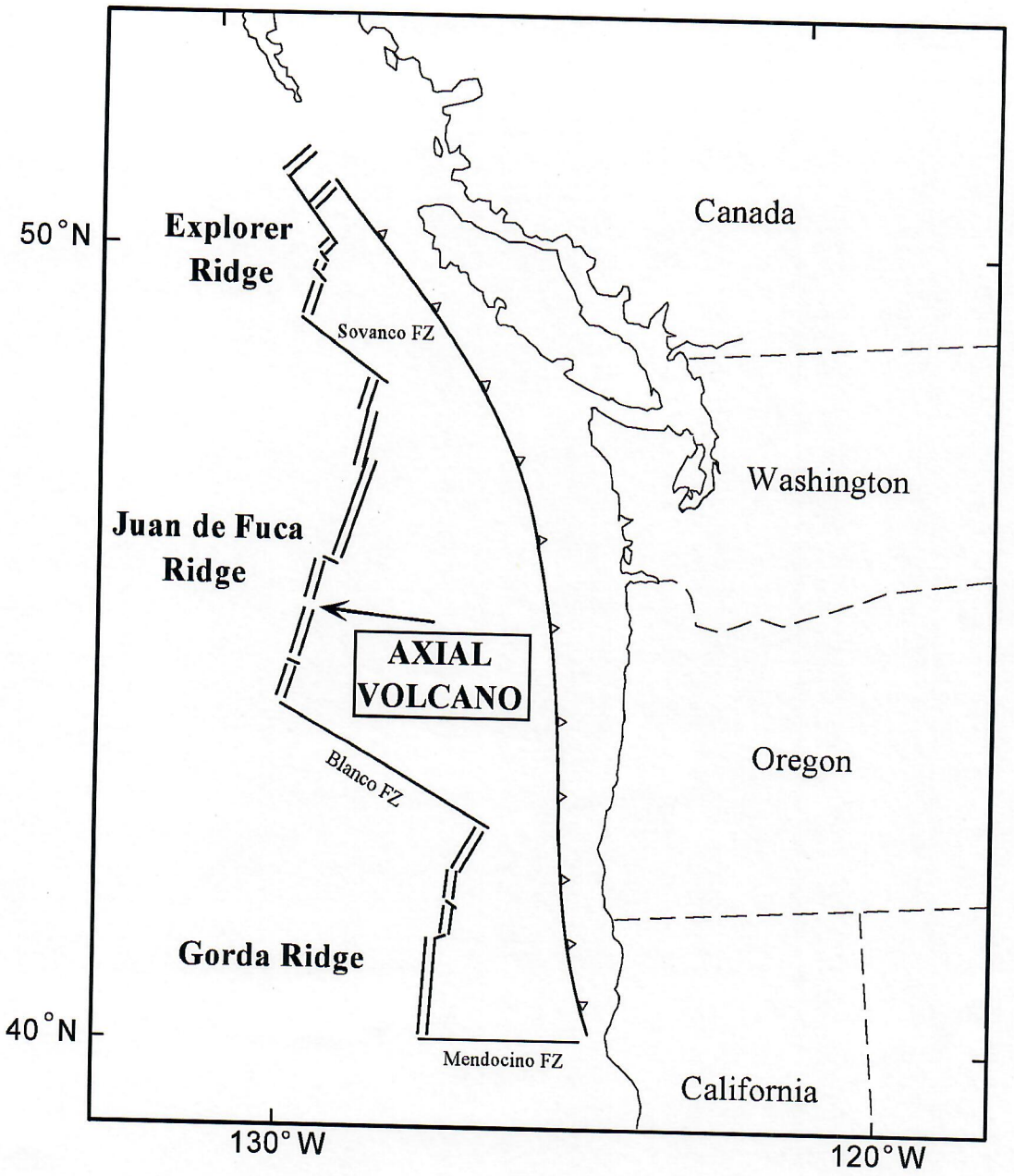

Figure 1. Map of the spreading centers in the Northeast Pacific, including the location of Axial Volcano.

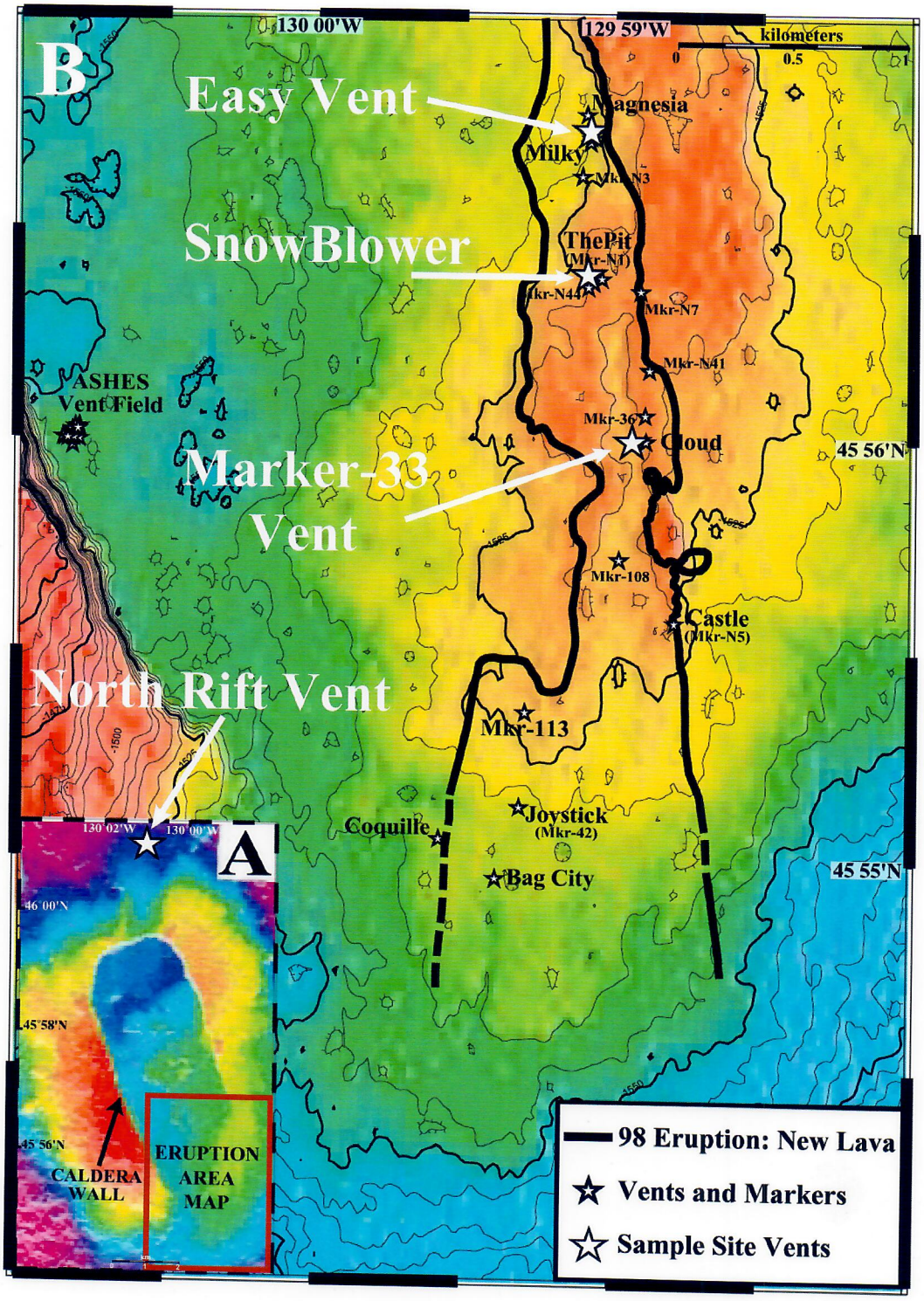

Figure 2. Map of Axial Volcano on the Juan de Fuca Ridge showing (A) summit caldera and (B) eruption area of southeast caldera with outline of new lava flow.

second and 1 to 2 m per second, respectively (Jannasch and Mottl, 1985). Temperature and hydrothermal flow at vent sites vary on the order from minutes to days to years on both large (kilometers) and small (meters) scales (Karl, 1995). After an eruptive event, the chemistry of the environment also changes over time. Shortly after the eruption, sulfur compounds (H_2S) are the most prevalent chemical components of hydrothermal vent fluids (Butterfield *et al.*, 1997). Energy capture in vent ecosystems is carried out by chemolithotrophic microorganisms that gain metabolic energy by using certain reduced inorganic compounds, including primarily sulfur and iron (Jannasch, 1995; Jannasch and Mottl, 1985; McCollom and Shock, 1997). These compounds may be present as a result of reactions between inorganic and organic compounds by mixing of the hydrothermal fluid with seawater. Another mechanism for producing these compounds is by mixing heated seawater with basaltic rocks at depth and circulating the hydrothermal fluids through the rocks (Jannasch and Mottl, 1985; McCollom and Shock, 1997).

The chemolithotrophic microbes found in hydrothermal vent environments may inhabit specific niches such as (i) microbial mats covering almost all areas exposed to diffuse flow, (ii) interstices associated with the vent fluids discharged from the deep subsurface, (iii) symbiotic associations with vent fauna (e.g., vestimentiferan tube worms, polychaete worms, and giant clams), or (iv) hydrothermal vent plumes (Karl, 1987; 1995). The metabolism of these microbes may be either aerobic to oxidize various chemical compounds such as H_2, H_2S, S^o, CH_4, Mn^{2+}, Fe^{2+}, and NH_4^+, or anaerobic to reduce S^o, SO_4^{2-}, CO_2, and Fe^{3+}. The use of any of these compounds as a source of metabolic energy depends upon the physical parameters of the environment, the concentration and diversity of potential electron donors

and electron acceptors, and the availability of free energy to make the reaction energetically favorable (Karl, 1987; McCollom and Shock, 1997). Generally, the biomass of vent biota also depends on whether or not the mixing of hot hydrothermal fluid with ambient, oxygenated, cold seawater is favorable for microbial chemosynthesis (Jannasch, 1995). Temperature is perhaps the single most important parameter controlling the chemistry of the hydrothermal fluids, followed by pressure, water/rock ratio, rock crystallinity, rock composition, and reaction time (Von Damm, 1995).

Recent studies have shown that bacterial diversity of some hydrothermal habitats is relatively low as compared with soil systems. Based on estimates of reannealing of soil DNA, it was found that forest soils contain around 4000 independent bacterial genomes in one gram of forest soil (Torsvik *et al.*, 1990) while only 12 phylogenetic types (phylotypes) were found by examining restriction pattern length polymorphism patterns of a microbial mat community from a deep sea hydrothermal vent (Moyer *et al.*, 1994). A hyperthermophilic pink filament community collected from Octopus Spring, Yellowstone National Park was found to contain only three phylotypes upon sequencing of the small subunit ribosomal RNA (SSU rRNA) genes obtained by PCR or polymerase chain reaction (Reysenbach *et al.*, 1994). However, 54 distinct phylotypes were recovered from a bacterial community in Obsidian Pool, a hot spring in Yellowstone National Park, increasing the depth of branching within several divisions of bacterial descent (Hugenholtz *et al.*, 1998b).

In February 1998, particle rich plumes at Axial Volcano were analyzed for microbial biomass (Cowen *et al.*, 1999). Unusual capsule forms as well as multi-cellular filaments, metal-encrusted sheathed clusters, and colonies enmeshed in a polymer-slime matrix were

found in the plumes, but were not present in the background samples. Interestingly, only a few sulfur rich particles and no sulfur encrusted filaments were found in the plume samples. However, massive amounts of white flocculated material resembling "snow" was observed in August 1998. This material was composed of elongated sulfur filaments with bacteria concentrated on the surface of the filaments (Delaney *et al.*, 1998; Feely *et al.*, 1999).

There are limitations to using enrichment cultivation to describe naturally occurring microbial communities (Amann *et al.*, 1995; Head *et al.*, 1998; Ward *et al.*, 1992; Ward *et al.*, 1998). Given these limitations, it is unlikely that collections of bacterial isolates are representative of *in situ* diversity and community structure since <1% of microorganisms observable in nature are typically cultivated using standard techniques (Amann *et al.*, 1995). Recently developed techniques in molecular biology can provide a culture-independent analysis of microorganisms (Amann *et al.*, 1995; Head *et al.*, 1998; Hugenholtz and Pace, 1996; Hugenholtz *et al.*, 1998a; Ward *et al.*, 1998).

Since ribosomal RNA (rRNA) molecules are ubiquitous in all cellular life forms, comparative analysis of their sequences can be used to infer phylogenetic relationships among organisms (Head *et al.*, 1998; Hugenholtz and Pace, 1996). Each type contains both highly conserved regions as well as variable regions unique to a particular population or closely related group. These SSU rRNAs, with their relatively slow rate of evolutionary change, have been widely used in ecological and phylogenetic studies. First, they are essential components of the protein synthesis machinery and are therefore functionally conserved and ubiquitously distributed in all organisms. Second, there has been no detection of horizontal gene transfer in this molecule as with other prokaryotic genes. Third, they can

be obtained directly from environmental DNA and are therefore readily isolated and identified. Finally, phylogenetic comparisons can be inferred over a broad range of evolutionary distances since highly variable regions, which are unique to a particular population or closely related group, are interspersed among highly conserved regions of the primary and secondary structure. These conserved regions allow for the design of oligonucleotide probes and PCR primers specific to various levels of phylogenetic diversity (Moyer *et al.*, 1998). A "universal" phylogenetic tree of life based on SSU rDNA sequences is illustrated in Figure 3. The Ribosomal Database Project II (RDP) contains over 16,000 SSU rRNA sequences in aligned format as well as more than 30,000 unaligned sequences (Maidak *et al.*, 2000). As the number of phylogenetically known SSU rDNA sequences grows, analyses of sequences derived directly from the environment may provide insights into the metabolic and trophic activity of various microbial taxa by suggesting possible metabolic pathways of specific populations (Amann *et al.*, 1995).

The goal of this study was to apply molecular methods to describe the community structure and diversity of bacterial communities inhabiting the microbial mats and floc-ejecta at Axial Volcano. The collection sites included vent habitats within the caldera on the new lava flow (Marker 33 Vent, Snow Blower, and Easy Vent), as well as a microbial community north of the caldera on an old lava flow (North Rift) and are shown in Figure 2. Clone libraries were generated by selectively amplifying bacterial SSU rDNA from purified genomic DNA (gDNA), where each clone was assumed to be representative of a stochastic first order approximation of populations contained in the sampled microbial community. Organismal diversity of Axial Volcano microorganisms in the domain B*acteria* (AXB) was

