Molecular Evidence for Microbially-Mediated Sulfur Cycling in the Deep Subsurface of the Witwatersrand Basin, South Africa

Leah Morgan
Senior Integrative Exercise
March 10, 2004

Submitted in partial fulfillment of the requirements for a Bachelor of Arts degree from Carleton College, Northfield, Minnesota.

Table of Contents

Molecular Evidence for Biologically-Mediated Sulfur Cycling in the Deep Subsurface of the Witwatersrand Basin, South Africa

Leah Morgan
Carleton College
Senior Integrative Exercise
March 10, 2004

Advisors:
Professor Jennifer L. Macalady, Carleton College
Thomas M. Gihring, Pacific Northwest National Laboratory

Abstract

The continental deep subsurface harbors a heterogeneous community of microorganisms that have yet to be well understood. The gold mines of the Witwatersrand Basin in South Africa provide economically feasible access to this environment. Molecular evidence for biologically-mediated sulfur cycling has been detected in fissure water in the Merriespruit mine and in groundwater from a dolomite aquifer in the Driefontein mine. PCR amplification, cloning, and sequencing of adenosine-5'-phosphosulfate reductase (APS) and 16S rRNA genes were used to assess the composition and diversity of sulfur-metabolizing microbial populations. Both APS reductase and 16S bacterial gene libraries from the Driefontein mine sample were dominated by sequences with high identity to *Desulfomicrobium baculatum*. The 16S archaeal library from Driefontein had a high percentage of sequences closely related to *Methanomicrobium mazei* and the ANME group of anaerobic methanotrophs. The 16S bacterial library from the Merriespruit mine sample was dominated by *Thiobacillus*-related species known to oxidize sulfur. The archaeal library showed a strong affiliation with *Methanobacterium curvum*. Most APS sequences from Merriespruit were from an undefined lineage but showed close association with clones from the mouse gastrointestinal tract; others were closely related to the sulfur-oxidizing bacterium *Allochromatium vinosum*. This study reports the first sulfate-reducing □-*Proteobacterial* sequences from the South African gold mines and greatly increases the known diversity of subsurface sulfur-metabolizing organisms. Geochemical data are consistent with molecular evidence for deep-subsurface sulfur cycling.

Keywords: biodiversity, biogeochemical cycles, geomicrobiology, subsurface, phylogeny, sulfur, Witwatersrand Supergroup, Transvaal Supergroup

Introduction

Microbial Investigations

As many as $5x10^{30}$ bacterial and archaeal organisms are believed to be currently living on Earth and contain as much carbon as all plant life combined. These microorganisms play significant roles in the cycling of carbon, sulfur, hydrogen, and other elements important to environmental and geological systems. Many microbes inhabit deep oceanic and subsurface environments that scientists are just beginning to investigate thoroughly (Madigan et al., 2003).

Until recently, standard microbial investigations have involved the culturing and isolation of novel species from environmental samples. Using this method, researchers subject a community of microbes to specific temperature, pH, redox, and nutrient conditions in order to determine the ideal growth situation for a particular species under which no other organisms can thrive. However, recent studies have shown that only 1% of all microorganisms are culturable using current enrichment techniques, leaving 99% of microbial diversity unexplored by these isolation methods (Hugenholtz, 2002).

New molecular techniques have bypassed these boundaries by allowing researchers to work directly with DNA extracted from environmental samples. Extracted DNA is subjected to a polymerase chain reaction (PCR), which creates many copies of a small section of the genome that codes for a particular function (a gene). Most work in this area has focused on the gene that codes for the 16S ribosomal RNA (rRNA) because it is highly conserved across the three domains of life: Bacteria, Archaea, and Eukarya (Fig. 1). This gene is therefore useful in determining the evolutionary history of organisms that are present in an environmental sample (Lane et al., 1985; Pace, 1997).

However, the 16S rRNA gene does have limitations. Although one might infer that closely related species are likely to have similar metabolic and physiological characteristics, the 16S rRNA gene cannot be used to definitively identify the metabolic capabilities involved in elemental cycling. To determine the occurrence and phylogeny of uncultivated

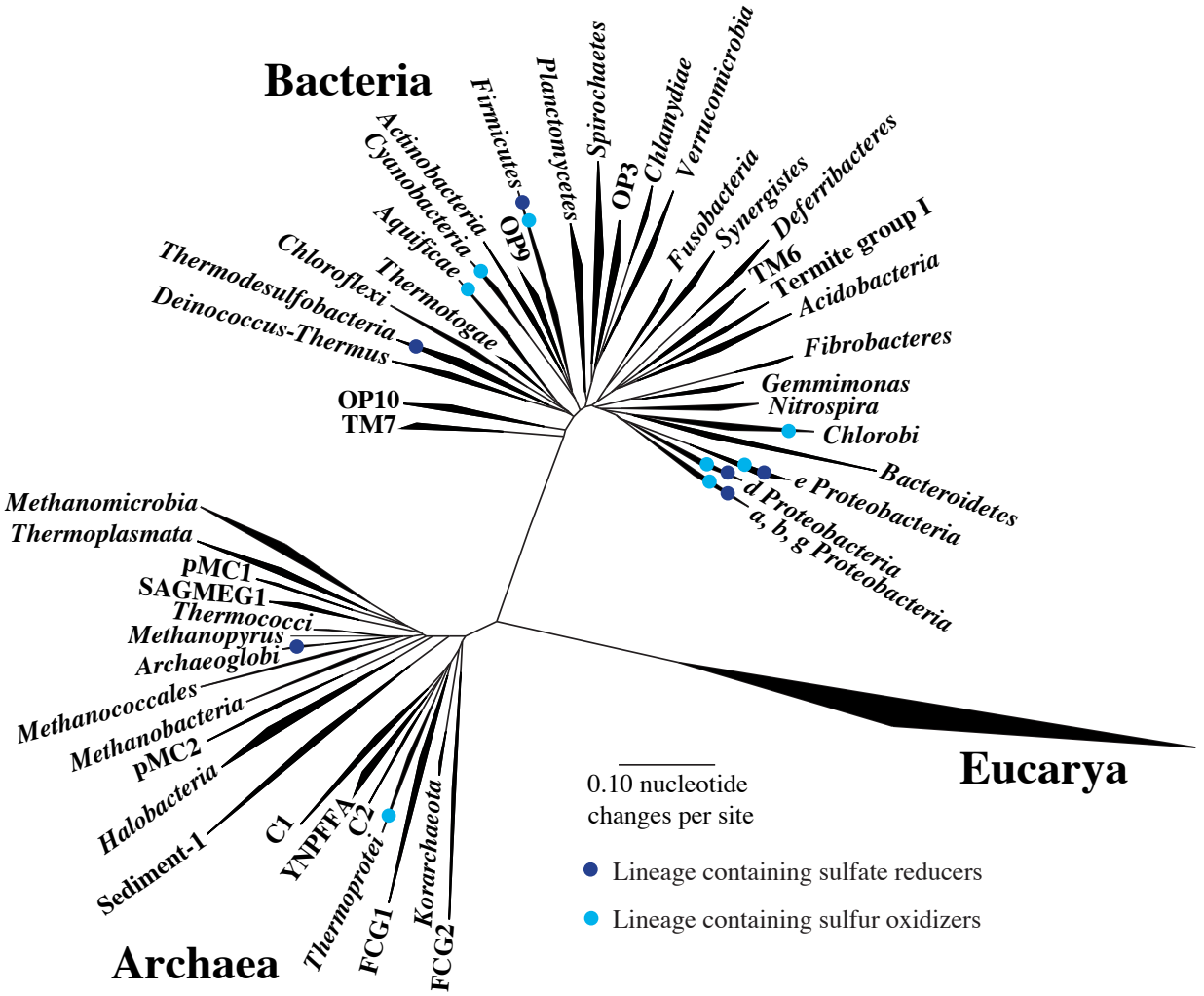


Figure 1: The three domains of life, according to 16S rRNA gene phylogeny. Lineages containing sulfate reducers and sulfur oxidizers are marked with circles. Tree modified from Macalady and Banfield, 2003.

organisms responsible for particular geochemical transformations, one must look for genes that code for proteins required for a particular metabolic pathway. Although specific protein-coding genes may not be present in all organisms within a community, their study gives insight into specific processes occurring in an environment.

Biological Sulfate Reduction and Sulfur Oxidation

Certain microbes harness energy for life processes by changing the redox state of sulfur compounds. Anaerobic sulfate-reducing prokaryotes (SRP) pass electrons from H_2 and organic acids to sulfate (SO_4^{2-}), producing sulfide (H_2S), elemental sulfur (S°), pyrite (FeS_2), and other reduced sulfur compounds (Fig. 2a). Culturable SRP are found in several phyla across the Bacterial and Archaeal domains, including *δ-, δ-, δ-, and δ-Proteobacteria, Firmicutes, Thermodesulfobacteria*, and *Archaeoglobi* (Fig. 1). Sulfur-oxidizing prokaryotes (SOP) pass electrons from reduced sulfur compounds to electron acceptors such as nitrate (NO_3) or oxygen (O_2) and produce sulfate as a byproduct (Fig. 2b) (Madigan et al., 2003). SOP are found across an even wider range of Bacterial and Archaeal lineages, including the *α-, β-, γ-, δ-, and ε-Proteobacteria, Chlorobi, Firmicutes, Cyanobacteria, Aquificae,* and *Thermoprotei*. (Fig. 1) (Friedrich, 1998; Hipp et al., 1997; Madigan et al., 2003).