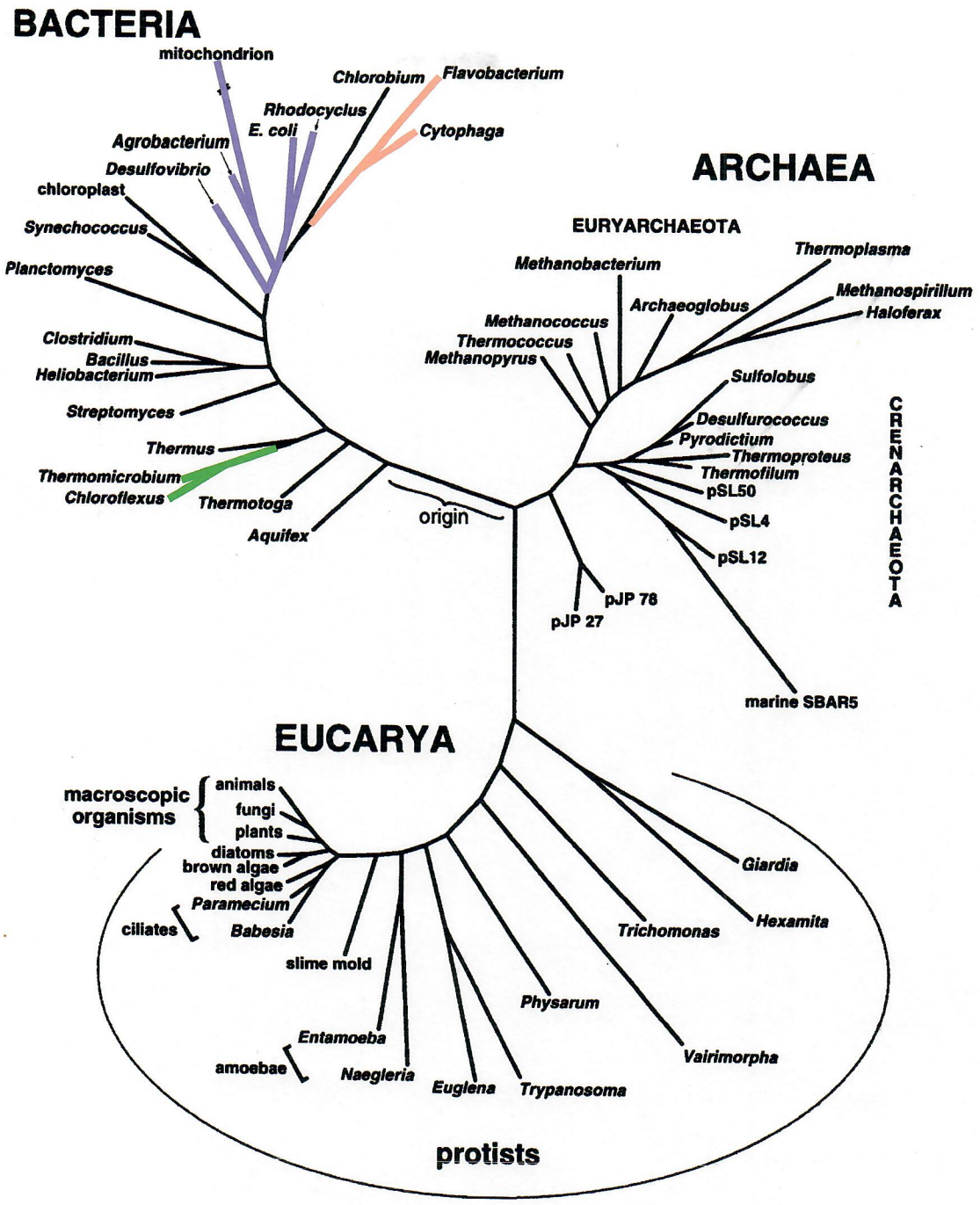

Figure 3. Diagrammatic "universal" phylogenetic tree of life based on SSU rDNA sequences. Redrawn from Barns and Nierzwicki-Bauer (1997). Based on analyses of Barns *et al.* (1996), Olsen *et al.* (1994b), and Sogin (1994). Highlighted lineages indicate dominant divisions detected at Axial Volcano vent sites. (Green = Green Non-Sulfur *Bacteria* and Relatives; Purple = *Proteobacteria*; Orange = Flexibacter-Cytophaga-Bacteroides)

determined by ARDRA or amplified ribosomal DNA restriction analysis (Heyndrickx *et al.*, 1996; Vaneechoutte *et al.*, 1992). Two treatments of two pairs of tetrameric restriction endonucleases were used to generate two restriction digests of SSU rDNA clones which were then analyzed by gel electrophoresis. Differences in restriction patterns indicated differences in the SSU rDNA sequences. Clones with the same restriction patterns were categorized into operational taxonomic units (OTUs; Moyer *et al.*, 1994). The use of four tetrameric restriction enzymes (a double-double digest) has been shown to differentiate among known phylotypes that have >98% sequence similarity (Moyer *et al.*, 1995) and has also been found to distinguish >99% of the bacterial taxa present within a modeled dataset of maximized diversity (Moyer *et al.*, 1996).

ARDRA is a useful tool in determining the phylogenetic and taxonomic information because it detects differences at the genotypic level in a rapid manner (Vaneechoutte *et al.*, 1992). Comparisons of ARDRA with known phylogenetic groupings of several lineages of *Bacteria* confirmed its efficacy as a useful identification technique (Heyndrickx *et al.*, 1996; Vaneechoutte *et al.*, 1992). In addition, only six of the 32 strains of *Acinetobacter* species were correctly identified using *rec*A restriction fragment length polymorphisms (*rec*A-RFLPs) while the ARDRA technique correctly identified all 32 strains (Jawad *et al.*, 1998).

Diversity is both a measure of the number of populations in a community and the genetic relatedness among the populations. Community structure was determined by the number of OTUs in a clone library and the distribution of clones among those OTUs. OTU richness (the number of OTUs detected from each sample) was estimated by rarefaction (Simberloff, 1978). Collector's curves and rank abundance diagrams were used to further

illustrate community structure relationships. SSU rDNAs from dominant OTUs were sequenced to determine their phylogenetic affiliations or phylotypes. These results were compared with the phylogeny of other microorganisms, including those from hydrothermal vents. This study is the first to examine the relationships among environmentally derived SSU rDNA clones in order to describe the community structure and diversity of Axial Volcano hydrothermal vent *Bacteria*.

MATERIALS AND METHODS

Collection of samples and site characteristics

Microbiological samples were collected at Axial Volcano at a depth of approximately 1500 meters using a suction sampler in conjunction with a rotating rosette bucket sampler attached to the remotely operated platform for ocean sciences or ROPOS (Shepherd and Juniper, 1997). Samples were collected on dive numbers 462 (Marker 33 Vent), 464 (Snow Blower), 467 (North Rift), and 476 (Easy Vent) during August and September of 1998. The four hydrothermal vent sites are shown in Figures 4 (Marker 33 and Snow Blower) and 5 (Easy and North Rift). Two-liter collection jars attached to the rotating rosette bucket sampler had 202 µm Nytex mesh covering their exhaust ports. Aboard ship, the samples were collected after the microbial mats had settled inside the two-liter jars and were then aseptically transferred into sterile 50 ml centrifuge tubes. Aliquants (1 ml) for microscopy were fixed with 50 µl glutaraldehyde (final concentration 2.5%) and stored at 4°C until examined. Samples collected in 50 ml centrifuge tubes were quick frozen in liquid nitrogen and kept on dry ice until they were returned to the laboratory and stored at -80°C until processed. Subsamples consisting of 1.0 ml mat or floc sample and 0.5 ml 80% glycerol were also cryopreserved.

Temperatures were measured using a thermistor probe attached to the robotic arm of ROPOS and manipulated from onboard the ship. Marker 33 (T = 37°C) and Easy (T = 8.0°C) Vents are diffuse venting sites with white, and dark orange with white microbial mats, respectively. There were many polynoids near the Marker 33 Vent location while the Easy Vent mats were not inhabited by many macrofauna. The North Rift sample (T = 4.5°C)

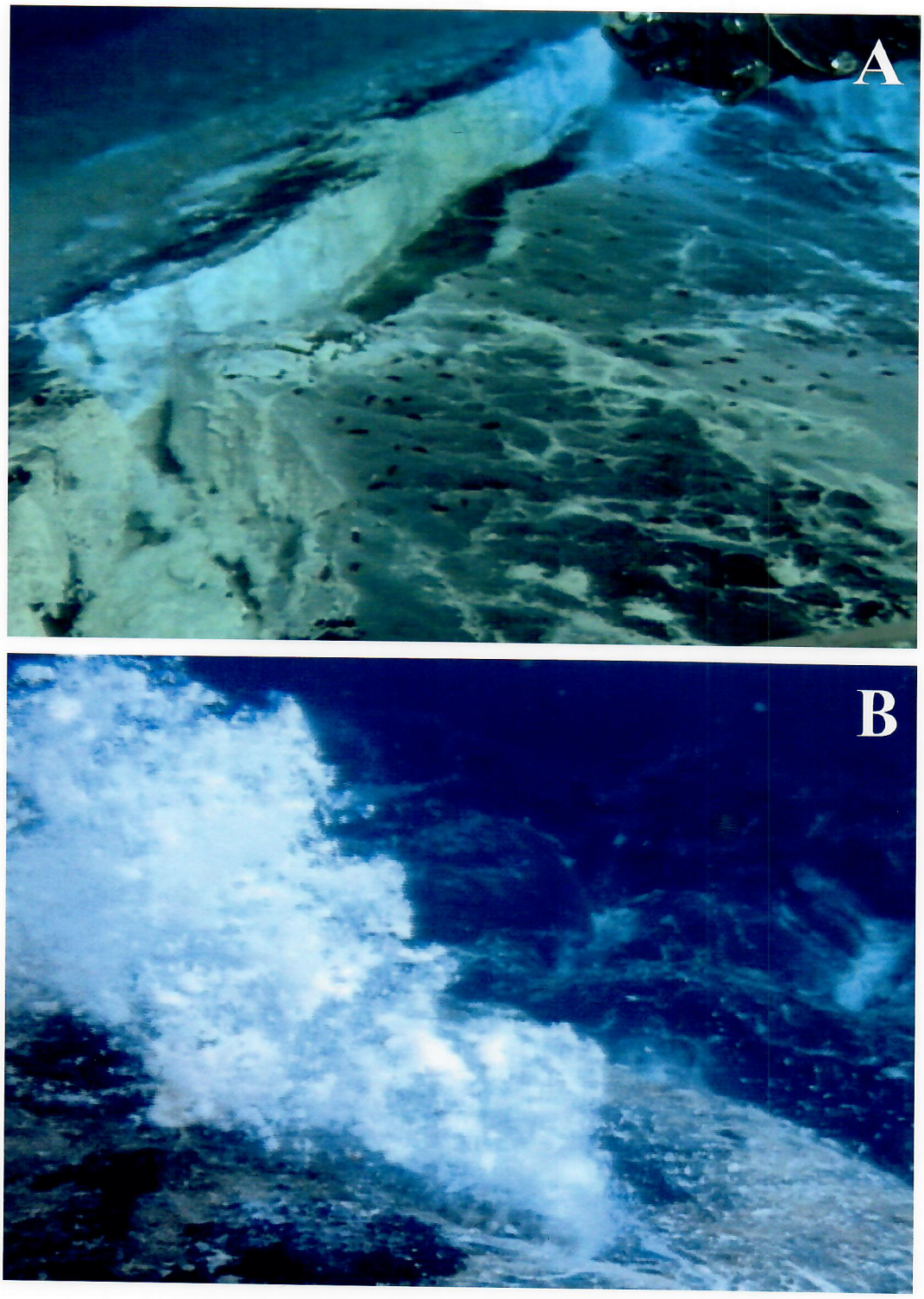

Figure 4. Diffuse venting site at (A) Marker 33 Vent and (B) Snow Blower Vent releasing microbial floc as seen from ROPOS during NeMO98 cruise.

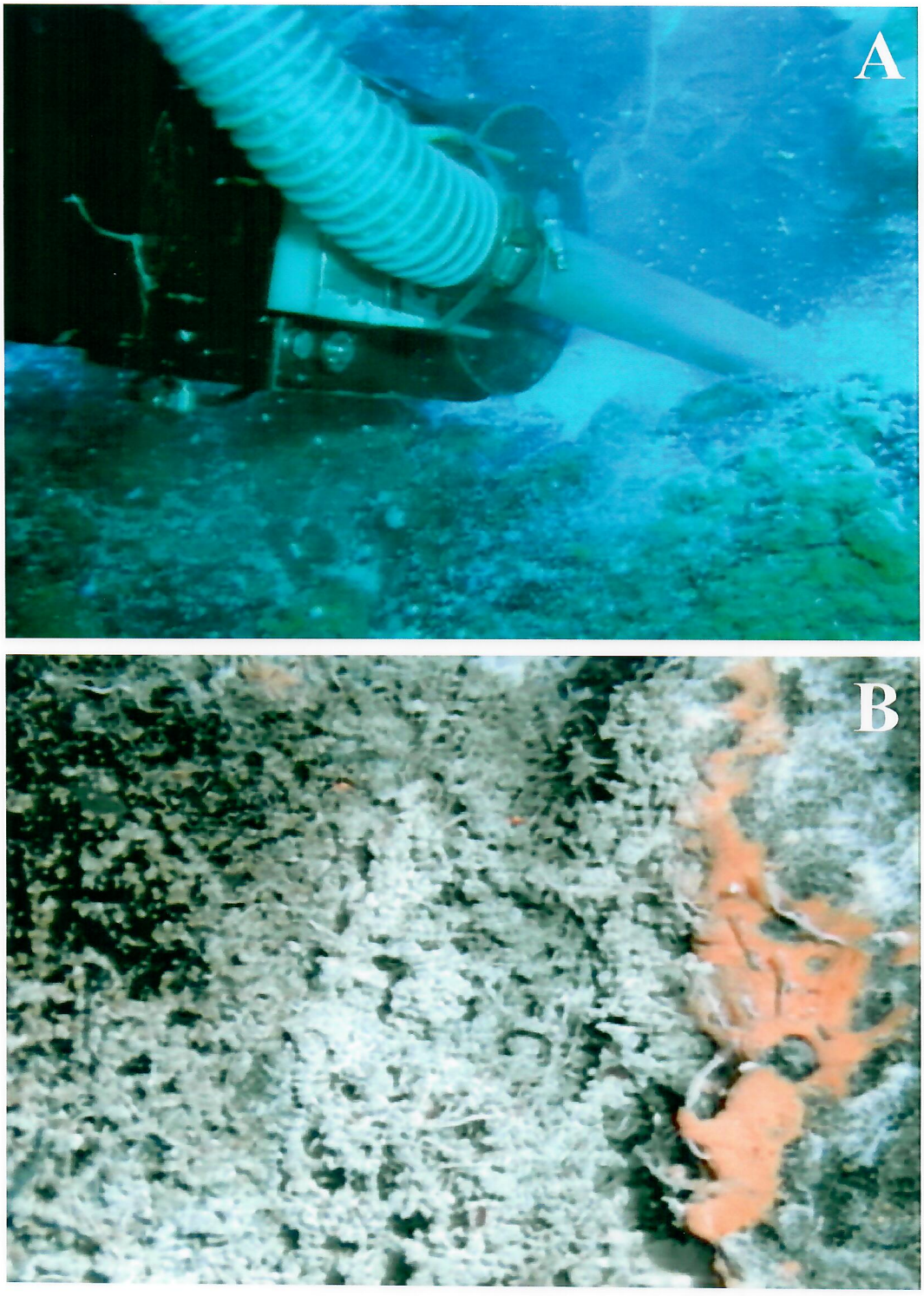

Figure 5. Diffuse venting sites at (A) Easy Vent and (B) North Rift Vent microbial mats as seen from ROPOS during NeMO98 cruise.

was a bright orange microbial mat found among many gastropods, polynoids, and tube worms, with minimal hydrothermal activity. The Snow Blower sample (T = 14.0°C) was taken when the skid plate of ROPOS punctured the seafloor, releasing white microbial debris from a subsurface cavity formed by caldera deflation from lava drainage. The physical and chemical characteristics of each sample site are listed in Table 1. Temperature and chemical data were recorded by a submersible system used to assess vented emissions or SUAVE mounted on ROPOS. This instrument has a high-resolution temperature sensor coupled to the intake valve and uses flow analysis-calorimetry to detect dissolved Mn, Fe (II and III), and H_2S every 5 seconds (Massoth *et al.*, 1991).

Genomic DNA extraction and purification

DNA was extracted and purified by using the UltraClean Soil DNA kit (MoBio Laboratories, Inc., Solana Beach, CA). Approximately 0.5 g (wet weight) of microbial mat material was added to the initial bead solution. The duration of bead beating was 1 minute at 3800 rpm in a Mini-BeadBeater (BioSpec Products, Inc., Bartlesville, OK), then 1 minute on ice, and was repeated twice for a total of 3 minutes of bead beating. The genomic DNA (gDNA) obtained by this protocol was PCR ready, requiring no further steps of purification. An aliquant of each sample was examined for size and purity by electrophoresis in a 1% SeaKem GTG agarose (BMA BioProducts, Rockland, ME) gel containing 0.4% (w/v) ethidium bromide against a λ-*Hind*III DNA standard (Life Technologies, Rockville, MD). Each sample was also quantified by an absorbance of 260 nm and analyzed for purity by an absorbance ratio of 260/280 nm with an HP model 8452A diode array spectrophotometer.

Table 1. Physical and chemical characteristics of Axial Volcano sample sites at the time of sampling.

Site	Temp (°C)	Depth (m)	Visible Microbial Material	H_2S (μM)	Fe (μM)	Mn (μM)
Marker 33 Vent	37.0	1524	White Mat	1000	40	18
Snow Blower	14.0	1521	White Floc	180	15	50
Easy Vent	8.0	1532	Dark Orange and White Mat	175	90	40
North Rift	4.5	1640	Bright Orange Mat	124	2	5

Sample yields were from 2.2 to 17.7 µg of DNA per gram of sample material (wet weight). The residual post extraction sample material was stored at -20°C until examined by acridine orange staining with epifluorescence microscopy to confirm cellular lysis efficiency.

PCR amplification of SSU rDNA

PCR was used to amplify the SSU rDNA with oligonucleotide primers that anneal to 3′ and 5′ conserved regions of bacterial SSU rRNA genes. The forward primer (5′-TNANACATGCAAGTCGRRCG) corresponds to positions 49 to 68 of *Escherichia coli* rRNA, and the reverse primer (5′-RGYTACCTTGTTACGACTT) corresponds to positions 1510 to 1492, where R = purine analog "K", Y = pyrimidine analog "P", and N = an equal mixture of both analogs at a single position (Moyer *et al.*, 1994; 1998). The 5′ ends of both primers contained a phosphalink amidite (PE Applied Biosystems, Foster City, CA) that aids in cloning efficiency.