Sulfate reduction follows one of two pathways. The assimilative pathway results in the formation and assimilation of organic sulfur-bearing compounds such as amino acids. The dissimilative pathway results in the excretion of reduced, inorganic sulfur compounds (Fig. 2a). Sulfur oxidation can also occur on two pathways. One pathway involves the direct oxidation of sulfite to sulfate, while the other involves the creation of the intermediate compound adenosine-5'-phosphosulfate and its subsequent oxidation to sulfite (Fig. 2b) (Madigan et al., 2003). These biogenic pathways involved in the sulfur cycle may give insight into Earth's earliest life forms because they are believed to have developed prior to

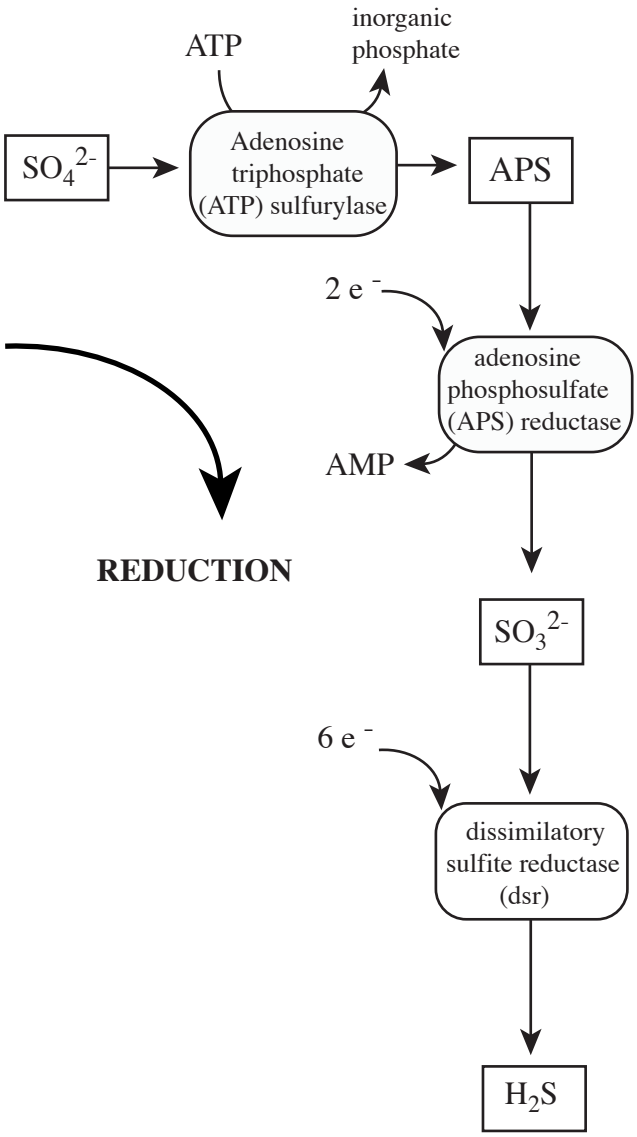


Figure 2a: The biochemical sulfate reduction pathway that includes APS reductase. Enzymes are shown in bubbles. Modified from Madigan, et al., 2003.

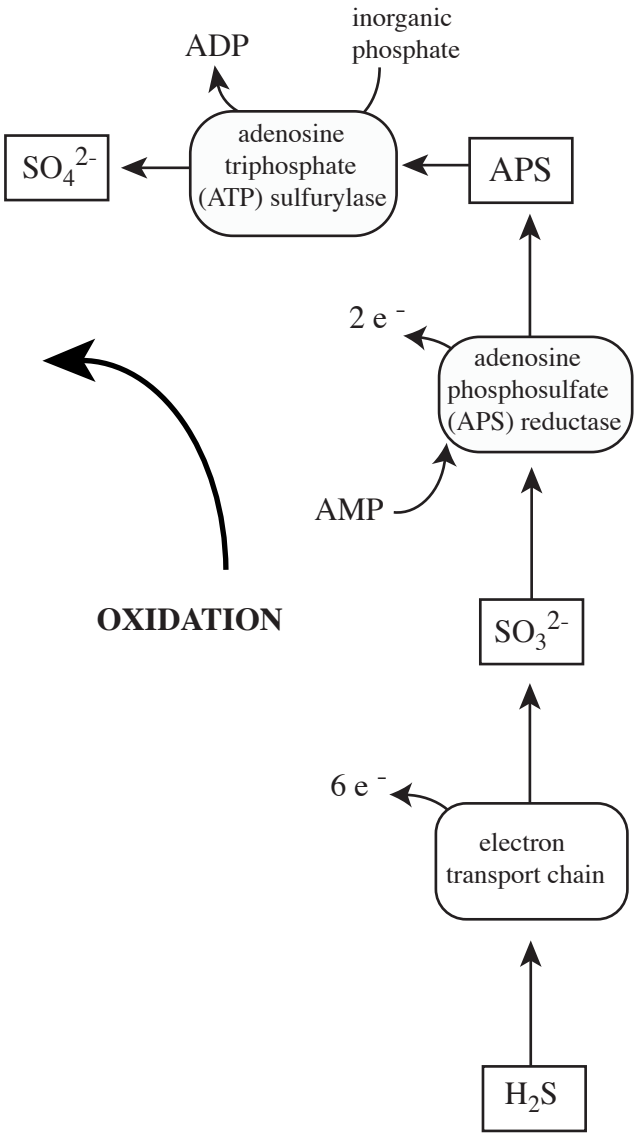


Figure 2b: The biochemical sulfur oxidation pathway that includes APS reductase. Enzymes are shown in bubbles. Modified from Madigan, et al., 2003.

both the development of photosynthesis and the divergence of the Archaeal and Bacterial domains (Canfield et al., 2000; Friedrich, 2002; Hipp et al., 1997; Wagner et al., 1998).

Adenosine-5'-phosphosulfate reductase (APS) is an enzyme on both the dissimilative sulfate reduction and the multi-step sulfur oxidation pathways and can catalyze both oxidative and reductive reactions between sulfate and sulfite (Fig. 2). The APS gene coding for this enzyme has been found in most cultured SRP (Friedrich, 2002) and several cultured SOP, including *Allochromatium vinosum* and *Thiobacillus denitrificans* (Fig. 1) (Hipp et al., 1997). Friedrich (2002) provides evidence for lateral gene transfer of the APS reductase gene between gram-positive and thermophilic lineages of SRP and suggests that APS gene phylogenies should be evaluated accordingly, as gene phylogenies may not accurately reflect organismal phylogenies.

Previous molecular work by Baker et al. (2003) on 16S rRNA and dissimilatory sulfite reductase (dsrAB) (Fig. 2a) genes has confirmed the presence of SRP closely related to *Desulfotomaculum* (*Firmicutes*) in fissure water of the Witwatersrand Supergroup. This study combines the use of the 16S rRNA and APS genes to investigate microbial diversity and sulfur cycling in the deep subsurface of the Witwatersrand Basin in South Africa. This thesis marks the first attempt to obtain and analyze APS gene sequences from deep subsurface samples collected in the South African gold mines.

Geological Setting

The Witwatersrand Supergroup, a 7 km thick gold-bearing quartzite conglomerate with interbedded carbon-rich layers, was deposited in the Witwatersrand Basin of South Africa between 3000 and 2700 Ma (Fig. 3). This supergroup overlies a 3400 Ma granite-greenstone basement complex. Sediments in the Witwatersrand are believed to be sourced from the paleo-Murchison Greenstone Range east of the basin (Fig. 3a) (Viljoen and Reimold, 2002). Subsequent greenschist to amphibolite facies metamorphism transformed the Witwatersrand sediments into a quartzite conglomerate. A debate still exists as to