Amplifications were performed in a reaction mixture containing 50-500 ng template DNA, 5 U *Taq* DNA polymerase (AmpliTaq Gold; PE Applied Biosystems), 5 µl 10X PCR reaction buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP, 10 µg acetylated bovine serum albumin (BSA; Promega, Madison, WI), 1 µM each forward and reverse primers, and sterile water to a total volume of 50 µl. Before template, BSA and *Taq* were added, the reaction mixtures were irradiated by UV light for 5 minutes to eliminate potential contaminating DNA templates (Sarkar and Sommer, 1990).

The reaction tubes were sealed and placed in a GeneAmp 9700 thermocycler (PE Applied Biosystems) under the following conditions: "hot start" at 95°C for 8 minutes,

30 cycles of denaturation (94°C for 1 minute), hybridization (55°C for 1.5 minutes), and elongation (72°C for 3 minutes), with a final 7 minute elongation at 72°C and a 4°C hold completing the amplification process. Aliquants of the amplified products were analyzed for size by electrophoresis on a 1% SeaKem GTG agarose gel containing 0.4% (w/v) ethidium bromide against a 1 kb-ladder DNA standard (Life Technologies). Every reaction series included a negative control which did not contain template DNA and did not yield any amplification products.

Construction of bacterial SSU rDNA clone libraries

Five individual PCR reactions were generated for each sampling site. These were then pooled, purified by size exclusion using Microcon 50 filters (Millipore, Bedford, MA), washed twice with 200 µl 0.1X Tris buffer (1.0 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), and recovered in 120 µl 0.1X Tris buffer. The purified rDNA PCR products were quantified by absorbance at 260 nm, ligated into the pT-Adv cloning vector (AdvanTAge PCR Cloning Kit; Clontech Laboratories, Inc., Palo Alto, CA), and transformed using competent TOP10F′ Epicurian Coli cells following the manufacturer's protocols.

All clones were screened by α-complementation using X-gal and IPTG (~1 mg/plate each) on LB agar containing 100 µg/ml ampicillin. Putative positive clones were streaked for isolation and then each isolated colony was transferred to another LB ampicillin plate. Each putative positive clone was then screened by PCR to determine the size of the plasmid insert. Reactions contained 2 µl 10X PCR buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP, 0.5 µM each primer (M13F and M13R), 4 µg acetylated BSA, 0.4 U *Taq* DNA polymerase,

and sterile water to a total volume of 20 µl. M13F (5' - TGTAAAACGACGGCCAGT) and M13R (5' - CAGGAAACAGCTATGACC) corresponds to positions on opposite sides of the vector cloning site. Using a sterile toothpick, a small portion of the freshly grown colony was transferred to the corresponding PCR reaction tube.

The PCR mixtures were then sealed and placed in a GeneAmp 9700 thermocycler with the following conditions: 94°C for 8 minutes, 30 cycles of denaturation at 94°C for 1 minute, hybridization at 55°C for 1.5 minutes, elongation at 72°C for 3 minutes, followed by an additional elongation at 72°C for 10 minutes and a 4°C hold. Amplified products were analyzed by electrophoresis on a 1% SeaKem GTG agarose gel containing 0.4% (w/v) ethidium bromide against a 1 kb-ladder DNA standard. Negative controls were also included and were shown not to yield any amplification products. The positive clones with the correct size inserts were grown overnight at 37°C in LB broth with 100 mg/ml ampicillin. Freeze cultures were cryopreserved using 1 ml broth culture and 500 µl sterile 80% glycerol, mixed, and quick frozen at -80°C. To purify the plasmids, the QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA) protocol was followed with the exception of using 100 µl Buffer EB instead of 50 µl to elute the DNA.

ARDRA

SSU rDNA was amplified using plasmids as templates for restriction analysis by amplified ribosomal DNA restriction analysis (ARDRA). Oligonucleotide primers that are proximal to the vector sequences were selected because ARDRA is potentially sensitive to the orientation of the cloned insert (Moyer, 2000). The forward primer (5′-

ACGGCCGCCAGTGTGCTG) and the reverse primer (5′-GTGTGATGGATATCTGCA) correspond to positions adjacent to opposite sides of the vector cloning site. The PCR reaction mixtures were as follows: 5 µl 10X PCR buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP, 1 µM each of forward and reverse primer, 10 µg acetylated BSA, 5 U *Taq* DNA polymerase, 2 µl plasmid template (~50 ng), and sterile water to a total volume of 50 µl.

The PCR mixtures were then sealed and placed in a GeneAmp 9700 thermocycler and incubated under the following conditions: 1 minute at 95°C, 30 cycles of denaturation (1 minute at 94°C), hybridization (1.5 minutes at 50°C), and elongation (3 minutes at 72°C), followed by an additional 7 minute elongation at 72°C and a 4°C hold. Aliquants of each PCR were analyzed for the correct size insert using gel electrophoresis on a 1% SeaKem GTG agarose gel containing 0.4% (w/v) ethidium bromide against a 1 kb-ladder DNA standard. The negative controls included with the PCR did not show any amplification.

Two sets of tetrameric enzymes were used in the restriction digests. The first treatment used 5 U each *Hha*I and *Hae*III (New England Biolabs, Beverly, MA), 3 µl 10X NEBuffer 2 (New England Biolabs), sterile water to a total volume of 15 µl, and 15 µl of each plasmid PCR reaction. The second treatment used 5 U each *Msp*I and *Rsa*I (New England Biolabs). These reagents and PCR products were mixed in microtiter dish wells, sealed with Seal Plate adhesive sealing films (Excel Scientific, Wrightwood, CA), and incubated for 16 hours at 37°C. After the incubation, 6 µl of 10X Orange G loading buffer, 15% (w/v) Ficoll Type 400 (Sigma Chemical Company, St. Louis, MO) and 0.25% (w/v) Orange G (Sigma Chemical Company), were then added to each restriction digest. DNA markers were prepared by mixing 2.5 µg of DNA Marker V (Roche Molecular Biochemicals,

Indianapolis, IN) with 3 µl Orange G loading buffer. Separation of restriction fragments was carried out in a cold room at 4°C with 3.5% MetaPhor agarose gels (BioWhittaker Molecular Applications, Rockland, MD) run at 5 volts/cm. The gels were then stained for one hour in about one liter of a 2.5 µg/ml ethidium bromide solution and destained in tap water for 30 minutes. The gels were then visualized by UV excitation and the images were captured using an 8 bit CCD camera (Ultralum, Paramount, CA).

Cluster analysis was performed on the restriction digest patterns using GelCompar version 4.0 (Applied Maths; Kortrijk, Belgium). The gels were digitally optimized and normalized to a single DNA marker to minimize gel-to-gel restriction pattern variability. Unweighted pair group analysis of Pearson product-moment correlations were carried out on all 249 clones from the four libraries. The clustering of the restriction pattern images was set at a correlation minimum of 75% by empirically visualizing the major clusters within each library. Clusters ≥75% were defined as separate OTUs (operational taxonomic units).

Rarefaction analysis

The rarefaction technique, a deterministic transform of the OTU richness data (Tipper, 1979), was performed on each clone library with the Matlab software (The Mathworks Inc., Natick, MA; Moyer *et al.*, 1998) using Simberloff's (1978) method. Rarefaction allows for the estimation of OTU richness as a function of OTU abundance data using unequal sample sizes and can be used to compare collections from communities that are taxonomically similar and from similar habitats (Tipper, 1979).

SSU rDNA sequencing

Representative bacterial SSU rDNA clones from OTUs that contained either three or more clones, or two clones found in different libraries, were completely sequenced using fluorescently labeled dideoxy terminators with an automated DNA Sequencer (PE Applied Biosystems). SSU rDNA templates were generated from a plasmid PCR using M13F and M13R primers. The PCR conditions and other reaction reagents were the same as those for the plasmid PCR for ARDRA except the hybridization temperature was 55°C. Amplification products were concentrated via Microcon 50 filters, washed twice with 200 µl 0.1X Tris buffer and once with 200 µl sterile water, then recovered in 100 µl sterile water. An aliquant of the purified amplification product was examined by electrophoresis in a 1% SeaKem GTG agarose gel containing 0.4% (w/v) ethidium bromide against a 1 kb-ladder DNA standard.

The cycle sequencing PCR was as follows: 4 µl of each DNA sample (100-200 ng), 4 µl Big Dye terminator ready reaction mix (PE Applied Biosystems), and 10 µmol of primer. The primers selected for this procedure were internal to the bacterial SSU rDNA as described by Lane (1991). The reaction conditions were 25-30 cycles of denaturation (10 seconds at 95°C), hybridization (5 seconds at 50°C), and elongation (4 minutes at 60°C) with a 4°C hold at the end of the cycles. The sequencing reactions were then precipitated using the isopropanol method outlined in the manufacturer's protocol, vacuum dried, and sequenced using an automated sequencer (PE Applied Biosystems). The software program GeneTool (BioTools Inc., Edmonton, Alberta, CA) was then used to transform the raw sequence data files generated from the automated DNA sequencer into contiguous SSU rDNA sequences for phylogenetic analysis.

Phylogenetic analysis

The SSU rDNA clone sequences were searched against sequences found in the Ribosomal Database Project II (RDP) using the SEQUENCE_MATCH function (Maidak *et al.*, 2000). This approach identifies any identical or closely related sequences existing in the database based on similarity. In addition, the level of dissimilarity between a potentially novel sequence and any previously recorded phylogenetic groups can be ascertained. Sequence data were also screened by the nearest neighbor based CHECK_CHIMERA function at RDP to minimize the chance of artefactual sequences described as nonexistent microorganisms or chimeras based on pairwise similarity analysis in the variable unaligned sequence domains (Robison-Cox *et al.*, 1995).

Sequences were then manually aligned to a collection of previously aligned sequences derived from RDP and Genbank using the Genetic Data Environment (GDE). Phylogenetic analyses were restricted to the comparison of highly to moderately conserved regions of the SSU rRNA gene. These nucleotide positions varied by analysis due to the different taxa selected for each comparison. All corresponding residues were based on the *Escherichia coli* numbering system. Positions 101 to 183, 219 to 451, 483 to 838, 848 to 1020, 1037 to 1041, 1043 to 1126, 1149 to 1165, 1171 to 1285, and 1287 to 1439 were used to construct the Green Non-Sulfur *Bacteria* and Relatives phylogenetic tree. The Flexibacter-Cytophaga-Bacteroides were compared with residues corresponding to positions 102 to 183, 219 to 418, 424 to 450, 483 to 833, 854 to 995, 999 to 1023, 1045 to 1165, 1167 to 1285, and 1287 to 1440. For the γ-*Proteobacteria*, the mask corresponded to positions 100 to 128, 130 to 183, 219 to 450, 480 to 836, 847 to 1024, 1045 to 1138, 1141 to 1362, and

1365 to 1445. The phylogenetic tree containing the δ-*Proteobacteria* included nucleotides corresponding to positions 101 to 183, 218 to 452, 483 to 838, 842 to 845, 849 to 1024, 1037 to 1129, 1141 to 1285, 1287 to 1435, and 1459 to 1480. Finally, the nucleotides used to construct the ϵ-*Proteobacteria* phylogenetic tree corresponded to positions 101 to 183, 219 to 450, 480 to 1024, 1035 to 1166, 1168 to 1285, 1287 to 1445, and 1460 to 1491.

Initial phylogenetic screening was conducted by using the neighbor-joining algorithm where distance matrix data estimate a single additive tree with a minimal overall tree length (Saitou and Nei, 1987). Final phylogenetic placement was conducted through maximum likelihood analyses with the fastDNAml program (version 1.1; Olsen *et al.*, 1994a). The final phylogenetic trees were constructed using jumbled orders for the addition of taxa and empirical transition/transversion ratios. In addition, the trees allowed for the global swapping of branches and were rooted to a known outgroup taxon. The search for an optimal tree was repeated using these parameters until the best log likelihood score was reached in at least three independent searches (Moyer *et al.*, 1995; 1998).

RESULTS

Construction of bacterial SSU rDNA clone libraries

The choice of putative positive clones was assumed to be stochastic because each clone was picked at random from the α-complementation screening plates. Each of the 249 clones examined had a ~1.5 kb insert as determined by M13F/M13R colony PCR screening and an additional amplification using proximal flanking vector sequences from the plasmid vector. A clone number was then assigned in ascending order. Clone libraries generated from samples taken at Snow Blower, Easy Vent, and North Rift contained 56 clones each, while 81 clones were analyzed from Marker 33 Vent.

ARDRA

The resulting dendrogram from the cluster analysis of restriction digest patterns for all 249 clones is shown in Figure 6. A total of 34 OTUs containing two or more clones (range n = 2 to 10) were found, comprising 47% of all the clones analyzed. There were fifteen OTUs containing three or more clones, or two clones found in different libraries, which were selected for sequence analysis. OTUs were numbered in increasing order as the number of clones within each OTU decreased (Table 2). The remaining OTUs (n = 132) contained only one clone and were not sequentially numbered. There were no OTUs found in all four libraries. However, five OTUs were found in two of the four libraries, and one OTU was found in three of the four libraries. The majority of OTUs 2 and 9 were found in Marker 33 Vent, but a few clones were also found at Easy Vent. There were also crossovers detected between Marker 33 Vent and Snow Blower involving OTUs 8, 10, and 15. OTU 4

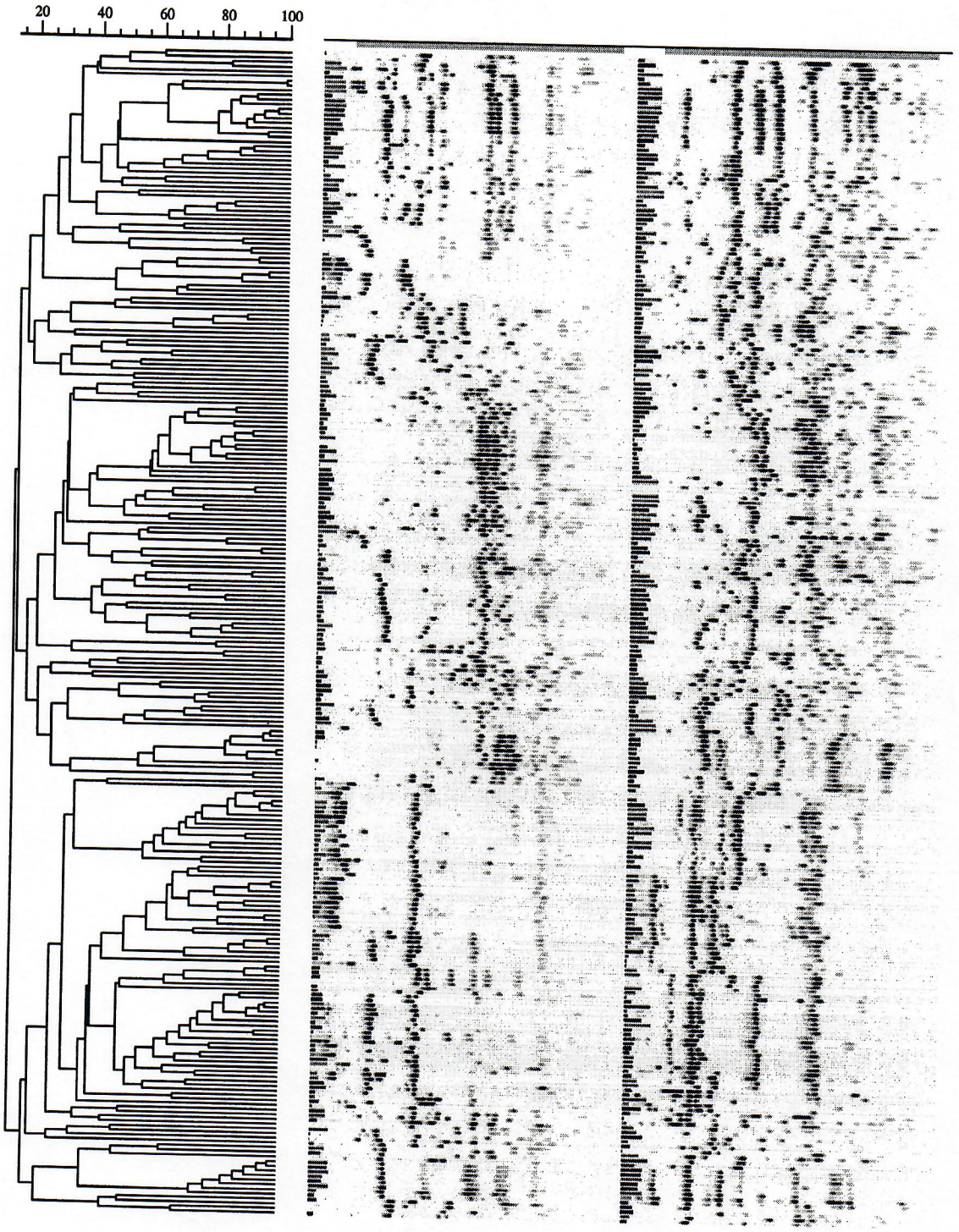

Figure 6. Cluster analysis dendrogram of ARDRA patterns for 249 bacterial SSU rDNA clones from Axial Volcano hydrothermal vents. Scale represents Pearson product-moment correlations. Bands above ARDRA patterns show range of data utilized to construct dendrogram.

Table 2. OTU and clone distribution for bacterial communities at Axial Volcano. The phylogenetic affiliations of OTUs 1 to 15 are indicated in parentheses. OTUs in bold occur in two or more sample sites. (FCB = Flexibacter-Cytophaga-Bacteroides; ϵ = ϵ-*Proteobacteria*; δ = δ-*Proteobacteria*; GNS = Green Non-Sulfur *Bacteria* and Relatives; γ = γ-*Proteobacteria*)

Operational Taxonomnic Unit (OTU)	Number of Clones				Total Clones per OTU
	Marker 33 Vent	Snow Blower	Easy Vent	North Rift	
AX*B* OTU 1 (FCB)				10	10
AX*B* OTU 2 (ϵ)	**6**		**3**		**9**
AX*B* OTU 3 (FCB)				8	8
AX*B* OTU 4 (ϵ)	**1**	**2**	**4**		**7**
AX*B* OTU 5 (δ)	7				7
AX*B* OTU 6 (GNS)		7			7
AX*B* OTU 7 (ϵ)		6			6
AX*B* OTU 8 (ϵ)	**3**	**1**			**4**
AX*B* OTU 9 (ϵ)	**3**		**1**		**4**
AX*B* OTU 10 (ϵ)	**1**	**2**			**3**
AX*B* OTU 11 (ϵ)		3			3
AX*B* OTU 12 (γ)			3		3
AX*B* OTU 13 (FCB)				3	3
AX*B* OTU 14 (FCB)				3	3
AX*B* OTU 15 (FCB)	**1**	**1**			**2**
AX*B* OTU 16	2				2
AX*B* OTU 17	2				2
AX*B* OTU 18	2				2
AX*B* OTU 19	2				2
AX*B* OTU 20	2				2
AX*B* OTU 21	2				2
AX*B* OTU 22	2				2
AX*B* OTU 23		2			2
AX*B* OTU 24		2			2
AX*B* OTU 25		2			2
AX*B* OTU 26		2			2
AX*B* OTU 27		2			2
AX*B* OTU 28			2		2
AX*B* OTU 29			2		2
AX*B* OTU 30			2		2
AX*B* OTU 31			2		2
AX*B* OTU 32			2		2
AX*B* OTU 33			2		2
AX*B* OTU 34				2	2
Single Clones	45	24	33	30	132
TOTAL	81	56	56	56	249

was detected in Marker 33 Vent, Snow Blower, and Easy Vent. All crossovers detected occurred among the new lava vents from within the Axial Caldera, no crossovers were detected with the sample collected from North Rift.

The number of clones examined, the number of OTUs found, the number of single occurrence OTUs, and the proportion of clones found in multiple OTUs (coverage; Good, 1953; McCaig *et al.*, 1999) are listed in Table 3 for each Axial Volcano *Bacteria* (AX*B*) clone library. The Easy Vent library had the lowest coverage (39%), while the Snow Blower library had the highest coverage (54%), indicating that between 39% and 54% of the overall organismal diversity in all four clone libraries was detected, using this methodological approach.

Rank abundance

The rank abundance diagrams for each of the four AX*B* clone libraries examined are shown in Figure 7. The most frequent operational taxonomic unit (OTU) for Marker 33 Vent library was OTU 5, followed by OTUs 2, 8, and 9. OTU 6 was the most abundant detected in Snow Blower's clone library, followed by OTUs 7 and 11. The most abundant OTU detected in Easy Vent's clone library was OTU 4, followed by OTUs 2 and 12. The most abundant OTU for North Rift, which was also the most abundant OTU detected overall, was OTU 1, followed by OTUs 3, 13, and 14. There were also six incidences of crossover detected among three of the four libraries (Table 2).

Table 3. Number of clones, total number of OTUs, and number of single-occurrence OTUs detected by ARDRA of Axial Volcano hydrothermal vent bacterial communities. Coverage indicates the proportion of clones found in multiple-occurrence OTUs.

Site	Number of Clones Examined (N)	Number of OTUs Found	Number of Single Occurrence OTUs[a] (s)	Coverage (1.00 - 0.00)[b] (C)
Marker 33 Vent	81	59	48	0.41
Snow Blower	56	36	26	0.54
Easy Vent	56	43	34	0.39
North Rift	56	35	30	0.46

[a]Within each library. Some single-occurrence OTUs were found in more than one library.
[b]Possible values for each library at a minimum (single OTU) and maximum (n = number of OTUs) diversity, respectively. Coverage equation is given in Appendix I.

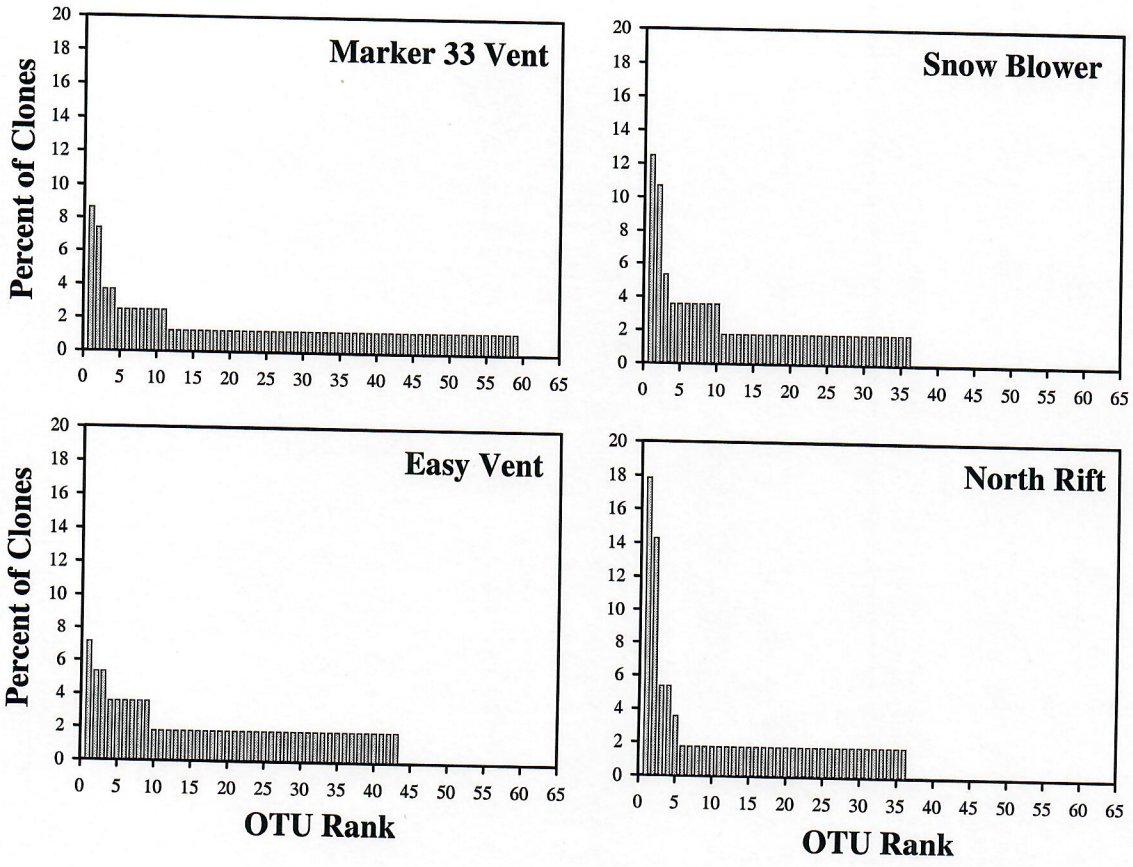

Figure 7. Rank abundance diagrams for bacterial community OTUs found at four Axial Volcano hydrothermal vent sample locations.

Collector's curves

A graphical estimation of the coverage of each bacterial SSU rDNA clone library was constructed by plotting the total number of OTUs detected as a function of the number of clones examined (McCaig *et al.*, 1999; Moyer *et al.*, 1994). As library coverage increases, the slope of the collector's curve decreases (Figure 8), indicating that more clones need to be examined before a new OTU can be found. The collector's curves for these four clone libraries show that coverage was relatively lower in both Easy Vent and Marker 33 Vent libraries and higher in both North Rift and Snow Blower. This result is consistent with the calculations of coverage in Table 3 (i.e., an inverse relationship).

Rarefaction

Rarefaction allows for the comparison of diversity from clone libraries of different sample size and estimates the number of phylotypes in a random sample of clones, sampled without replacement from a community of finite size. Estimates of the number of OTUs with the corresponding standard deviations are plotted as a function of the number of clones examined for each clone library are shown in Figure 9. For all four clone libraries, the maximum number of clones plotted in Figure 9 is two clones less than the actual value as a result of the iterative process used by the computer algorithm (Simberloff, 1978). The number of OTUs predicted gives an estimate of OTU richness, and was highest for Easy and Marker 33 Vent libraries and lower for Snow Blower and North Rift after the examination of 56 clones in Easy Vent, Snow Blower, and North Rift and 81 clones in Marker 33 Vent libraries. Standard deviations for Easy and Marker 33 Vent curves overlapped as well as standard deviations for Snow Blower and North Rift curves.

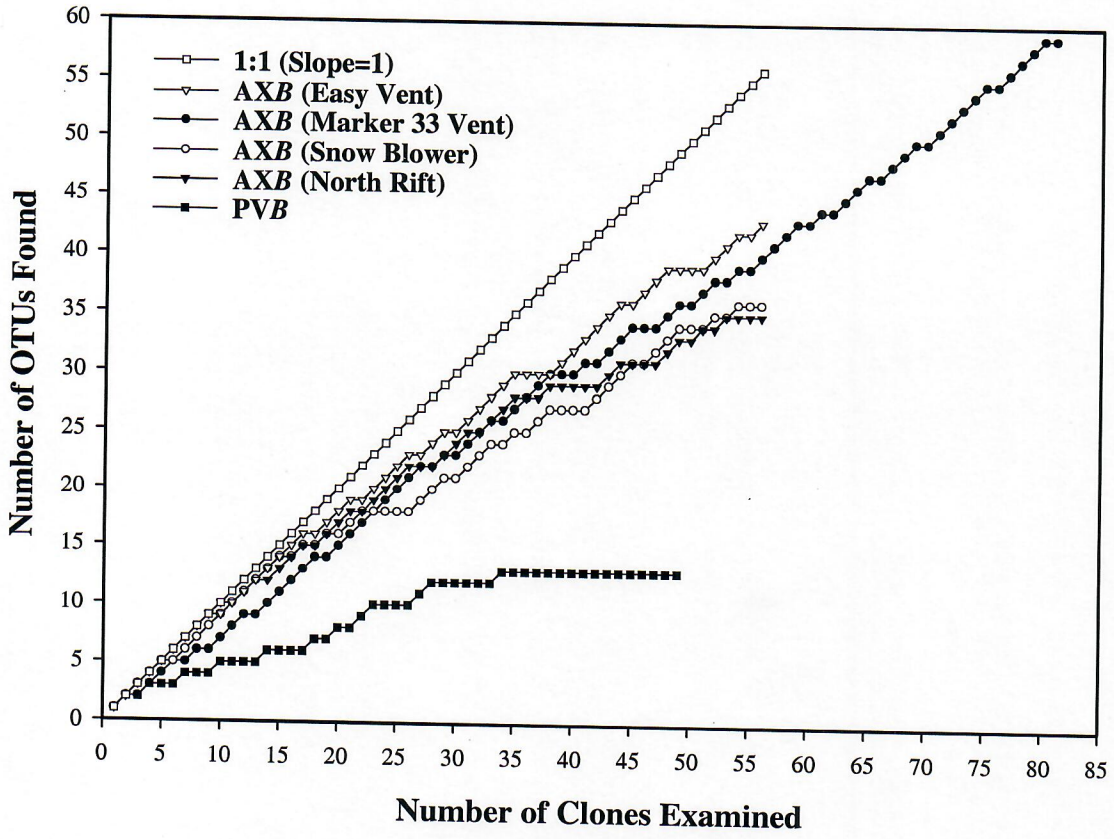

Figure 8. Collector's curves for bacterial OTUs from four Axial Volcano hydrothermal vent sites. Pele's Vent *Bacteria* (PV*B*) data are shown for comparison (Moyer *et al.*, 1994). Theoretical maximum diversity is represented by slope = 1.

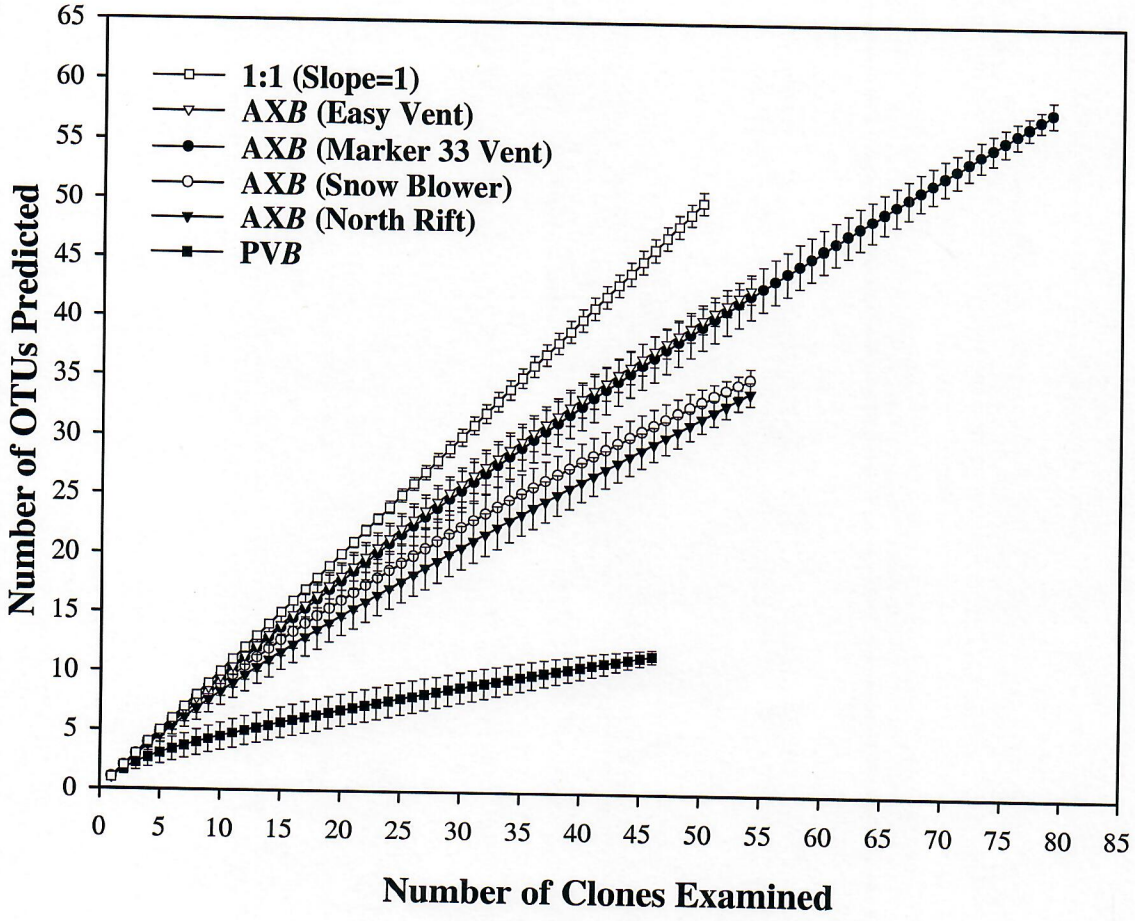

Figure 9. Rarefaction analysis curves for four Axial Volcano hydrothermal vent sites. Pele's Vent *Bacteria* (PV*B*) data are shown for comparison (Moyer *et al.*, 1994). Theoretical maximum diversity is represented by slope = 1.