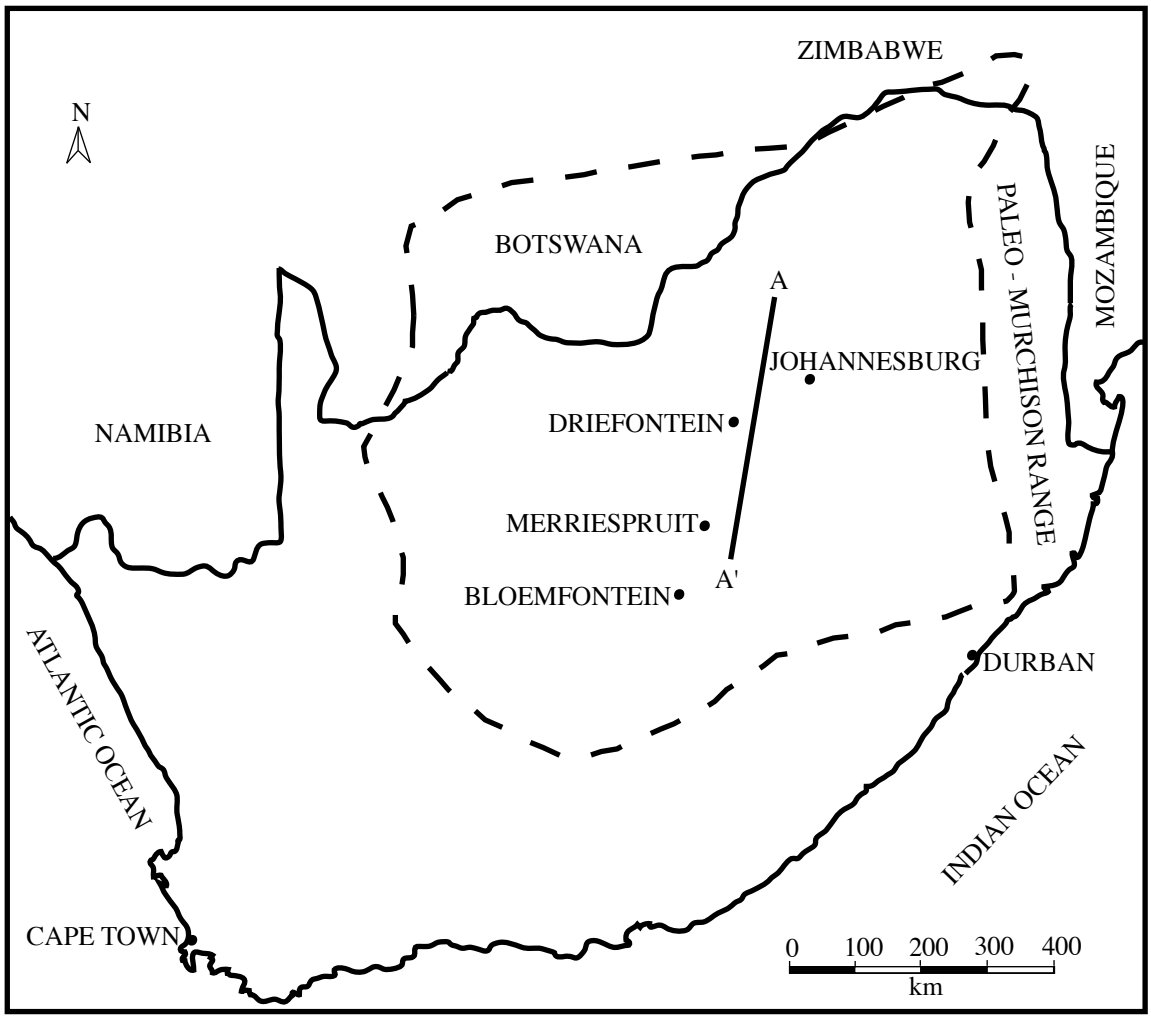


Figure 3a: Map of South Africa showing sampling locations and major cities. Line A - A' refers to cross section in figure 3b. Location of paleo-Witwatersrand Basin is shown with dashed lines. Modified from Viljoen and Reimold, 2002.

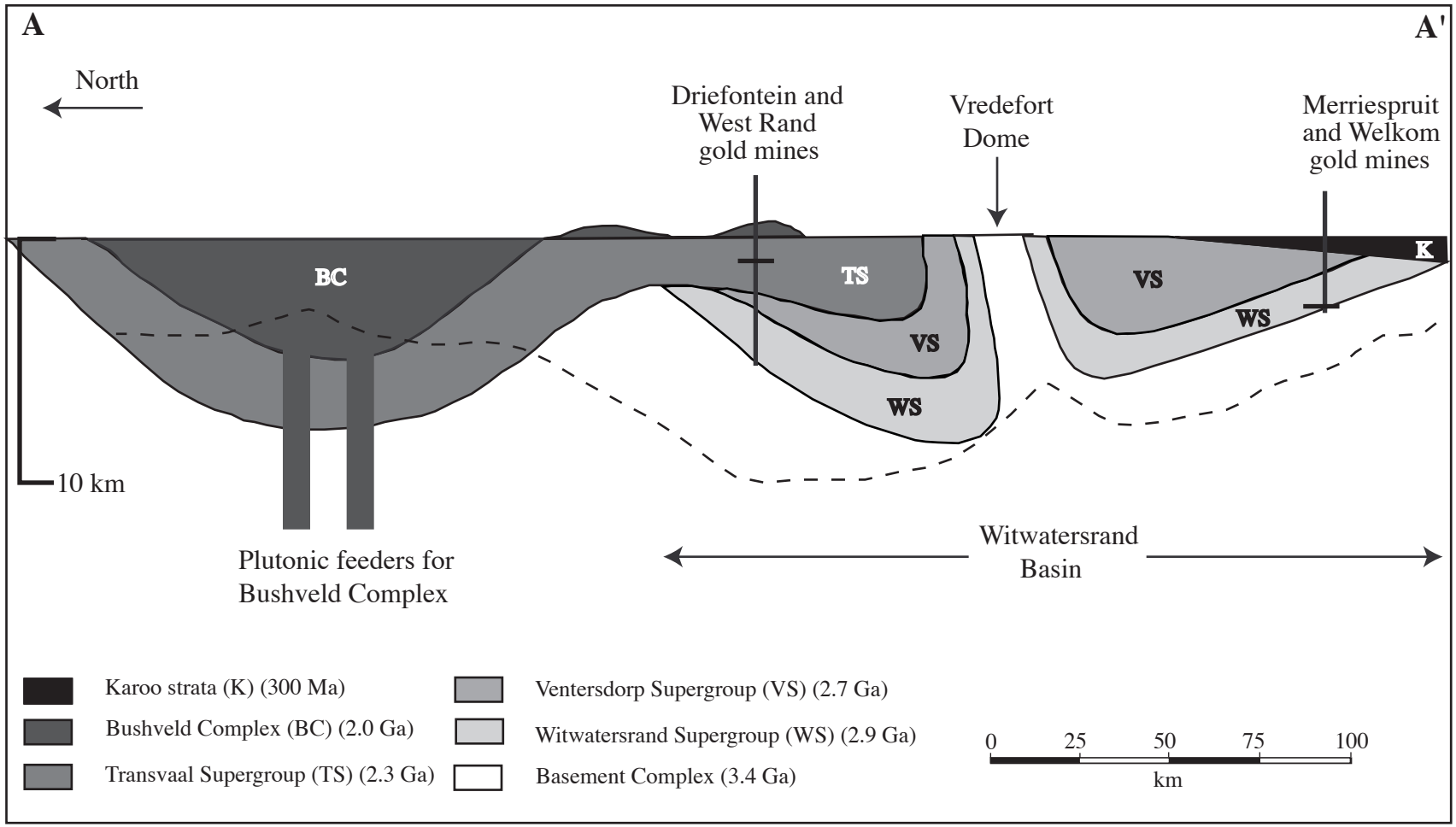


Figure 3b: Cross-section of Witwatersrand Basin showing sampling locations and geological formations. Dashed line represents isogeotherm for 120°C. Horizontal lines in Driefontein and Merriespruit mine shafts indicate sampling depths. Cross-section line A - A' is shown on figure 3a. Modified from Onstott, unpublished.

whether gold, uraninite, pyrite, and hydrocarbons were derived from original sediments or whether they migrated into the basin with hydrothermal fluids during metamorphism (Drennan et al., 1999). The Witwatersrand Supergroup is overlain by the Ventersdorp lavas in the south and the stromatolitic Malmani dolomite formation of the Transvaal Supergroup (2600-2250 Ma) in the north. The Vredefort meterorite impact at 2020 Ma created the Vredefort Dome structure (Fig. 3b) (Viljoen and Reimold, 2002). Dikes of the Pilansberg Complex (1400 Ma) compartmentalize meteoric groundwater in the Malmani aquifer and fissure water in the Witwatersrand Supergroup. Water in these formations has residence times of 13 kyr and 1-123 Myr, respectively (Lippmann et al., 2003; Omar et al., 2003; Onstott, 2004a).

The Witwatersrand Supergroup has been mined for gold since the 1880's and has produced nearly half of all gold ever mined in the world (Viljoen and Reimold, 2002). The deep shafts and tunnels dug by the mining industry (Fig. 3b) have created an opportunity for microbiologists and geochemists to easily sample fissure water from the deep subsurface, where microorganisms are believed to live chemolithotrophically on the energy from redox disequilibria (Onstott et al., 2003). The high temperatures and pressures of the deep subsurface have been proposed as analogs for early terrestrial and extraterrestrial conditions (Teske et al., 2003). The gold mines in South Africa are among the deepest in the world and provide relatively easy access to the subsurface while avoiding many of the high costs of drilling and reaching even deeper depths (>4km). Since 1997, a group led by Tullis C. Onstott of Princeton University and funded by the Life in Extreme Environments (LExEn) program of the National Science Foundation has been sampling and investigating fissure water from the gold mines in South Africa (Baker et al., 2003; Lippmann et al., 2003; Moser et al., 2003; Onstott et al., 2003; Takai et al., 2001). An extensive geochemical and microbial database has been compiled, but many questions regarding microbial metabolism, spatial distribution, and phylogeny remain unanswered.