Phylogenetic analysis

The phylogenetic affiliations of each of the fifteen AX*B* OTUs sequenced when searched against Ribosomal Database Project II (RDP; Maidak *et al.*, 2000) sequences are listed in Table 4. AX*B* OTUs 1, 3, 13, 14, and 15 are contained in the Flexibacter-Cytophaga-Bacteroides (FCB) division. AX*B* OTU 1 and OTU 3 are contained in the Cytophaga group I with S_{ab} values of 0.544 and 0.677 to their respective nearest matches. AX*B* OTU 13 is phylogenetically similar (S_{ab} = 0.590) to a clone in the Cytophaga group II while AX*B* OTUs 14 and 15 are members of the Bacteroides group with similarity coefficients of 0.675 and 0.556, respectively. AX*B* OTU 5 was the only OTU found in the δ-*Proteobacteria* and had a similarity coefficient of 0.806 to *Desulfocapsa sulfoexigens*. AX*B* OTU 12 was contained in the γ-*Proteobacteria* with an S_{ab} value of 0.693 to *Methylomonas* strain LW21 as its nearest neighbor. AX*B* OTU 6 was most closely related to Environmental Clone WCHB1-31 group, with the nearest match to clone SJA-68 (S_{ab} = 0.565). The remaining seven AX*B* OTUs were contained in the ϵ-*Proteobacteria* lineage, two of which were found in the Campylobacter group with S_{ab} values of 0.577 and 0.648 for AX*B* OTUs 7 and 10A, respectively. AX*B* OTU 2 was found to have a similarity coefficient of 0.752 when compared to a symbiont of the shrimp *Rimicaris exoculata*, a member of the Thiovulum group. AX*B* OTUs 4 and 11 were also contained in the Thiovulum group with similarities to Pele's Vents *Bacteria* clones 73 (S_{ab} = 0.843) and 55 (S_{ab} = 0.893), respectively. The final two OTUs, AX*B* OTUs 8 and 9, are similar to strains found in the *Alvinella pompejana* symbionts subgroup (Thiovulum group) with similarity coefficients of 0.741 and 0.697, respectively. No fully sequenced SSU rRNA genes were

Table 4. Phylogenetic affiliations of sequenced Axial Volcano SSU rDNA phylotypes.

AX*B* OTU Number	Sequenced Clone Number	Phylogenetic Affiliation[a]	Nearest Match	Similarity Coefficient[b]
AX*B* OTU 1	NR11	Bacteroides and Cytophaga Cytophaga group I RDP#2.15.1.3	*Cytophaga fucicola* str. NN015860	0.544
AX*B* OTU 2	M11	ϵ-*Proteobacteria* Thiovulum group RDP#2.28.5.2	Symbiont of *Rimicaris exoculata*	0.752
AX*B* OTU 3	NR50	Bacteroides and Cytophaga Cytophaga group I RDP#2.15.1.3	Activated sludge clone BA2	0.677
AX*B* OTU 4	EZ19	ϵ-*Proteobacteria* Thiovulum group RDP#2.28.5.2	Pele's Vents *Bacteria* clone PV*B*73	0.843
AX*B* OTU 5	M34	δ-*Proteobacteria* Desulfobulbus propionicus assemblage RDP#2.28.4.11	*Desulfocapsa sulfoexigens* str. SB164P1	0.806
AX*B* OTU 6	SB5	Green Non-Sulfur *Bacteria* & Relatives Environmental Clone WCHB1-31 group RDP#2.18	Contaminated aquifer clone SJA-68	0.565
AX*B* OTU 7	SB24	ϵ-*Proteobacteria* Campylobacter group RDP#2.28.5.3	*Sulfurospirillum arachonense* str. F1F6 DSMZ 9755	0.577
AX*B* OTU 8	M2	ϵ-*Proteobacteria* Thiovulum group RDP#2.28.5.2	Marine sediments JTB315	0.741
AX*B* OTU 9	M8	ϵ-*Proteobacteria* Thiovulum group RDP#2.28.5.2	Marine sediments NKB9	0.697
AX*B* OTU 10A AX*B* OTU 10B	M26 SB12	ϵ-*Proteobacteria* Campylobacter group RDP#2.28.5.3	Marine sediments BD1-5	0.648 0.733
AX*B* OTU 11	SB26	ϵ-*Proteobacteria* Thiovulum group RDP#2.28.5.2	Pele's Vents *Bacteria* clone PV*B*55	0.893
AX*B* OTU 12	EZ17	γ-*Proteobacteria* Methylomonas group RDP#2.28.3.10	*Methylomonas* str. LW21	0.693
AX*B* OTU 13	NR55	Bacteroides and Cytophaga Cytophaga group II RDP#2.15.1.4	Mid-Atlantic Ridge clone VC2.1Bac22	0.590
AX*B* OTU 14	NR70	Bacteroides and Cytophaga Bacteroides group RDP#2.15.1.2	Austrian oil field clone R1	0.675
AX*B* OTU 15	SB1	Bacteroides and Cytophaga Bacteroides group RDP#2.15.1.2	Marine sediments clone Sva1038	0.556

[a]As described by the RDP, version 8.0 (Maidak *et al.*, 2000). Excludes sequences containing less than 1000 nucleotides and the unclassified phylotypes.
[b]S_{ab} value which is the number of unique oligomers shared between the sequence and a given RDP sequence divided by the lower number of unique oligomers in either of the two.

determined to be chimeras when checked with the CHECK_CHIMERA function at RDP. However, there may be potential chimeras in the single-occurrence clones which were not sequenced.

Since there were five bacterial lineages encompassing the OTUs initially determined by similarity search on RDP, five phylogenetic trees were constructed to determine the genetic diversity of the clones sequenced from Axial Volcano microbial communities. The phylogenetic affiliations as well as the relative abundance of each OTU is shown in Table 5. AX*B* OTU 6 is found in the Chloroflexus subdivision, which is contained within the Green Non-Sulfur *Bacteria* and Relatives division (Figure 10). This OTU accounts for 12.5% of the SSU rRNA clones recovered from Snow Blower, which had 39.4% of its OTUs categorized by phylotype, and is unique to that site (Table 5). *Thermotoga maritima* and OPB45 were used in addition to *Aquifex pyrophilus* as the outgroup, to help define the division.

Five AX*B* OTUs are related to the Bacteroides and Cytophaga subdivision (Figure 11). AX*B* OTUs 1 and 3 are most closely related to members of Cytophaga group I and account for 17.9 and 14.3% of the sequences detected at the North Rift site (Table 5), respectively. North Rift had 43.0% of its clones categorized by phylotype (Table 5). AX*B* OTU 14 accounts for 5.4% of the phylotypes from North Rift and is most closely related to members of Bacteroides group (Table 5). The other phylotype associated with the Bacteroides group is AX*B* OTU 15 which is a crossover between Marker 33 Vent (1.2%) and Snow Blower (1.8%). AX*B* OTU 13 had a relative abundance of 5.4% in the North Rift site and its closest relative is a member of Cytophaga group II (Table 5). *Thermonema lapsum*

Table 5. Summary of phylogenetic affiliations and percent recoveries of bacterial SSU rRNA genes from microbial communities at Axial Volcano.

Phylogenetic Affiliation[a]	Phylotype(s)[b]	Clones Recovered (%)[c]			
		Marker 33 Vent	Snow Blower	Easy Vent	North Rift
Green Non-Sulfur *Bacteria* and Relatives	AX*B* OTU 6		12.5		
Flexibacter - Cytophaga - Bacteroides	AX*B* OTU 1				17.9
	AX*B* OTU 3				14.3
	AX*B* OTU 13				5.4
	AX*B* OTU 14				5.4
	AX*B* OTU 15	1.2	1.8		
γ-*Proteobacteria*	AX*B* OTU 12			5.4	
δ-*Proteobacteria*	AX*B* OTU 5	8.6			
ϵ-*Proteobacteria*	AX*B* OTU 2	7.4		5.4	
	AX*B* OTU 4	1.2	3.6	7.1	
	AX*B* OTU 7		10.7		
	AX*B* OTU 8	3.7	1.8		
	AX*B* OTU 9	3.7		1.8	
	AX*B* OTU 10	1.2	3.6		
	AX*B* OTU 11		5.4		
TOTAL		27.0	39.4	19.7	43.0

[a]As determined by RDP, version 8.0 (Maidak *et al.*, 2000).
[b]The phylotypes used in maximum likelihood analyses.
[c]Calculated by dividing the number of group-specific bacterial clones by the total number of clones analyzed in the clone library.

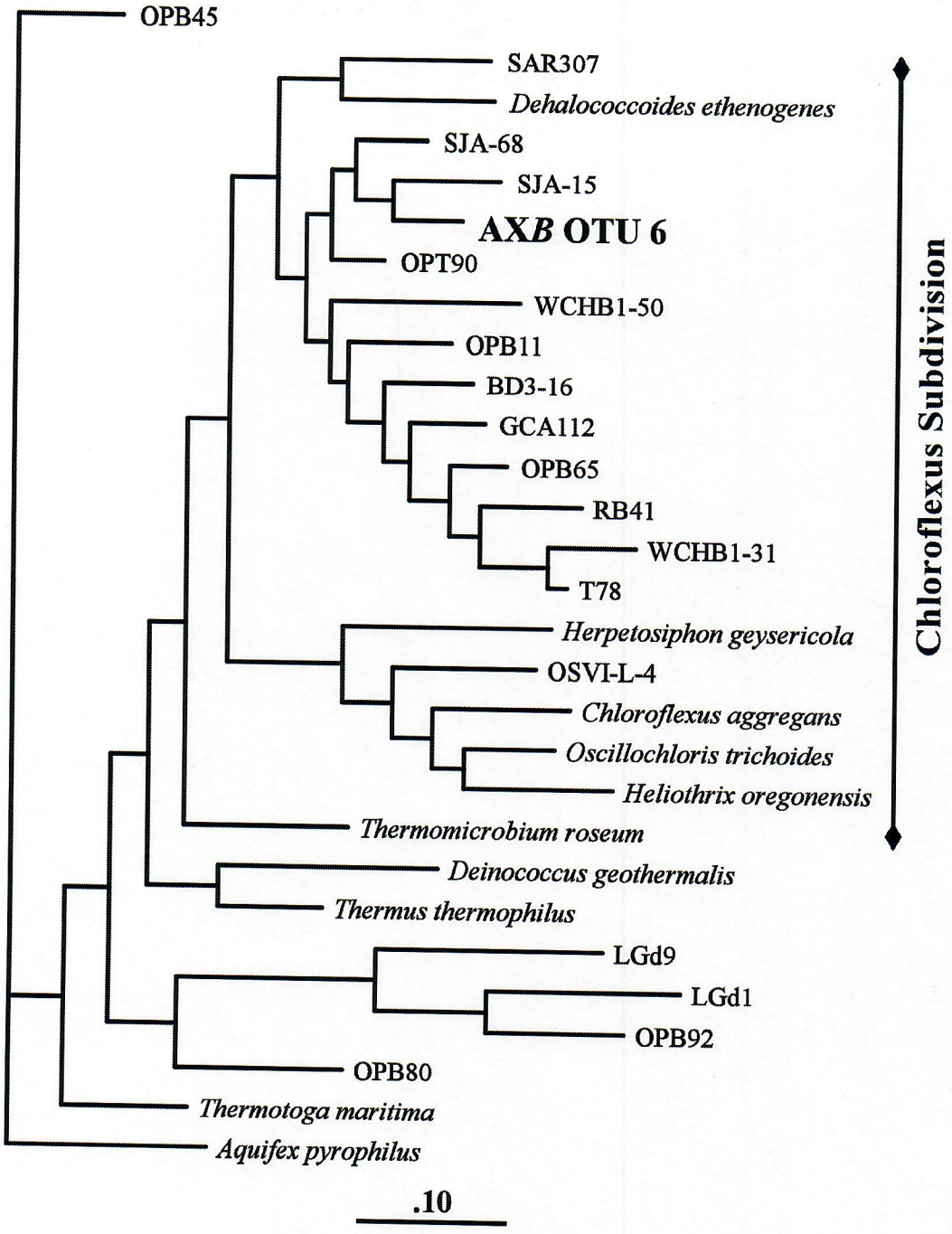

Figure 10. Phylogenetic tree demonstrating the relationships of AX*B* OTU 6 with members of the Green Non-Sulfur *Bacteria* and Relatives as determined by maximum likelihood analysis of SSU rDNA sequences. An outgroup is represented by *Aquifex pyrophilus* SSU rDNA sequence data. Sequences not determined in this study were provided by RDP. Scale bar represents 0.10 fixed mutations per nucleotide position.

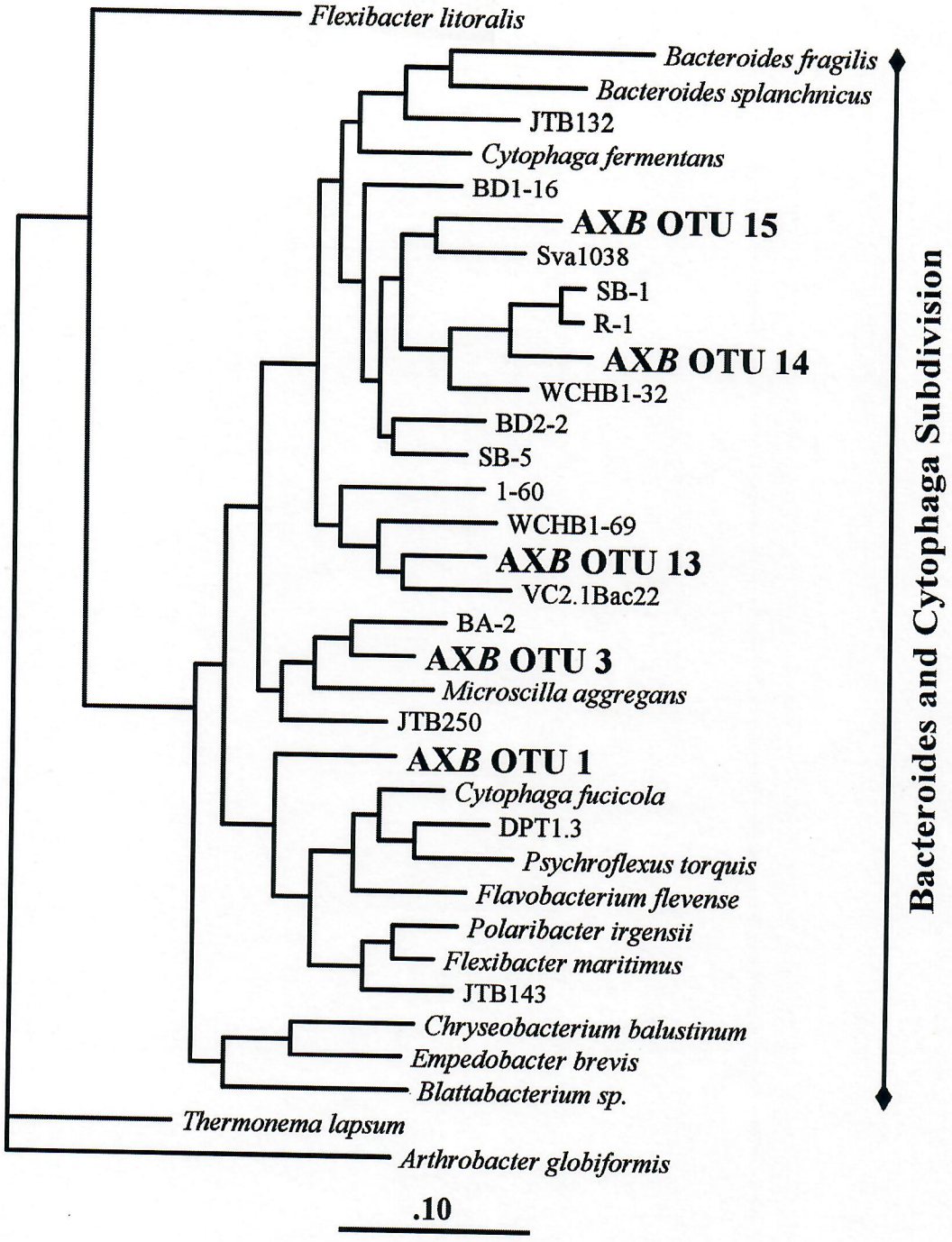

Figure 11. Phylogenetic tree demonstrating the relationships of AX*B* OTUs 1, 3, 13, 14, and 15 with members of the Flexibacter-Cytophaga-Bacteroides division as determined by maximum likelihood analysis of SSU rDNA sequences. An outgroup is represented by *Arthrobacter globiformis* SSU rDNA sequence data. Sequences not determined in this study were provided by RDP. Scale bar represents 0.10 fixed mutations per nucleotide position.

and *Flexibacter litoralis* were used to help define this subdivision of the Flexibacter-Cytophaga-Bacteroides.

The γ- and δ-*Proteobacteria* each contained one phylotype from Easy Vent and Marker 33 Vent, respectively. One phylotype (AX*B* OTU 12) was contained in the γ-*Proteobacteria* (Figure 12) and accounted for 5.4% of the SSU rRNA clones recovered from Easy Vent, which was categorized 19.7% by phylotype (Table 5). AX*B* OTU 12 is most closely related to mussel symbionts in the Methylomonas group contained in the γ-*Proteobacteria*. Members of the ϵ-*Proteobacteria* were used to aid in defining this phylogenetic tree. The other phylotype, AX*B* OTU 5, is most closely related to *Desulfocapsa sulfoexigens* contained in the δ-*Proteobacteria* (Figure 13) and had a relative abundance of 8.6% (Table 5).

The majority of AX*B* OTUs detected in this study were contained in the ϵ-*Proteobacteria* (Figure 14). AX*B* OTU 2 is most closely related to a symbiont of *Rimicaris exoculata*, with AX*B* OTUs 8 and 9 being ancestrally related to that symbiont. Marker 33 Vent, which had 27.0% of its OTUs categorized by phylotype, and Snow Blower had representatives from AX*B* OTU 8 at clone recoveries of 3.7 and 1.8%, respectively (Table 5). Easy Vent and Marker 33 Vent had representatives from both AX*B* OTUs 2 and 9. AX*B* OTU 2 was recovered at 7.4% at Marker 33 Vent and 5.4 % at Easy Vent, while AX*B* OTU 9 accounted for 3.7% and 1.8% at Marker 33 Vent and Easy Vent, respectively (Table 5). Two AX*B* OTUs were closely related to Pele's Vent *Bacteria* (PV*B*) OTUs. AX*B* OTU 4, which accounted for 1.2%, 3.6%, and 7.1% of the clones detected at Marker 33 Vent, Snow Blower, and Easy Vent, respectively, was most closely affiliated with

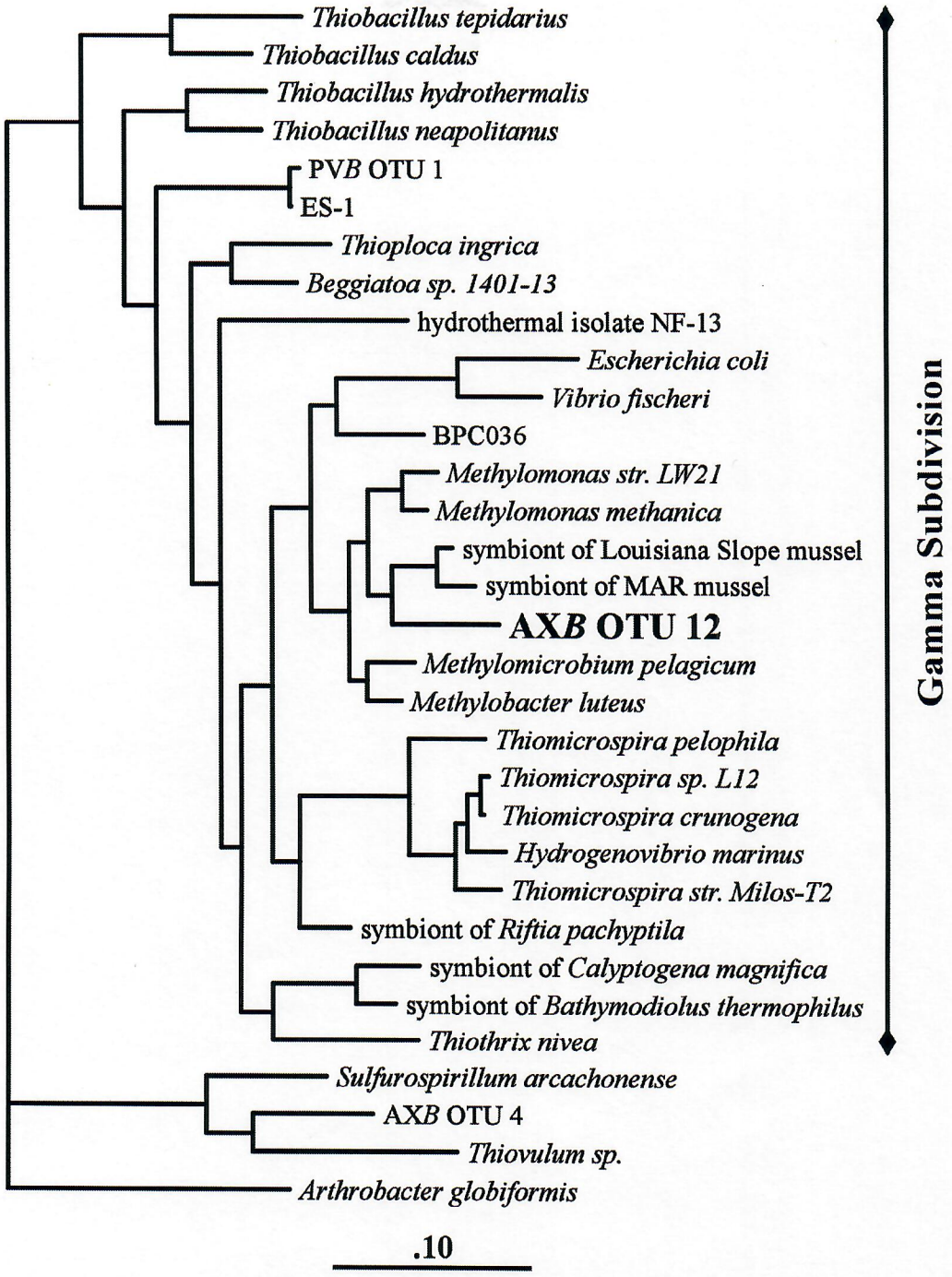

Figure 12. Phylogenetic tree demonstrating the relationships of AX*B* OTU 12 with members of the γ-*Proteobacteria* as determined by maximum likelihood analysis of SSU rDNA sequences. An outgroup is represented by *Arthrobacter globiformis* SSU rDNA sequence data. Sequences not determined in this study were provided by RDP. Scale bar represents 0.10 fixed mutations per nucleotide position.

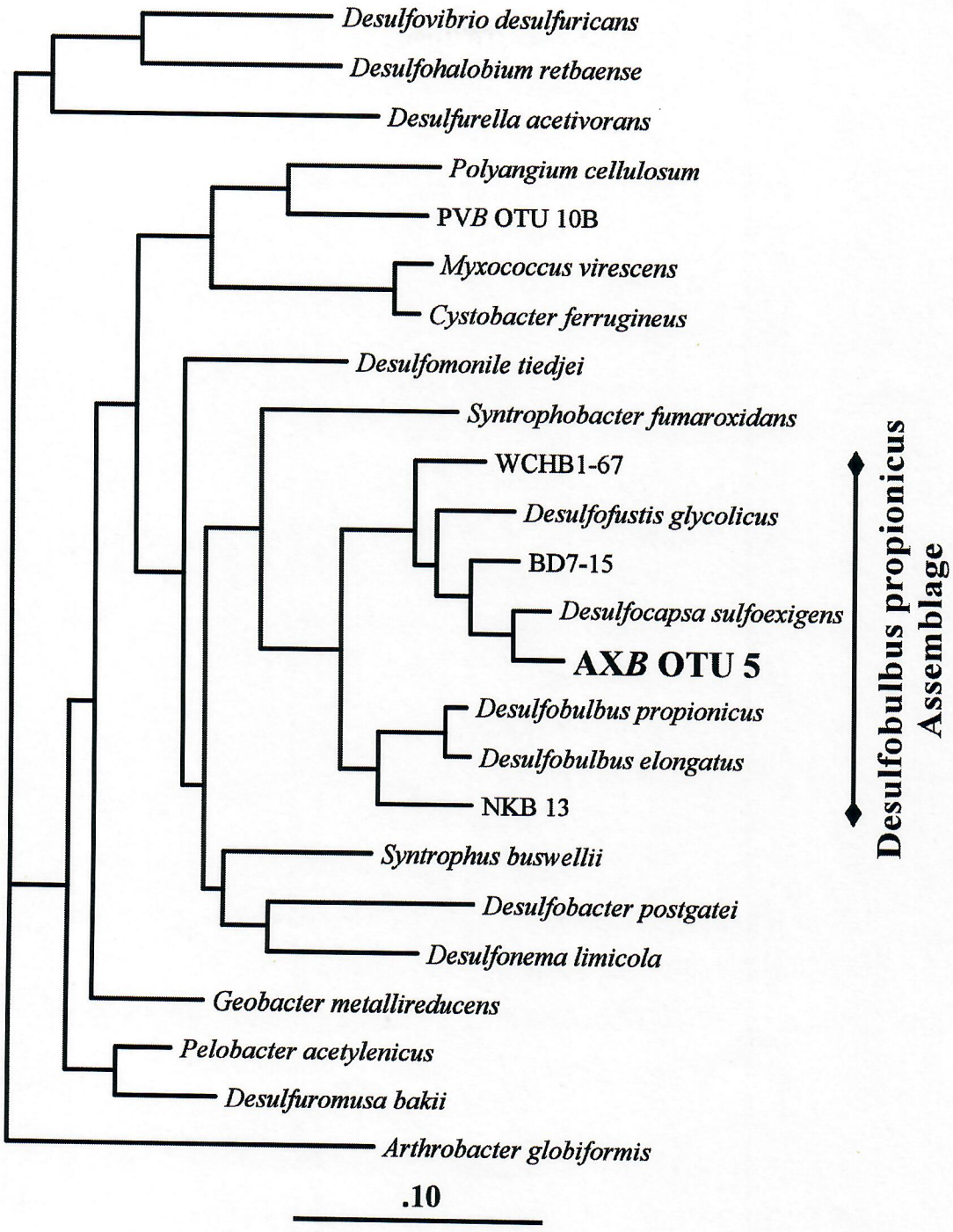

Figure 13. Phylogenetic tree demonstrating the relationships of AX*B* OTU 5 with members of the δ-*Proteobacteria* as determined by maximum likelihood analysis of SSU rDNA sequences. An outgroup is represented by *Arthrobacter globiformis* SSU rDNA sequence data. Sequences not determined in this study were provided by RDP. Scale bar represents 0.10 fixed mutations per nucleotide position.

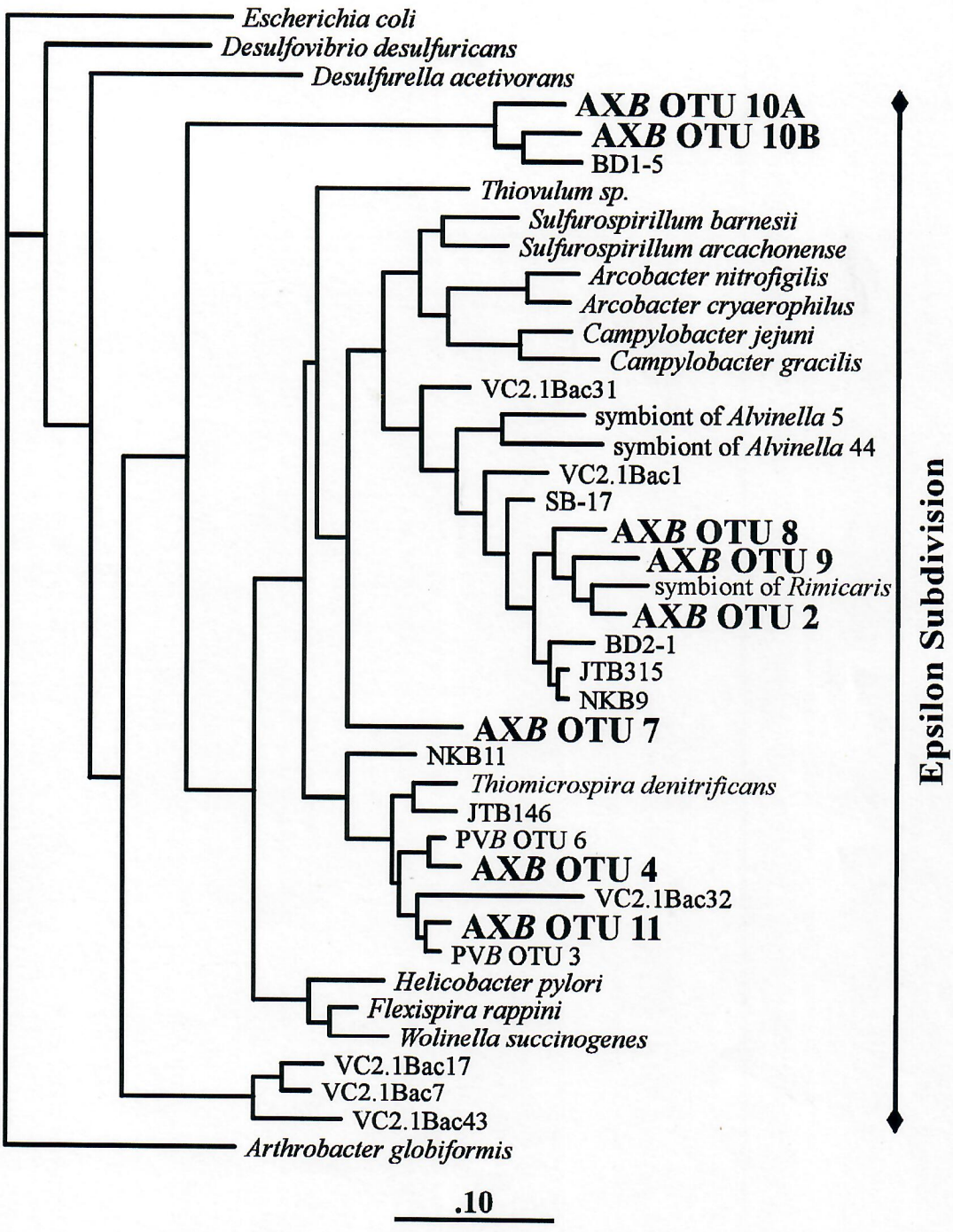

Figure 14. Phylogenetic tree demonstrating the relationships of AX*B* OTUs 2, 4, 7, 8, 9, 10A, 10B, and 11 with members of the ϵ-*Proteobacteria* as determined by maximum likelihood analysis of SSU rDNA sequences. An outgroup is represented by *Arthrobacter globiformis* SSU rDNA sequence data. Sequences not determined in this study were provided by RDP. Scale bar represents 0.10 fixed mutations per nucleotide position.

PV*B* OTU 6. The closest relative to AX*B* OTU 11 was PV*B* OTU 3 and was detected only in Snow Blower's clone library at 5.4% (Table 5). AX*B* OTU 7, which accounted for 10.7% of the clones recovered from Snow Blower, is most closely related to a *Thiovulum* sp. The final AX*B* OTU affiliated with the ϵ-*Proteobacteria,* OTU 10, had two members sequenced and with BD1-5, formed a novel deeply-rooted lineage within the ϵ-*Proteobacteria.* The secondary structures of AX*B* OTUs 10A and 10B are shown in Appendix II. Members of the γ- and δ-*Proteobacteria* were also included in this phylogenetic tree to yield better definition to the ϵ subdivision of the *Proteobacteria.*

DISCUSSION

A microbial community is an assemblage of co-occurring populations of microorganisms interacting at a given location or habitat (Atlas and Bartha, 1998). The goal of this study was to first, examine the community structure, or number of populations in a community and their relative abundance, and second, determine the phylogenetic diversity, or genetic relatedness among the dominant populations from four distinct vent sites at Axial Volcano which erupted approximately seven months prior to our initial sampling. The Axial *Bacteria* (AX*B*) communities were characterized using amplified ribosomal DNA restriction analysis (ARDRA) and phylogenetic sequence analyses.

As with any sampling-detection procedure, there are limitations when using these molecular methods, principally PCR, which may result in over- or under-estimation of taxon diversity. PCR selection (mechanisms that favor the amplification of certain templates), unequal binding of PCR primers to different bacterial groups, or lysis bias may underestimate diversity. PCR drift (stochastic variation when amplification proceeds largely from the genomic templates), or low G+C templates may cause diversity to be overestimated (McCaig *et al.*, 1999; Polz and Cavanaugh, 1998; Suzuki and Giovannoni, 1996; Wagner *et al.*, 1994). In order to minimize PCR selection and drift, several replicate PCR amplifications were combined and fewer cycles of amplification were used in this study. Degeneracies were designed into the primer sequences selected for the amplification of SSU rDNA in order to accommodate the sequence variation seen across the *Bacteria* at those positions (e.g., Lane, 1991).