Methods

Sampling

Water samples were obtained from exploratory boreholes that intercept subsurface groundwater and fissure water, accessed through the ultra-deep (>4 km) gold mines of the Witwatersrand Basin in South Africa. Samples reported in this study were collected at the Driefontein and Merriespruit gold mines. The Driefontein sample (DR9IPCH1101602; referred to here as VV) was collected at a depth of 0.9 km below surface (kmbls) within the groundwater aquifer of the Malmani dolomite formation of the Transvaal Supergroup. The Merriespruit sample (MS149062003BH1; referred to here as MS) was collected at a depth of 1.998 kmbls from isolated fissure water in the quartzite conglomerates of the Witwatersrand Supergroup (Fig. 3).

Samples were obtained aseptically and anaerobically with the use of a packer, which is placed into the boreholes. A seal on the packer forces all water through the packer and into sterile sampling equipment, preventing contact with mine air. An autoclaved telescoping packer constructed of Delran plastic and equipped with quick-connect release valves attached to sterile Tygon tubing with a syringe tip was used to collect molecular and geochemical samples. Water for molecular analysis was sampled aseptically and anaerobically and stored in 12 L canisters for transport to the lab, where the canisters were vacuum-pumped through Gelman Supor Polysulfone filters (0.2 μm Pall Corp., East Hills, NY) to collect cellular material. Dissolved anion, cation, and total Fe samples were filtered (22 μm Nylon Acrodisk, Gelman) into 15 mL disposable centrifuge tubes (Falcon). The centrifuge tubes for the cation and total Fe samples were acid washed and preloaded with 2.5 mL concentrated HNO_3^- and 2 ml concentrated HCl, respectively. Samples for dissolved NH_3 were filtered into 120 ml serum vials, preserved with 50 μl concentrated H_2SO_4 and crimp-sealed without head space. Unfiltered samples included total NH_3 (20 to 120 ml serum vials, preserved with 50 μl conc. H_2SO_4 and crimp sealed without head space), and sulfide species (45 ml amber glass vials, preloaded with 500 μl of 2 M Zn

acetate and crimp sealed without head space). The anion and phosphate sample bottles were only partially filled, frozen immediately upon return to the field lab and kept frozen until analysis. The sulfide species samples were split into two serum vials in an anaerobic glove bag with 10%H_2/90%N_2 gas mixture, crimp sealed and frozen until analysis (Moser et al., 2003).

Geochemical field measurements included pH, temperature, Eh (mV), conductivity (mS), O_2 (0.1-1,1-10 ppm), total Fe (1-10 ppm), sulfide (0.1-10 ppm), and NH_3 (1-10 ppm), using the pH, temperature, and conductivity probe and Eh probe HI98201 (Hanna Instruments, Woonsocket, RI) and Chemet Kits K-7510, K-6010, K-9510, K-1501, respectively (Chemetrics, Inc., Calverton, VA) (Moser et al., 2003).

DNA Extraction

DNA was extracted from the DNA filters using the MOBIO Ultraclean Soil DNA Kit (MOBIO Labs, Inc., Solana Beach, Calif.). This process isolates DNA from cellular material, as required for subsequent procedures. DNA extraction was performed at the University of the Free State (Bloemfontein, South Africa), by members of the Research Experiences for Undergraduates program (MS149062003BH1) and at Pacific Northwest National Laboratory by Duane P. Moser (DR9IPCH1101602).

Polymerase Chain Reaction (PCR) and Thermal Cycling

PCR is a method for replicating and isolating a specific gene in a sample. It involves subjecting a reaction mixture to a precise sequence of temperatures in the presence of a DNA polymerase in order to imitate the events of DNA replication within a living cell. Reagents in the mixture include extracted DNA template; Taq polymerase, an enzyme that replicates DNA strands; primers, short DNA strands that match with conserved regions near the ends of the desired gene; dNTPs, extra nucleotides (adenine, thymine, guanine, and cytosine) to create new copies of the DNA template; and a buffer, which maintains a correct

pH and salt concentration for the reaction. This PCR mixture is then exposed to thermal cycling, a specific series of temperatures under which the reagents operate to replicate DNA. The precise thermal cycle varies depending on the desired gene, but the general cycle involves an initial denaturing step to unbind the double-stranded DNA, followed by 35 cycles of denaturing, annealing (binding), and extension. Each cycle provides the conditions required to duplicate every strand of DNA in the sample. A final extension period rebinds the DNA, which is then stored at 4°C to prevent degradation.

PCR was performed using *ExTaq* polymerase and the provided buffer (Takara Bio, Inc, Japan). Forward and reverse primers (Sigma Genosys) used for each amplified gene are summarized in Table 1a. PCR reagents were combined with 1 μL template to a final volume of 25 μL at the following final concentrations: 1X *ExTaq* buffer, 200 μM dNTPs, 0.2 μM forward primer, 0.2 μM reverse primer, and 1.5units/25 μL *ExTaq*. Positive and negative amplification controls were run with every PCR reaction. Thermal cycling was performed with a PTC-225 Peltier Thermal Cycler (MJ Research, Waltham, Mass.), as shown in Table 1b.

Gel Electrophoresis

Products of the PCR reactions were run on agarose gels to determine the presence or absence of the amplified gene. By subjecting negatively charged DNA to an electric current, the amplified PCR product was forced through the gel towards the positive electrode and separated according to length. Smaller fragments move more easily through pores in the agarose and thus run father than longer fragments, creating bands at different positions on the gel that represent DNA of different lengths. The DNA stain ethidium bromide was added to the gel and allows bands to be viewed under ultraviolet light. PCR products of 16S rRNA and APS reductase genes that showed amplification were then ready for cloning.

a.)

Desired Gene	Primer	Sequence	Citation
APS	APS-FW	5'-TGGCAGATMATGATYMACGGG–3' *	Deplancke 2000
APS	APS-RV	5'-GGGCCGTAACCGTCCTTGAA–3'	Deplancke 2000
16S archaea	21F	5'-TTCYGGTTGATCCYGCCRGA-3'	Moyer 1998
16S archaea	1492R	5'-GGTTACCTTGTTACGACTT-3'	Reysenbach 2000
16S nested archaea	21F	5'-TTCYGGTTGATCCYGCCRGA-3'	Moyer 1998
16S nested archaea	915R	5'-GTGCTCCCCCGCCAATTCCT-3'	Stahl 1991
16S bacteria	27F	5'-AGAGTTTGATCMTGGCTCAG-3'	Braker 2001
16S bacteria	1492R	5'-GGTTACCTTGTTACGACTT-3'	Reysenbach 2000
Sequencing primer	M13F	5' -GTAAAACGACGGCCAG- 3'	
Sequencing primer	M13R	5' -CAGGAAACAGCTATGAC- 3'	

b.)

Primer	Initial Denaturing		Denaturing (x35)		Annealing (x35)		Extension (x35)		Final Extension		Storage
	Time (m:s)	Temp (deg C)	Time (m:s)	Temp (deg C)	Time (m:s)	Temp (deg C)	Time (m:s)	Temp (deg C)	Time (m:s)	Temp (deg C)	Time (m:s)
APS	5:00	95	0:30	62	1:00	72	1:00	72	20:00	4	forever
16S	5:00	95	0:30	50	1:00	72	1:30	72	20:00	4	forever
M13	5:00	95	0:30	50	1:00	72	1:30	72	20:00	4	forever

Table 1: (a) Primers used to amplify each gene in this study. * APS-FW was published incorrectly and should not include the final "G." **(b)** Thermal cycling for each primer set.

Cloning

Cloning consists of two steps, ligation and transformation, which are performed to isolate variations of homologous genes from each other. This is required because PCR amplification of a particular sample yields many copies of different versions of the desired gene present in the environment. In order to obtain sequences, copies of the gene with different sequences must be isolated from the others. Ligation is the insertion of the PCR-amplified gene into a plasmid by binding together the ends of the insert with the ends of the plasmid. A plasmid is a circular piece of double-stranded DNA that contains an insertion site into which the desired gene is spliced. This plasmid is then subjected to a transformation reaction, where it is inserted into a host cell for replication.

Cloning was completed with the Invitrogen TA Cloning Kit (Invitrogen, San Diego, Calif). APS and 16S rRNA genes were inserted into the pCR2.1 plasmid vector (Invitrogen) (Fig. 4). The resulting product was then transformed into chemically competent TOP10 *Escherichia coli* cells (Invitrogen) using the manufacturer's instructions, forcing the cells to incorporate the plasmid. Clones were then plated onto Petri dishes with LB agar and incubated at 37°C overnight, providing the proper nutrients and temperature to stimulate cell replication. Growth was controlled in several ways. The cloning vectors contain genes for antibiotic resistance and kanamycin was therefore used to prevent the growth of both *E. coli* cells that were not transformed with an insert. Additionally, the plates were treated with X-GAL for blue-white screening of colonies. Clones without a plasmid insert are colored blue by this process. Clone libraries were constructed by picking 48 white colonies from each sample from the plates and growing them individually in microcentrifuge tubes with LB media and kanamycin.