Generation of chimeras is another potential problem with the ecological application

of SSU rDNA methods. These chimeras are thought to arise stochastically from the interaction of different SSU rRNA genes during PCR (Kopczynski *et al.*, 1994) and usually result when partial length rDNA fragments are present in low-molecular-weight genome DNA preparations (Amann *et al.*, 1995). The formation of chimeras is anticipated during the initial amplification of SSU rDNA (Amann *et al.*, 1995; Kopczynski *et al.*, 1994). To minimize the formation of chimeras, the extracted microbial SSU rDNA was analyzed to confirm a relatively high molecular weight (≥6.5 kb). For this study, all operational taxonomic units (OTUs) containing three or more clones, or two clones found in more than one library, were sequenced and analyzed by the software program CHECK_CHIMERA available from the RDP and no chimeric sequences were discovered. However, it is likely that there were chimeric sequences present in the 132 single occurrence OTUs that were not sequenced because it is extremely improbable that more than two identical chimeras will be formed.

There are also limitations when interpreting the results of molecular data. When the SSU rRNAs are used to estimate diversity, there are biases toward physiologically active microorganisms. When using SSU rDNA as a result of PCR amplifications, genetic diversity can be estimated for potentially slow growing or dormant microorganisms also present in the community (Moyer *et al.*, 1994). Microorganisms may contain multiple copies of the rRNA gene per genome and perhaps their RNA sequences are preferentially selected for in the construction of clone libraries (Farrelly *et al.*, 1995; Ward *et al.*, 1998). However, results from a multi-template PCR study did not support the preferential selection of RNA genes or genomes due to their copy number (Polz and Cavanaugh, 1998). With the application of

ARDRA, aberrant patterns can be produced by a single missing or extra restriction site (Vaneechoutte *et al.*, 1992), which may result in different groupings of single-occurrence OTUs in the cluster analysis performed by the software package GelCompar (Heyndrickx *et al.*, 1996).

Molecular methods were used to characterize the diversity of microbial mats from four sites and fifteen OTUs were detected among 249 bacterial SSU rDNA clones examined. No single phylotype dominated all sites at Axial Volcano. In addition, the most dominant bacterial OTU found in Marker 33, Snow Blower, and North Rift were unique to those sites (Table 2; Table 5). When the cumulative OTU distribution of 249 clones (Figure 8) and the calculated rarefaction curves (Figure 9) were analyzed, no saturation effect was detected, indicating that total organismal diversity was high. However, even with the high diversity detected in the Axial Volcano samples, coverage estimates (39 - 54%; Table 3) suggest that a substantial fraction of the bacterial community was partially revealed and that there were no major biases occurring in the extraction, amplification, and cloning procedures. In addition, the total OTU richness might reflect the potential flexibility within the bacterial community to respond to rapid changes in environmental conditions.

ARDRA has infrequently been used to characterize deep-sea hydrothermal vent bacterial communities and a broad comparison of the trends observed in these studies to characterize other vent communities is as yet premature. However, the collector's curve and rarefaction analysis for PV*B*, shown in Figures 8 and 9, indicate that taxa diversity of the microbial mat collected from Pele's Vents was considerably lower than that detected in any of the four samples collected from Axial Volcano. The bacterial communities collected from

the four sites at Axial Volcano were not dominated by a single OTU to the same degree, as had been previously found at Pele's Vents (Moyer *et al.*, 1994). The most dominant phylotype, which comprised 25% of all clones analyzed at Pele's Vents (Moyer *et al.*, 1994; 1995), has since been isolated and characterized as a lithotrophic, microaerophilic iron-oxidizing bacterium (Emerson and Moyer, 1997). However, there were an abundance of potentially sulfur-oxidizing ϵ-*Proteobacteria* found in the Axial Volcano samples related to *Thiovulum* sp. and *Thiomicrospira denitrificans*, as well as a deeply-rooted lineage demonstrating an ancestral divergence to the cultured and environmental isolates.

Assuming that the SSU rDNA clone libraries are first approximations representing populations from bacterial mat communities, three divisions of the *Bacteria* are dominant throughout the four Axial Volcano sites selected for this study (Figure 3). The first and deepest division detected at Axial was the Green Non-Sulfur *Bacteria* and Relatives, which consists mostly of anoxygenic photosynthetic thermophilic bacteria (Wise *et al.*, 1997). The representatives in culture have a wide range of phenotypes including anoxygenic photosynthesis (*Chloroflexus*) and thermophilic organotrophy (*Thermomicrobium*) (Hugenholtz *et al.*, 1998b). AX*B* OTU 6 (Figure 10) is grouped phylogenetically with the environmental isolates, and not with the majority of the cultured isolates. *Dehalococcoides ethenogenes*, an anaerobe involved in the reductive dechlorination of tetrachloroethene (Maymo-Gatell *et al.*, 1997) clustered with SAR 307, a mesophilic bacterioplankton obtained from an oligotrophic region in the Sargasso Sea (Giovannoni *et al.*, 1996). The group of environmental isolates also included microorganisms collected from Obsidian Pool, Yellowstone National Park (OPT90, OPB11, and OPB65; Hugenholtz *et al.*, 1998b); a

contaminated aquifer in Michigan (WCHB1-31, WCHB1-50; Dojka *et al.*, 1998); marine sediment from the Rhukyu Trench (BD3-16; Li *et al.*, 1999b); hydrocarbon seep sediment (GCA112; O'Neill *et al.*, 2000); sediment collected from a South Carolina bay (RB41; Wise *et al.*, 1997); and activated sludge from a wastewater treatment plant (T78; Snaidr *et al.*, 1997). The environmental isolates (SJA-15 and SJA-68) that were most closely related to AX*B* OTU 6, were detected from an anaerobic trichlorobenzene-transforming microbial consortium from an aquifer contaminated with hydrocarbons and chlorinated solvents (von Wintzingerode *et al.*, 1999). This deep subsurface taxon (AX*B* OTU 6) is most likely growing anaerobically and is possibly responsible for the transformation of halogenated organic compounds, and degradation of hydrocarbons.

The second division detected in Axial Volcano samples is the Flexibacter-Cytophaga-Bacteroides, which has been reclassified several times in recent years (Johansen *et al.*, 1999). Isolates from this division are common members of the bacterial community in coastal regions, especially on the surface of marine benthic macroalgae, where they are involved in the degradation of organic matter (Johansen *et al.*, 1999). Five OTUs in this division detected from Axial Volcano are distributed within the Bacteroides and Cytophaga subdivision (Figure 11). AX*B* OTU 15, detected in low frequencies from Marker 33 Vent and Snow Blower, is most closely related to a microorganism in the Bacteroides group collected from Arctic Ocean sediment anoxic below a depth of ~8 mm (Sva1038; Ravenschlag *et al.*, 1999). The other four OTUs were unique and detected only at North Rift. The most frequently encountered OTU, AX*B* OTU 1, was an outgroup to a lineage of microorganisms which include both cultured and uncultured environmental isolates. The two

environmental isolates are DPT1.3, collected from the Ligurian Sea water column at a depth of 1800 meters (Giuliano *et al.*, 1999) and JTB143, a clone from sediment within the *Calyptogena* clam community in the Japan Trench cold seep (Li *et al.*, 1999c). Two closely related cultured isolates, *Psychroflexus torquis* (Bowman *et al.*, 1998) and *Polaribacter irgensii* (Gosink *et al.*, 1998) are from Antarctic sea ice. Another close relative, *Cytophaga fucicola*, a gliding, aerobic, yellow/orange pigmented bacterium, was isolated from the surface of a marine macroalga in Danish waters (Johansen *et al.*, 1999). AX*B* OTU 3 is most closely affiliated with a clone discovered in activated sludge (BA2; Snaidr *et al.*, 2000). AX*B* OTU 13 clusters with several environmental isolates collected from a Mid-Atlantic Ridge (MAR) *in situ* growth chamber or vent cap (VC2.1Bac22; Reysenbach *et al.*, 2000); a contaminated aquifer in Michigan (WCHB1-69; Dojka *et al.*, 1998); and a dominant clone (1-60) covering a microscopic nematode, which actively moved toward high sulfide concentrations, found in a Bermuda sandflat (Polz *et al.*, 1999). AX*B* OTU 14 groups with environmental clones from an Austrian oil field (R1; Friedrich *et al.*, 2000), two clones associated with a benzene-degrading and sulfate-reducing consortium from Guaymas Basin sediment (SB-1 and SB-5; Phelps *et al.*, 1998), and another clone from the contaminated aquifer in Michigan (WCHB1-32; Dojka *et al.*, 1998). The North Rift bacterial community is completely dominated by members of the Bacteroides and Cytophaga subdivision which were not detected in the other three microbial communities.

The third division present at Axial Volcano is the *Proteobacteria*, of which three subdivisions are found. The first subdivision detected was the γ-*Proteobacteria*, which includes chemolithotrophic sulfur oxidizing and methanotrophic endosymbionts, as well as

free-living methylotrophs, *Beggiatoa* and *Thiomicrospira.* Methylotrophic bacteria are a diverse group of microorganisms whose sole energy sources come from one-carbon compounds more reduced than CO_2. Methanotrophs are a subset of methylotrophs and are only able to grow on methane (Bratina *et al.*, 1992). AX*B* OTU 12 (Figure 12), the only dominant γ-*Proteobacteria* OTU detected from Axial Volcano, was an outgroup to two methanotrophic mussel symbionts found from the MAR (Distel *et al.*, 1995) and the Louisiana Slope (Distel and Cavanaugh, 1994). These symbionts, which exist within specialized cells of the animals' gill epithelia (Distel *et al.*, 1995), provide the hosts with a large amount of their nutritional carbon and energy needs while the host provides necessary substrates such as O_2, H_2S, or CH_4 (Distel and Cavanaugh, 1994). These symbionts are clustered among cultured isolates within the Methylomonas group. *Methylomonas methanica* and strain LW21, both type I methanotrophs, were collected from freshwater sediments in Washington State (Costello and Lindstrom, 1999). *Methylomicrobium pelagicum* is a strict aerobic chemoheterotroph which utilizes methane and methanol (Bowman *et al.*, 1995) and another methylotroph is *Methylobacter luteus* (Bratina *et al.*, 1992). Several species of *Thiomicrospira*, chemolithotrophic sulfur-oxidizers, have been frequently isolated from hydrothermal vent samples (Brinkhoff *et al.*, 1999; Muyzer *et al.*, 1995; Wirsen *et al.*, 1998). However, none of the samples collected from Axial Volcano were dominated by any *Thiomicrospira* spp. In addition, relatives of *Thioploca*, a mixotrophic sulfide oxidizer, and *Beggiatoa*, a filamentous sulfide oxidizer, were not found among the dominant OTUs at Axial Volcano.

The δ-*Proteobacteria* was the second subdivision of *Proteobacteria* discovered at

Axial Volcano. AX*B* OTU 5 (Figure 13), found exclusively in the microbial mat sample collected from Marker 33 Vent, is a member of the Desulfobulbus propionicus assemblage which consists of two closely related lineages. The first lineage includes environmental isolates collected from a contaminated aquifer in Michigan (WCHB1-67; Dojka *et al.*, 1998), sediment from the Japan Trench at a depth of 6379 meters (BD7-15; Li *et al.*, 1999b), and AX*B* OTU 5. Cultured isolates from this lineage include *Desulfofustis glycolicus*, a strict anaerobe (Friedrich *et al.*, 1996) and *Desulfocapsa sulfoexigens*, a mesophilic anaerobe which grows exclusively by the disproportionation of inorganic sulfur compounds (Finster *et al.*, 1998). The second lineage within the Desulfobulbus propionicus assemblage consists of a clone from Nankai Trough sediments at a depth of 3843 meters (NKB13; Li *et al.*, 1999a) as well as two cultured *Desulfobulbus* isolates. *Desulfobulbus propionicus* is a dissimilatory sulfate-reducing bacterium (Devereux *et al.*, 1989) and *Desulfobulbus elongatus* disproportionates sulfur compounds in the presence of ferric hydroxide (Janssen *et al.*, 1996). Disproportionation is an inorganic fermentation in which sulfur compounds with intermediate oxidation states serve as both electron donors and electron acceptors in an energy-generating redox process (Finster *et al.*, 1998).

The third subdivision of the *Proteobacteria* identified and most abundant within samples collected from within the caldera of Axial Volcano is the ϵ-*Proteobacteria*. Seven AX*B* OTUs (Figure 14) clustered into four different phylogenetic groups. The first group, containing AX*B* OTUs 4 and 11, also contained two Pele's Vent *Bacteria* clones (PV*B* OTUs 3 and 6; Moyer *et al.*, 1995), a clone from the vent cap community collected from MAR (VC2.1Bac32; Reysenbach *et al.*, 2000), clones collected from the Japan Trench

(JTB146; Li *et al.*, 1999c), clones from Nankai Trough sediment within a *Calyptogena* clam community (NKB11; Li *et al.*, 1999a), and *Thiomicrospira denitrificans*, a misnamed strict anaerobe which grows as an obligate chemolithotroph by the oxidation of sulfide and thiosulfate (Muyzer *et al.*, 1995; Ruby *et al.*, 1981). The second group includes environmental isolates from the MAR vent cap (VC2.1Bacs1 and 31; Reysenbach *et al.*, 2000); symbionts of *Alvinella pompejana*, polychaetous annelids that colonize walls of actively venting high temperature chimneys along the East Pacific Rise vent field (Haddad *et al.*, 1995); a clone associated with a benzene-degrading and sulfate-reducing consortium, possibly scavenging for hydrogen after the fermentation process, collected from Guaymas Basin sediment (SB-17; Phelps *et al.*, 1998); an ectosymbiont of *Rimicaris exoculata*, a shrimp dominating the macrofaunal community at MAR hydrothermal vents (Polz and Cavanaugh, 1995); an epibiont-related clone from Suruga Bay sediment (BD2-1; Li *et al.*, 1999b); another member from the bacterial community from Japan Trench sediment (JTB315; Li *et al.*, 1999c); three clones from sediment collected from the Nankai Trough (NKB9; Li *et al.*, 1999a); and AX*B* OTUs 2, 8, and 9. The third group is AX*B* OTU 7 which grouped as an ancestor to the symbionts and cultured isolates, and is closely related to *Thiovulum* sp., a mesophilic obligate chemolithoautotroph which grows in veils and webs held together by a polysaccharide matrix (Jorgensen and Revsbech, 1983). The fourth group consisted of AX*B* OTUs 10A and 10B, along with a clone detected from Suruga Bay sediment (BD1-5; Li *et al.*, 1999b), and is deeply-rooted to members of the ϵ-*Proteobacteria*. AX*B* OTUs 10A and 10B are clones incorporated into the same OTU based on ARDRA. Two clones from this OTU were sequenced and included in the phylogenetic

analysis due to their high level of dissimilarity to the other ϵ-*Proteobacteria* AX*B* OTUs and to their exceptionally deep-rooted phylogenetic positioning. Another deep branch of the ϵ-*Proteobacteria* was found at MAR hydrothermal vents which included VC2.1Bac7, 17, and 43 (Reysenbach *et al.*, 2000). Overall, ϵ-*Proteobacteria* OTUs consisted of 14.5% of the total number of clones found at Axial Volcano.

Although physiology cannot be directly assumed from phylogeny, in many cases the physiology can be cautiously inferred from the phylogenetic results. Potentially important metabolisms of AX*B* phylotypes are indicated in Table 6. For the Marker 33 Vent community, the clustering of AX*B* OTUs 2, 8, and 9 (14.8%; Table 5) with the ectosymbionts of *Rimicaris exoculata* (Polz and Cavanaugh, 1995), which are chemolithotrophic sulfur oxidizers, provides evidence that sulfur oxidation is important. Another indication that sulfur oxidation is potentially occurring was the presence of AX*B* OTU 4 (1.2%), a close relative of the sulfur-oxidizing *Thiomicrospira denitrificans* (Muyzer *et al.*, 1995). The dominance of sulfur-oxidizing microorganisms is most likely driven by the high concentration of H_2S (ca. 1000 μM; Table 1). The white color of the microbial mat is also indicative of the presence of elemental sulfur and sulfur compounds. In addition, the abundance of AX*B* OTU 5 (8.6%), which is closely related to *Desulfocapsa sulfoexigens* (Finster *et al.*, 1998) and was recovered solely from this microbial mat, serves as evidence that the chemolithotrophic process of sulfur disproportionation is potentially occurring in this community. There was also a member of the Flexibacter-Cytophaga-Bacteroides division (AX*B* OTU 15; 1.2%) indicating that additional modes of metabolism, such as chemoorganotrophy, may be present. All ϵ-*Proteobacteria* phylotypes found at

Table 6. Summary table including phylogenetic subdivisions, potentially important metabolisms, and OTU distribution for Axial Volcano *Bacteria* phylotypes. Colored boxes indicate the presence of at least one clone from a particular OTU at that site. Dark-colored boxes refer to the most dominant OTU at that site. Diamonds in center of box represent an OTU unique to that particular site.

Phylotypes	Phylogenetic Subdivisions	Potentially Important Metabolisms	Sample Sites			
			M33	SB	EZ	NR
OTU 1	Bacteroides-Cytophaga	chemoorganotrophy				◆
OTU 2	ϵ-*Proteobacteria*	sulfur oxidation				
OTU 3	Bacteroides-Cytophaga	chemoorganotrophy				◆
OTU 4	ϵ-*Proteobacteria*	sulfur oxidation				
OTU 5	δ-*Proteobacteria*	sulfur disproportionation	◆			
OTU 6	Chloroflexus	possible transformation of halogenated organics and hydrocarbon degradation		◆		
OTU 7	ϵ-*Proteobacteria*	sulfur oxidation		◆		
OTU 8	ϵ-*Proteobacteria*	sulfur oxidation				
OTU 9	ϵ-*Proteobacteria*	sulfur oxidation				
OTU 10	ϵ-*Proteobacteria*	sulfur oxidation				
OTU 11	ϵ-*Proteobacteria*	sulfur oxidation		◆		
OTU 12	γ-*Proteobacteria*	methylotrophy and possibly methanotrophy			◆	
OTU 13	Bacteroides-Cytophaga	chemoorganotrophy				◆
OTU 14	Bacteroides-Cytophaga	chemoorganotrophy				◆
OTU 15	Bacteroides-Cytophaga	chemoorganotrophy				

Marker 33 Vent were also detected at other new lava vent sites. AX*B* OTUs 2 and 9 were also detected at Easy Vent and AX*B* OTUs 4 and 10 were found at the Snow Blower Vent site. The member of the Bacteroides and Cytophaga subdivision (AX*B* OTU 15) present at Marker 33 Vent was also found at the Snow Blower Vent.

Sulfur oxidation is also indicated in the Snow Blower floc-ejecta by the abundance of (i) AX*B* OTU 7 (10.7%; Table 5), which is closely related to the *Thiovulum* sp. (Jorgensen and Revsbech, 1983), (ii) AX*B* OTU 8 (1.8%), a relative of the ectosymbiont of *Rimicaris exoculata* (Polz and Cavanaugh, 1995), and (iii) AX*B* OTUs 4 and 11 (9.0%), as close relatives of *Thiomicrospira denitrificans* (Muyzer *et al.*, 1995). Two members of the ϵ-*Proteobacteria*, AX*B* OTUs 7 and 11, were unique to this library, while AX*B* OTUs 4 and 8 were also found in the bacterial mat collected from Marker 33 Vent. AX*B* OTU 6 (12.5%), detected only in this bacterial community, is a member of the Green Non-Sulfur *Bacteria* and Relatives, a deeply-rooted branch in the domain *Bacteria*. This group exhibits a variety of physiological differences and the closest relative in culture is *Dehalococcoides ethenogenes* (Maymo-Gatell *et al.*, 1997). OTU 6 is most likely an anaerobic microorganism, possibly originating in the deep subsurface. In addition, the two clones sequenced as subsets of AX*B* OTU 10, detected at Marker 33 (AX*B* OTU 10A; 1.2%) as well as Snow Blower (AX*B* OTU 10B; 3.6%) Vents, are deeply-rooted ancestors to both cultured isolates as well as environmental isolates collected from the Atlantic and Pacific Oceans. AX*B* OTU 15, which made up 1.8% of the sequenced OTUs from Snow Blower and was also detected at Marker 33 Vent (1.2%), indicated that members of the Bacteroides and Cytophaga subdivision, representing potential heterotrophs, were also present in these microbial

communities.

The abundance of AX*B* OTUs 2, 4, and 9 (14.3%; Table 5) located within the ϵ-*Proteobacteria* is an indication that sulfur oxidation may also be an important process in the Easy Vent bacterial community since close relatives of the ectosymbiont of *Rimicaris exoculata* (Pulz and Cavanaugh, 1995) as well as *Thiomicrosprira denitrificans* (Muyzer *et al.*, 1995) were detected. AX*B* OTU 12 (5.4%), which was only found at this site, clustered with methylotrophs, providing evidence that methylotrophy and perhaps methanotrophy is an important mode of metabolism here as well. All of the ϵ-*Proteobacteria* found at this location were present in samples collected from either Marker 33 Vent or Snow Blower. AX*B* OTU 4 was found at both Marker 33 and Snow Blower, while AX*B* OTUs 2 and 9 were present at the Marker 33 Vent site, indicating once more that sulfur oxidation was important in the new lava area of Axial Caldera after a recent eruption. The dark orange color of this microbial mat was also indicative of the presence of iron compounds when viewed microscopically (data not shown). This site had the highest measurable presence of iron compounds among new lava vent sites (ca. 90 μM; Table 1), however no iron-oxidizers were detected among the sequenced phylotypes.

The OTUs sequenced from the orange microbial mat located at the North Rift Vent site were all unique to that site and were all contained within the Bacteroides and Cytophaga subdivision. The most dominant OTU, AX*B* OTU 1 (17.9%; Table 5) was deeply-rooted to many physiologically disparate microorganisms including those collected from Antarctic sea ice, the Mediterranean Sea water column, the surface of an alga, and marine sediment (Bowman *et al.*, 1998; Gosink *et al.*, 1998; Giuliano *et al.*, 1999; Johansen *et al.*, 1999; Li

et al., 1999c). The physiology of AX*B* OTU 1 cannot be inferred beyond potential chemoorganotrophy based on phylogeny. There were no cultured isolates phylogenetically similar to AX*B* OTU 13 (5.4%), however a similar clone was detected from a MAR vent cap community, indicating the presence of this lineage in both the Atlantic and Pacific Oceans (Reysenbach *et al.*, 2000). The remaining OTU sequenced from the North Rift community was AX*B* OTU 14 (5.4%) and again cannot be physiologically described since there are no related cultured isolates. However, the similarity of this OTU to clones detected in a contaminated aquifer, an oil field, and a sulfate-reducing consortium from Guaymas Basin sediment suggests a cosmopolitan distribution of this OTU in various habitats (Dojka *et al.*, 1998; Friedrich *et al.*, 2000; Phelps *et al.*, 1998). Secondary production by heterotrophic microorganisms is potentially occurring in this unusual bright orange microbial mat growing on top of white microbial mat surrounded by macrofauna at this site. The combination of greater depth, lower venting temperature, lesser amount of bioenergetically available compounds, and location on an old lava flow north of the caldera could be responsible for the unique microbial community at this vent site (Table 1).

Molecular-based methods were used to study community structure and diversity of bacterial communities from Axial Volcano. It was determined that organismal diversity of the four hydrothermal vent microbial mats at Axial Volcano was relatively high and only partially revealed, as determined by rarefaction and coverage estimates. The ϵ-*Proteobacteria* found at Axial Volcano comprised an extremely phylogenetically diverse group of related phylotypes. The proximity of vent locations occurring on new lava, and chemistry of the vent fluid could be responsible for the abundance of ϵ-*Proteobacteria* inside the Axial

Caldera. The majority of AX*B* OTUs were found in this subdivision, spanning both inside and outside of the *Thiovulum* sp. and *Thiomicrospira denitrificans* lineages, which have now been detected in several hydrothermal vent habitats. Symbiotic bacteria on the carapace and extremities of the shrimp *Rimicaris exoculata* and free-living communities were isolated from the Snake Pit Vent site on the Mid-Atlantic Ridge (Polz and Cavanaugh, 1995; Reysenbach *et al.*, 2000). *Alvinella pompejana* epibionts were detected from the Elsa Vent site 13°N on the East Pacific Rise (Haddad *et al.*, 1995). The majority of the OTUs found at hydrothermal vents within Loihi Volcano near the Hawaiian islands were ϵ-*Proteobacteria* (Moyer *et al.*, 1994; 1995). In addition, hydrothermal vents in Guaymas Basin, Mexico have yielded several OTUs closely related to the *Rimicaris exoculata* symbiont (Kleinkauf, 2000). OTUs detected from Axial Volcano on the Juan de Fuca Ridge further expand the known cosmopolitan distribution and ecological importance of ϵ-*Proteobacteria* at deep sea hydrothermal vent habitats. The rapid changes in heat flux output and composition of hydrothermal fluids following volcanic eruptive events may be directly responsible for microbiological production at hydrothermal vents.

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APPENDIX I

Coverage Equation:

$$C = \frac{N - s}{N}$$

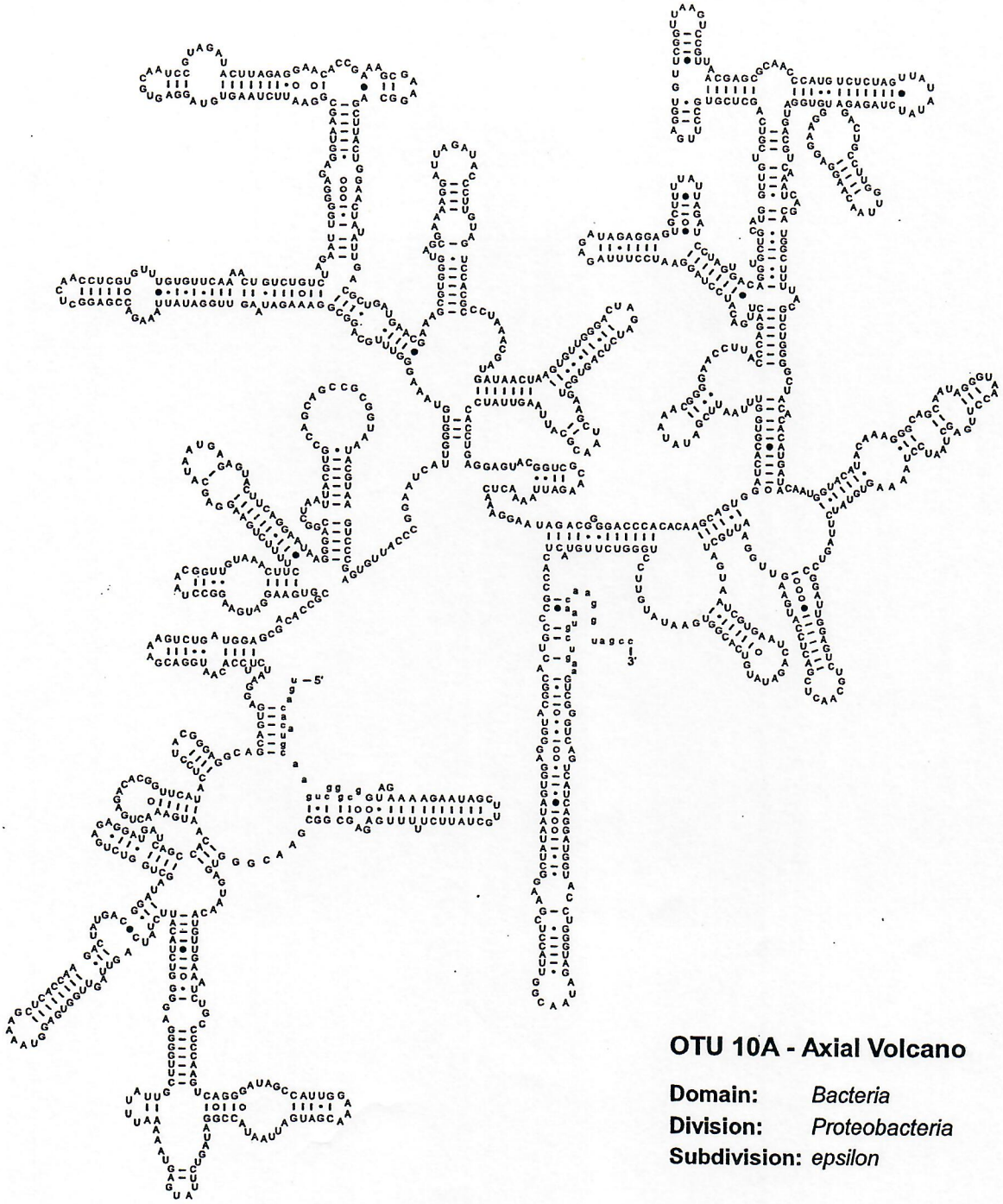
Where N = number of clones examined and s = number of single-occurrence OTUs within a library.

APPENDIX II

SSU rRNA SECONDARY STRUCTURE MODELS

The following pages constitute SSU rRNA secondary structure models for AX*B* OTUs 10A and 10B.

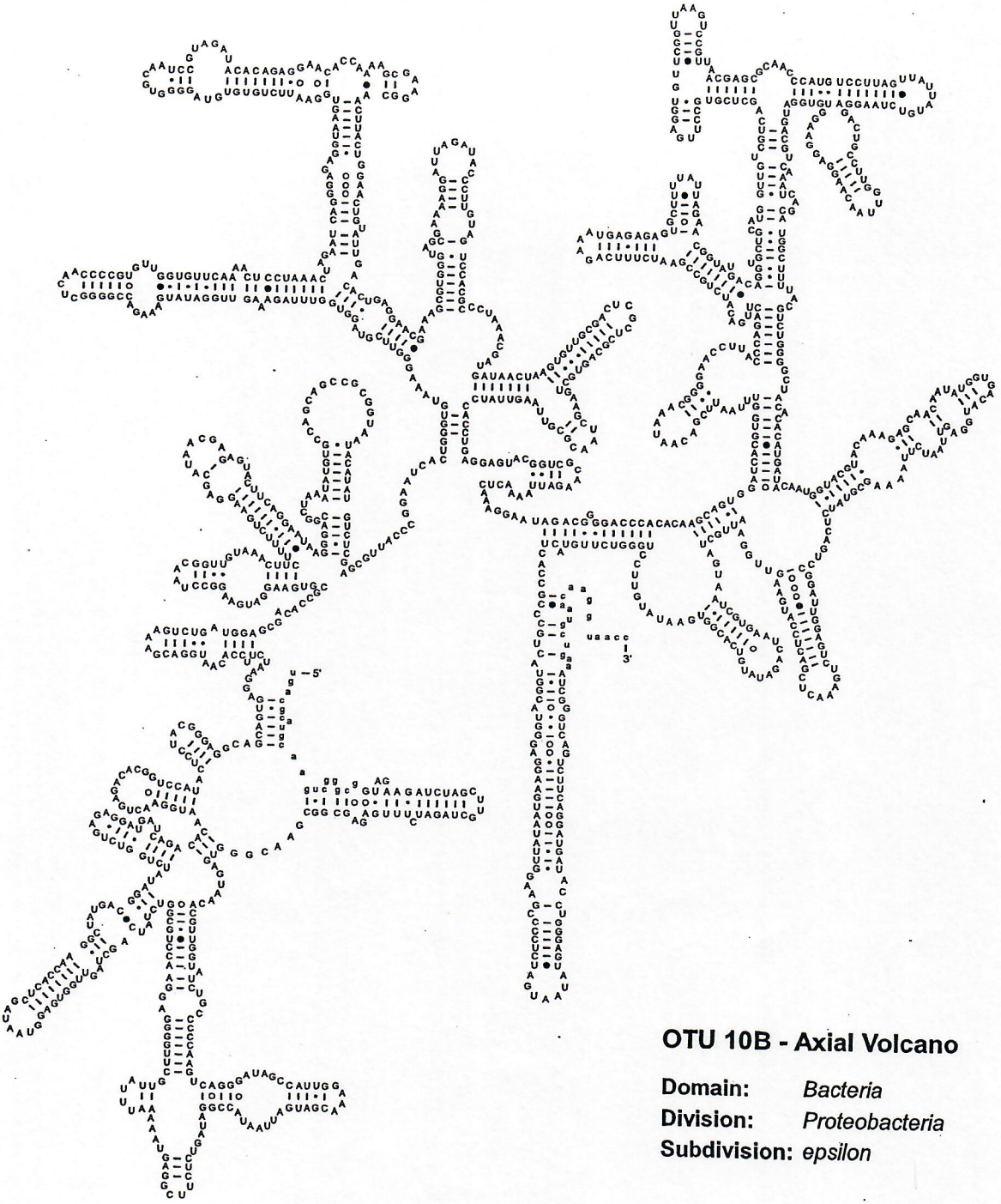
Secondary Structure: small subunit ribosomal RNA



OTU 10A - Axial Volcano

Domain: *Bacteria*
Division: *Proteobacteria*
Subdivision: *epsilon*

Secondary Structure: small subunit ribosomal RNA



OTU 10B - Axial Volcano

Domain: *Bacteria*
Division: *Proteobacteria*
Subdivision: *epsilon*