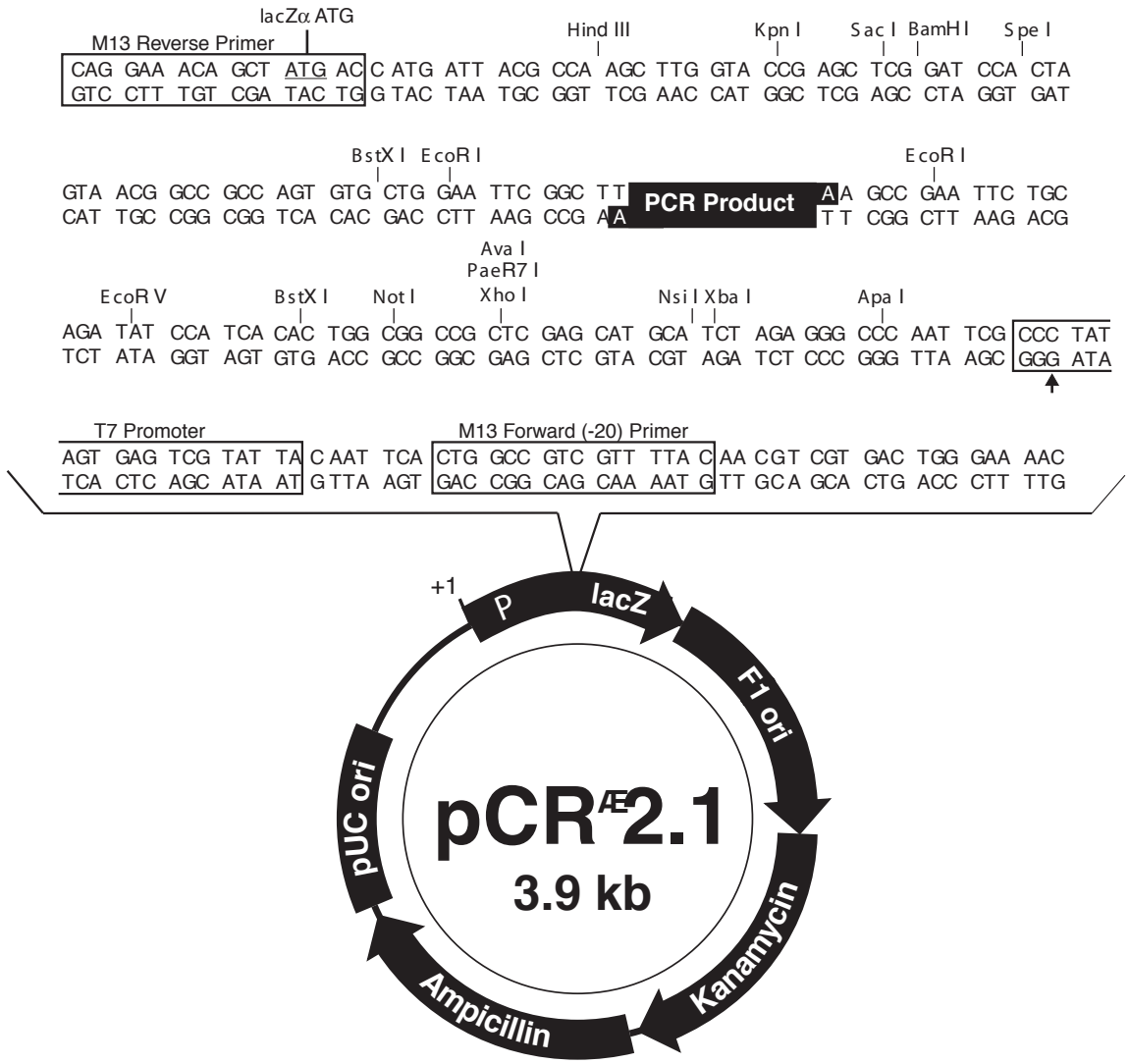


Figure 4: Map of Invitrogen cloning plasmid vector pCR 2.1 used in ligation reactions.

M13 PCR

Amplification of the desired, isolated gene was completed by performing a PCR reaction using the M13F and M13R primers (Table 1a) corresponding to sites found on the pCR2.1 plasmid vector surrounding the inserted gene (Fig. 4). This is required to isolate the desired gene from the plasmid that it was inserted into during ligation and to amplify the gene for furthur analyses. 1 μL of template was added to a mixture of 19.3 μL H20, 2.6 μL *ExTaq* buffer (10X), 2.1 μL dNTPs, 0.05 μL M13F and M13R primers (100 μM), and 0.1 μL *ExTaq*. This mixture was then subjected to thermal cycling (Table 1b) with the PTC-225 Peltier Thermal Cycler (MJ Research, Waltham, Mass.) or the Perkin Elmer 9600 GeneAmp PCR System (Perkin Elmer, Wellesley, Mass.).

Restriction Digest

Restriction Fragment Length Polymorphism (RFLP) fingerprinting was performed to determine which clones had identical inserted genes so as not to waste time and money sequencing every clone. This is done by combining M13 PCR product with restriction enzymes, which cut DNA when they recognize specific sequences. Identical genes are cut in the same places by the restriction enzymes and thus produce an identical pattern of fragments when run on a gel.

Restriction digests were performed with two four-base cutters, HhaI and HaeIII, and their corresponding buffers (New England Biolabs, Beverly, Mass.). HhaI digests were set-up with 2 μL template, 8 μL Buffer 4 (10X), 54.2 μL H_20, 0.8 μL BSA, and 1.0 μL HhaI. HaeIII digests included 2 μL template, 8 μL Buffer 2, 54.0 μL H_20, and 2.0 μL HaeIII. These reactions were incubated overnight at 37°C. The digests were viewed on 3.5% low-melt agarose gels with TBE buffer. Each HhaI digest was mixed with its corresponding HaeIII digest and the two were loaded onto the gel together. Combining these two four-base restriction enzymes created a highly sensitive screening process. The resulting gels were photographed on a Kodak ImageStation 440CF with Kodak 1D v.3.6 software (Fig.

5a). Identical patterns were identified as containing replicate sequences and grouped together (Fig. 5b). One clone from each group was selected for sequencing and purified with the MOBIO PCR Purification Kit (MOBIO Labs, Inc., Solana Beach, Calif.). The laboratory work explained above (PCR, electrophoresis, cloning, M13PCR, and RFLP) was performed by Leah Morgan and Thomas Gihring with the help of Cody Simonson at the Pacific Northwest National Laboratory, Environmental Microbiology Group (Richland, Washington).

Sequencing

DNA sequencing was performed using an Applied Biosystems 3100 genetic analyzer with primers M13F and M13R (Table 1a) at the Florida State University sequencing facility in Tallahassee, Florida. Sequence fragments were assembled to yield a complete copy of each gene sequenced. Sequences were checked for errors in Sequencher v3.1.1 (Gene Codes Corporation, Ann Arbor, Mich.) by Mike Dollhopf and David Balkwill at Florida State University in Tallahassee.

Phylogenetic Analysis

Phylogenetic analysis and the creation of phylogenetic trees involves two major steps. First, sequences for inclusion on a tree must be aligned to each other with the goal of maximizing the number of identical regions. Second, the alignment is turned into a tree by one of many algorithms used by phylogeneticists. Various algorithms incorporate different evolutionary assumptions, and a robust phylogeny should produce similar results across several different algorithms. Algorithms used in this study include maximum-likelihood, neighbor-joining, and Bayesian analysis.

Gene sequences were searched for the primer sequences in Bioedit (Hall, 2001b) and trimmed to exclude plasmid sequences. The National Center for Biotechnology Information BLAST (Basic Local Alignment Search Tool) search algorithm (Altschul et al.,

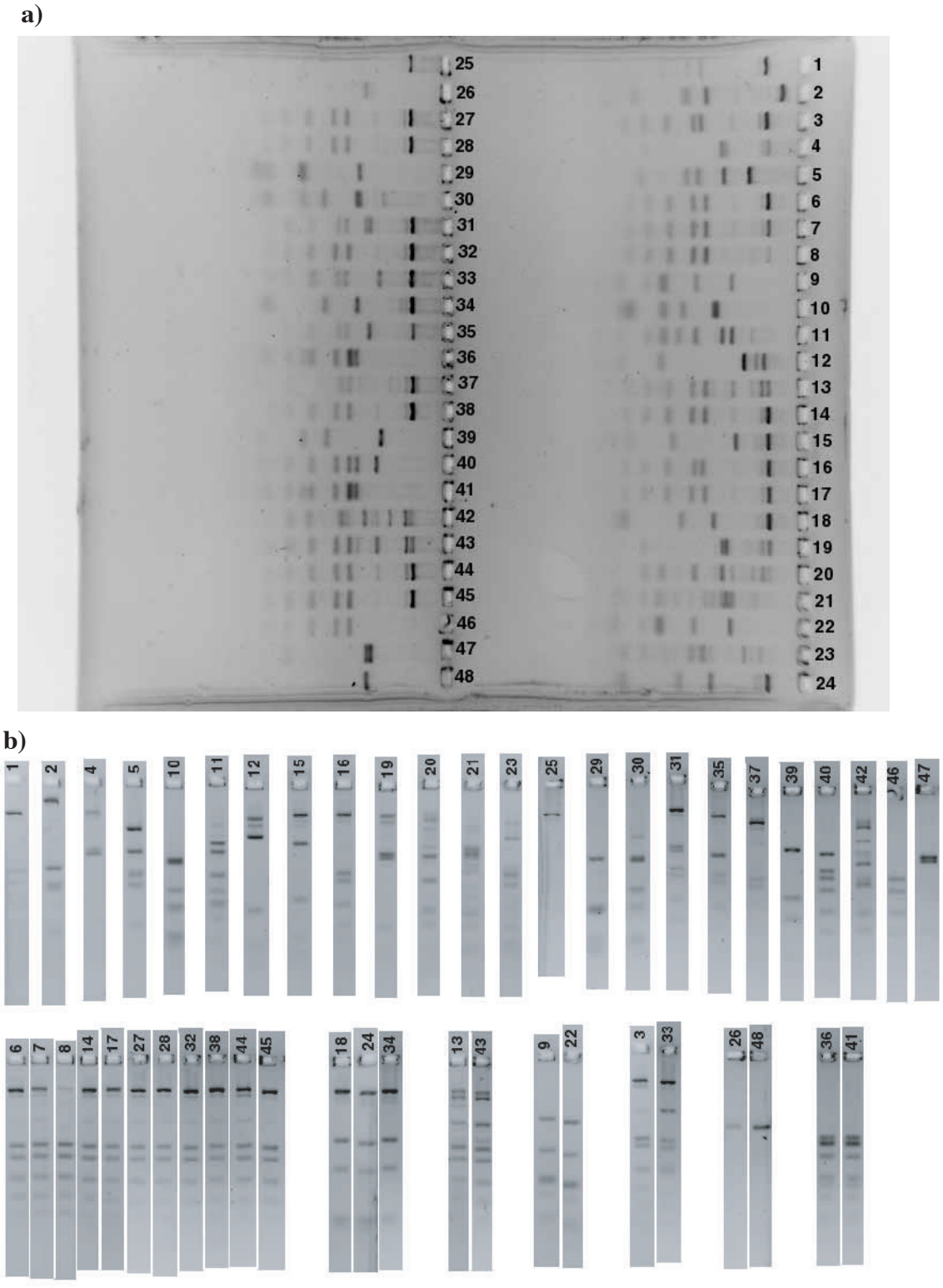


Figure 5: (a) Photo of RFLP gel, sample APS-VV. **(b)** RFLP analysis of APS-VV clones. Gel photos were imported into Adobe Photoshop and manipulated to compare patterns. Clones with similar patterns were grouped, and one from each group was selected for sequencing. All unique clones were sequenced.

1997) was used to search for closely related sequences in public databases and to confirm the presence of the desired gene. Closest BLAST matches were retrieved and included in alignments. 16S rDNA sequences were aligned to a master database with the quick aligner in ARB (v. 2.0) (Ludwig et al., 2002) and checked manually. APS sequences were aligned in Clustal X (Thompson et al., 1997) using parameters suggested by Hall (Hall, 2001a).

16S rDNA sequences (900 to 1600 base pairs) were checked for chimeras using Bellerophon (Huber et al., 2004) and the Ribosomal Database Project's ChimeraCheck (Cole et al., 2003). A chimera is an artifact of PCR that is formed of pieces of the desired gene from different organisms, creating the appearance of highly divergent, novel sequences. Additionally, each clone library was manually searched for chimeras in Bioedit (Hall, 2001b). APS sequences (~450 base pairs) were too short for analysis in Bellerophon and were checked manually. Several chimeras were identified in each clone library and were excluded from analyses.

Alignments for Bacterial and Archaeal 16S rDNA genes were imported into MrBayes (Huelsenbeck, 2000) for Bayesian analysis. MrBayes was run for 100,000 generations with a sample frequency of 100 and a gamma rate correction. The "burn in" value was set to remove the first 350 trees from the phylogeny. Alignment for the APS gene was imported into ARB (Ludwig et al., 2002) and used to create a neighbor-joining tree.

To assess the quality of the alignments and consistency of phylogeny, multiple treeing algorithms were used to check for congruence. ARB was used to create maximum-likelihood trees for VV 16S rDNA bacterial and archaeal sequences. MS 16S rDNA trees were created in neighbor-joining (archaea) and maximum-likelihood (bacteria). An APS tree was constructed in MrBayes as described above for 16S rDNA tree. Additionally, bootstrapping (likelihood for MrBayes trees) was performed to assess the reliability of constructed trees. Bootstrapping is a statistical method that tests how well the tree reflects the data; a higher percentage reflects a more reliable cluster of sequences.

Geochemistry

Anion and cation samples were analyzed at Princeton University. The anion samples were measured by ion chromatography (DX-320, Dionex, Sunnyvale, Calif.) using EG40 and LC25 columns and an AS40 autosampler. Detection limits for SO_4^{2-}, NO_3, acetate, formate, and propionate were 0.1 ppm. The relative standard deviation ranged between 10 to 20% based on a 4-point calibration curve. The cations were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (ICP 4300 DV Optima, Perkin-Elmer, Wellesley, MA). The relative standard deviation ranged between 4 and 25% based on a 4-point calibration curve. Select trace metal cation concentrations were measured by ICP-MS (Activation Laboratories Ltd., Ontario, Canada). The sulfide content was determined by saturating the sample with 22% Zn-acetate and then re-dissolving the subsequent ZnS precipitate with 0.1 M HCl, before measuring the Zn content by ICP-AES. Given the Zn detection limit of 0.05 ppm the calculated equivalent detection limit for dissolved sulfide was 0.025 ppm (Moser et al., 2003).

Results

Phylogeny

Phylogenetic diversity of 16S rDNA and APS gene clones from the Driefontein and Merriespruit samples are summarized in Table 2 and Fig. 6. Driefontein sequences (VV) showed slightly higher diversity across lineages than Merriespruit, even with fewer total sequences.

Merriespruit 16S rDNA sequences (Table 2a; Fig. 7a, b) showed an association to SOP (*Thiobacillus*), SRP (*Desulfotomaculum, Desulfomonas*), and a nitrate-reducing bacterium (*Sterolibacterium denitrificans*). Other 16S rDNA sequences were closely related to clones from the Tomsk-7 deep well in Siberia, hydrothermal deposits in the Guaymas Basin, Mono Lake, a contaminated aquifer, and the SAGMA (South African Gold Mine Archaea) lineage. The Bacterial clone library was well distributed across all lineages

Table 2a: Summary of Merriespruit (MS) 16S and APS clones and their inferred taxonomies.

Clone	Division (subdivision)	Nearest taxon	Nearest environmental clone	% of library
16S bacteria				
MS2-13	*Firmicutes*	*Desulfotomaculum australicum*	Mono Lake clone, AY245489	11.1
MS2-30	*Firmicutes (low G+C)*	*Anaerobranca bogoriae*	Mono Lake clone, AF507877	11.1
MS2-38	*Nitrospira*	*Magnetobacterium bavaricum*	Wetland clone, AF534005	22.2
MS2-2	*Proteobacteria (beta or gamma)*	*Thiobacillus baregensis*	Sulfur River Cave clone, AF047619	11.1
MS2-5	*Proteobacteria (beta or gamma)*	*Thiobacillus baregensis*	Sulfur River Cave clone, AF047619	11.1
MS3-17	*Proteobacteria (beta)*	*Sterolibacterium denitrificans*	P+ sludge clone, AF204243	11.1
MS3-31	*Proteobacteria (delta)*	*Desulfomonas species*	Anaerobic clone, AF447133	11.1
MS3-19	*Proteobacteria (gamma)*	*Thiobacillus species*		11.1
16S archaea				
MS2-46	*Methanobacterium*	*Methanobacterium curvum*		31.3
MS2-33	SAGMA	SAGMA-F	Siberian deep well clone, AJ583385	6.3
MS2-3			Siberian deep well clone, AJ583382	6.3
MS2-10			Guaymas Basin clone, AF419624	6.3
MS2-11		SAGMA-S	Mono Lake clone, AY2245466	6.3
MS2-34			Contaminated aquifer clone, AY501682	43.8
APS				
MS2-36	*Proteobacteria (gamma)*	*Allochromatium vinosum*		1.6
MS3-10/MS3-7	*Proteobacteria (gamma)*	*Allochromatium vinosum*		3.1
MS3-24	*Proteobacteria (gamma)*	*Allochromatium vinosum*		1.6
MS3-14	*Proteobacteria (gamma)*	*Allochromatium vinosum*		1.6
MS2-14	*Archaea*	*Archaeoglobus fulgidus*	mouse gut clone, AF196341	1.6
MS2-10			mouse gut clone, AF196334	3.1
MS2-2			mouse gut clone, AF196334	39.1
MS3-4			mouse gut clone, AF196334	9.4
MS3-25			mouse gut clone, AF196334	1.6
MS3-30			mouse gut clone, AF196334	1.6
MS3-41			mouse gut clone, AF196334	1.6
MS2-29			mouse gut clone, AF196334	1.6
MS3-1			mouse gut clone, AF196334	15.6
MS3-37			mouse gut clone, AF196334	1.6
MS2-3			mouse gut clone, AF196334	7.8
MS3-39			mouse gut clone, AF196334	1.6
MS3-11			mouse gut clone, AF196334	1.6
MS3-23			mouse gut clone, AF196334	1.6

Table 2b: Summary of Driefontein (VV) 16S and APS clones and their inferred taxonomies.

Clone	Division (subdivision)	Nearest taxon	Nearest environmental clone	% of library
16S bacteria				
VV-10	*Chlorobia*	*Chlorobium limicola*	uncultured bacterium, AY340841	2.6
VV-7	*Firmicutes*	*Desulfotomaculum alkaliphilum*	uncultured bacterium, AY122603	17.9
VV-30	*Firmicutes*	*Desulfotomaculum aeronauticum*		10.3
VV-41	*Firmicutes*	*Bacillus joetgali*		2.6
VV-48	*Planctomycetes*	*Planctomyces species*		2.6
VV-9/VV-44	*Proteobacteria (delta)*	*Desulfomicrobium baculatum*		51.3
VV-43	*Proteobacteria (delta)*	*Desulfosarcina variabilis*	uncultured bacterium, AY050605	5.1
VV-12/VV-14	*Proteobacteria (gamma)*	*Acinetobacter junii*	other South Africa clones	5.1
VV-1	Termite Group I		termite gut clone, D63633	2.6
16S archaea				
VV-132	*Thermoprotei*	*Cenarchaeum symbiosum*	estuary sediment clone DOUR04, AF201358	3.3
VV-139	*Methanomicrobia*	*Methanomicrobium mazei*	h.th. vent clone, ANME group AB019758	50
VV-115	*Methanobacterium*	*Methanobacterium bryantii*	UntArch91	40
VV-111	SAGMEG-1		SAGMA-J	6.7
APS				
VV-15	*Proteobacteria (gamma)*	*Allochromatium vinosum*		4.3
VV-1	*Proteobacteria (delta)*	*Desulfomicrobium baculatum*	mouse gut clone, AF196336	52.2
VV-16	*Proteobacteria (delta)*	*Desulfomicrobium baculatum*	mouse gut clone, AF196336	4.3
VV-37	*Proteobacteria (delta)*	*Desulfovibrio africanus*		4.3
VV-29	*Proteobacteria (delta)*	*Desulfonema ishimotonii*		4.3
VV-30	*Proteobacteria (delta)*	*Desulfonema ishimotonii*		4.3
VV-10	*Proteobacteria (delta)*	*Desulfonema ishimotonii*		4.3
VV-9	*Proteobacteria (delta)*	*Desulfofrigus oceanense*		8.7
VV-19	*Proteobacteria (delta)*	*Desulfofrigus oceanense*		4.3
VV-11	*Proteobacteria (delta)*	*Desulfobacter curvatus*		4.3
VV-20	*Proteobacteria (delta)*	*Desulfobacter curvatus*		4.3

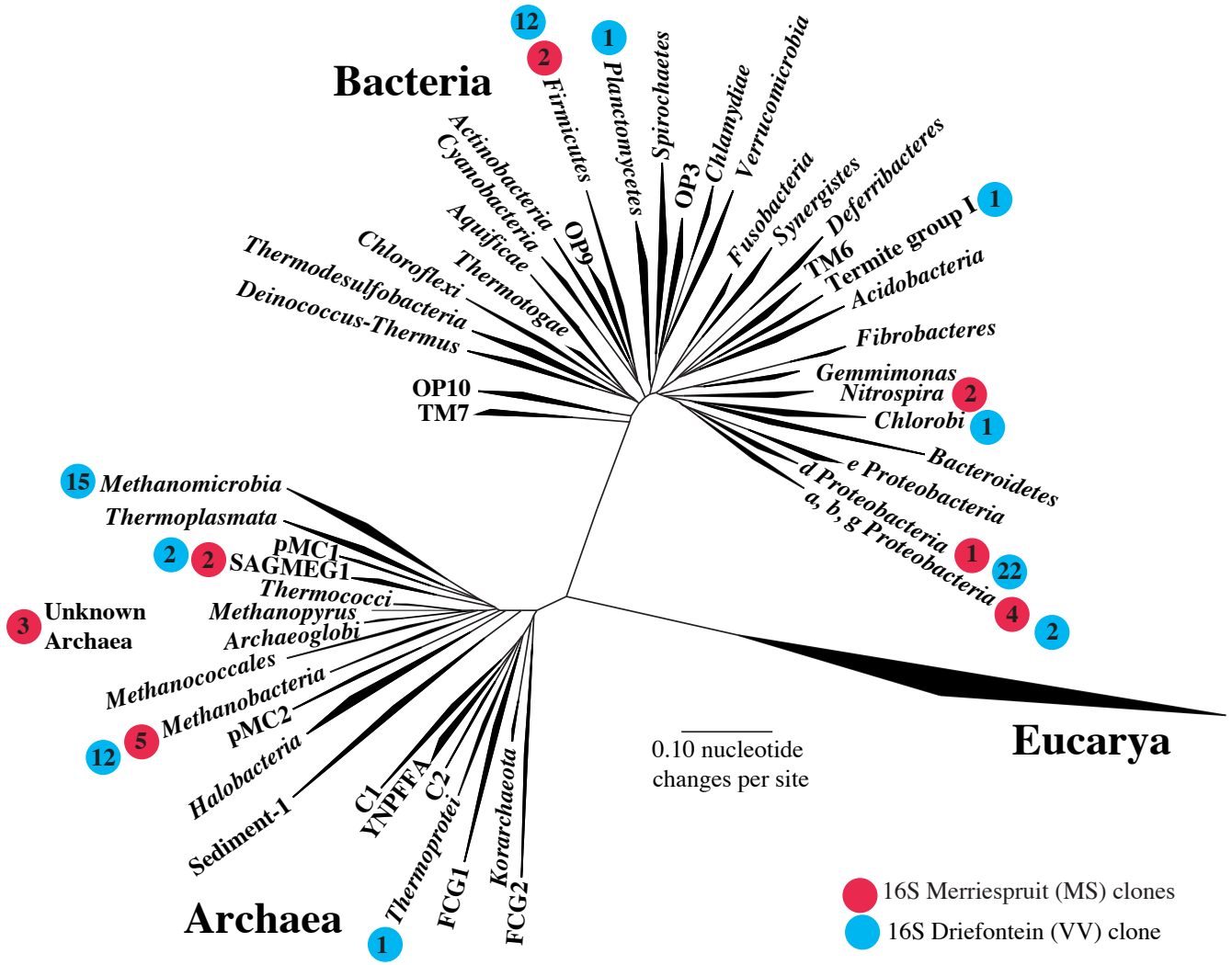


Figure 6a: Distribution of MS (Merriespruit) and VV (Driefontein) 16S rDNA clones within the three domains. Clones are shown as a dot on the tree. Numbers represent the number of sequences from each lineage. Modified from Macalady and Banfield, 2003.

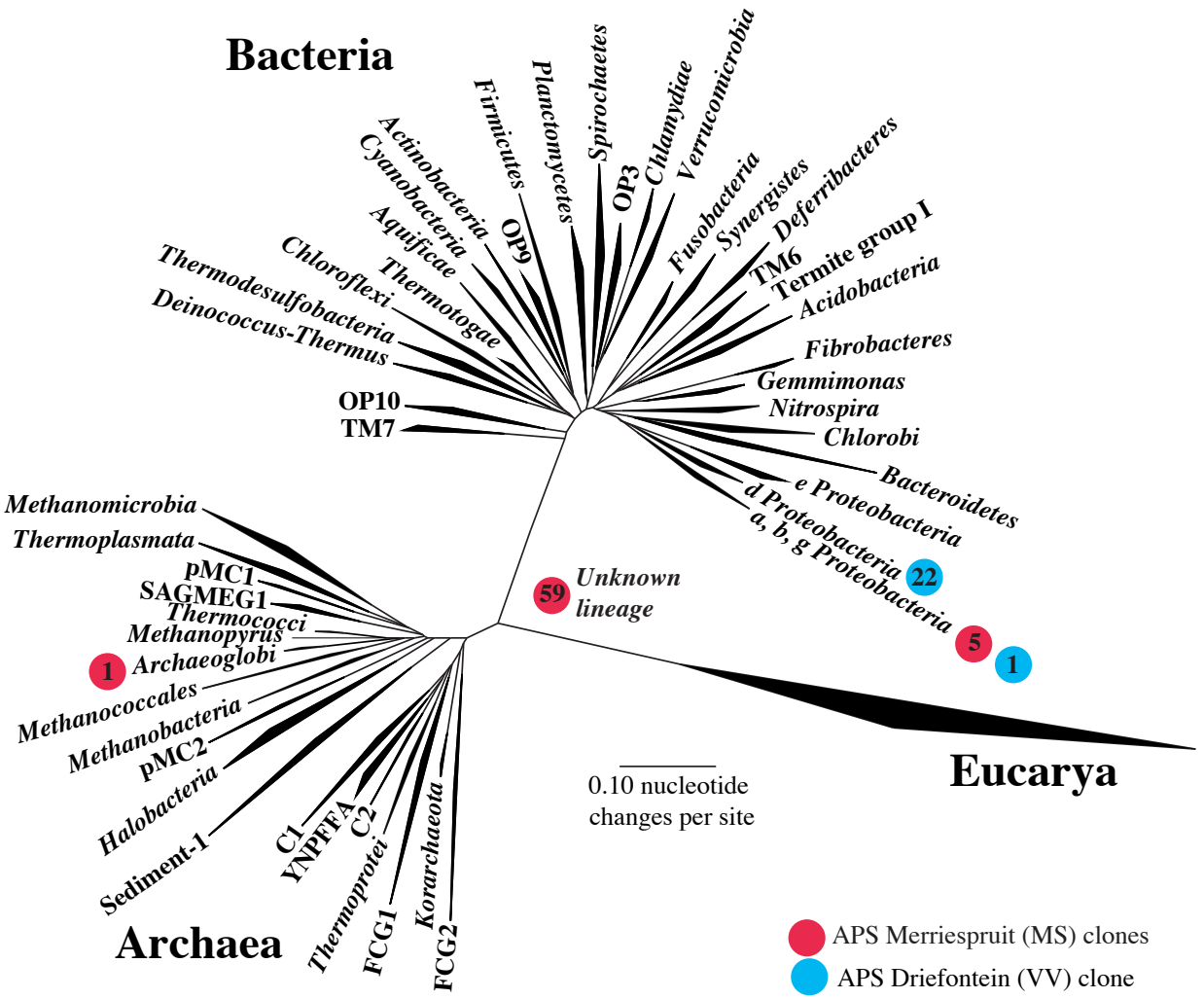
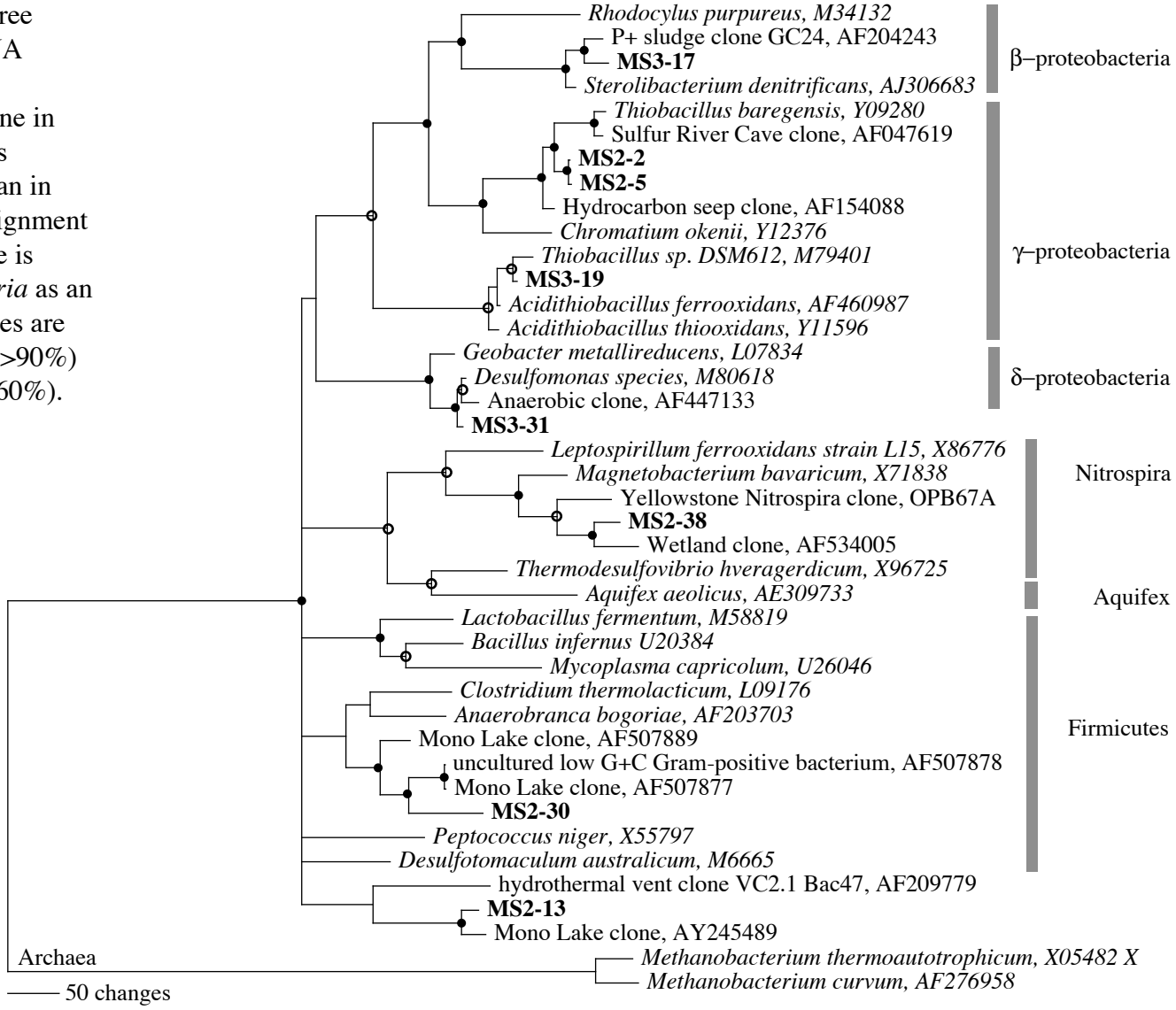


Figure 6b: Distribution of MS (Merriespruit) and VV (Driefontein) APS clones within the three domains. APS clones are shown as a dot on the tree. Numbers represent the number of sequences from each lineage. Modified from Macalady and Banfield, 2003.

Figure 7a: Phylogenetic tree based on bacterial16S rDNA nucleotide sequences from Merriespruit (MS) gold mine in South Africa. The tree was constructed by Leah Morgan in MrBayes using an ARB alignment and viewed in PAUP. Tree is rooted with *Methanobacteria* as an outgroup. Likelihood values are indicated by filled circles (>90%) and open circles (<90%, >60%). No circles indicates <60% bootstrap value.



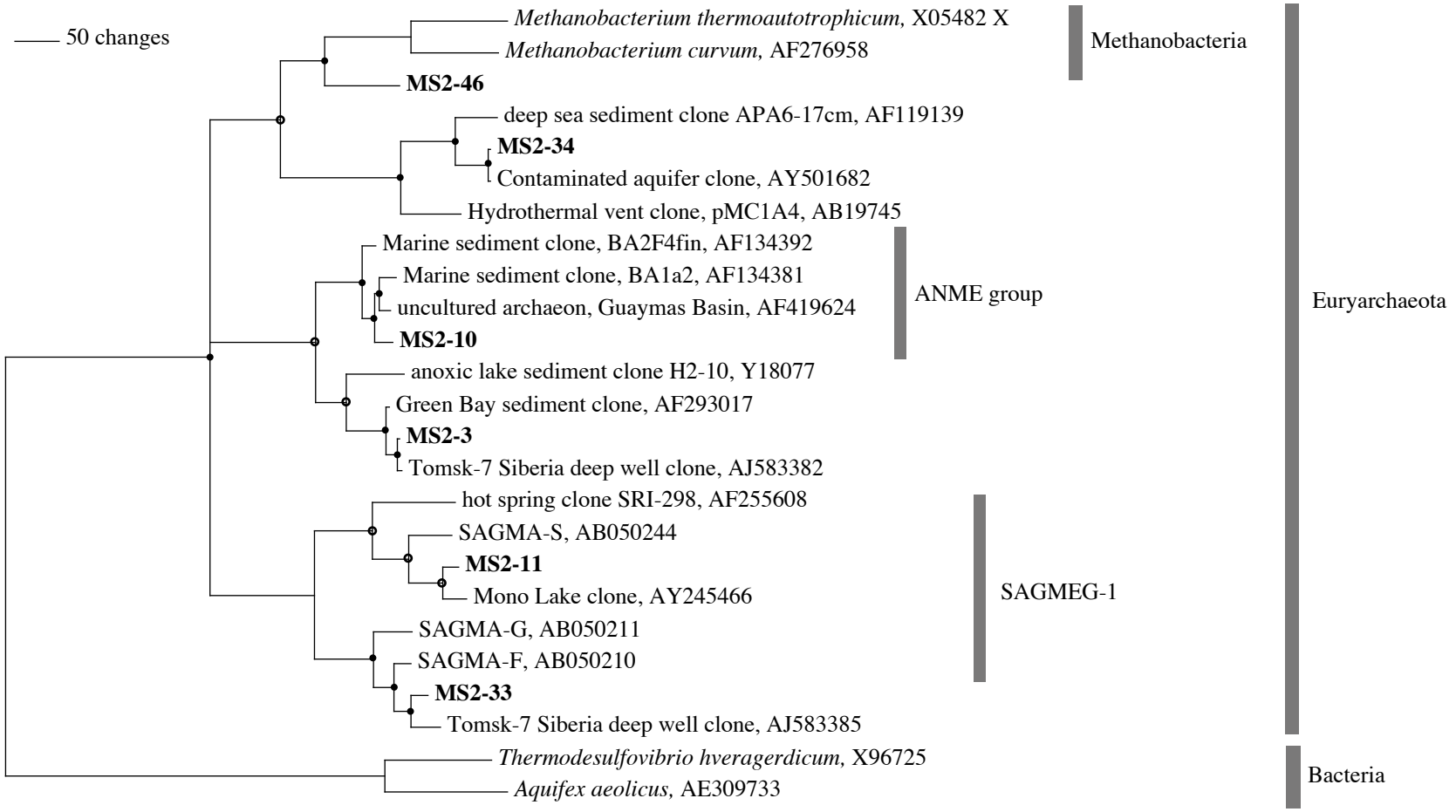
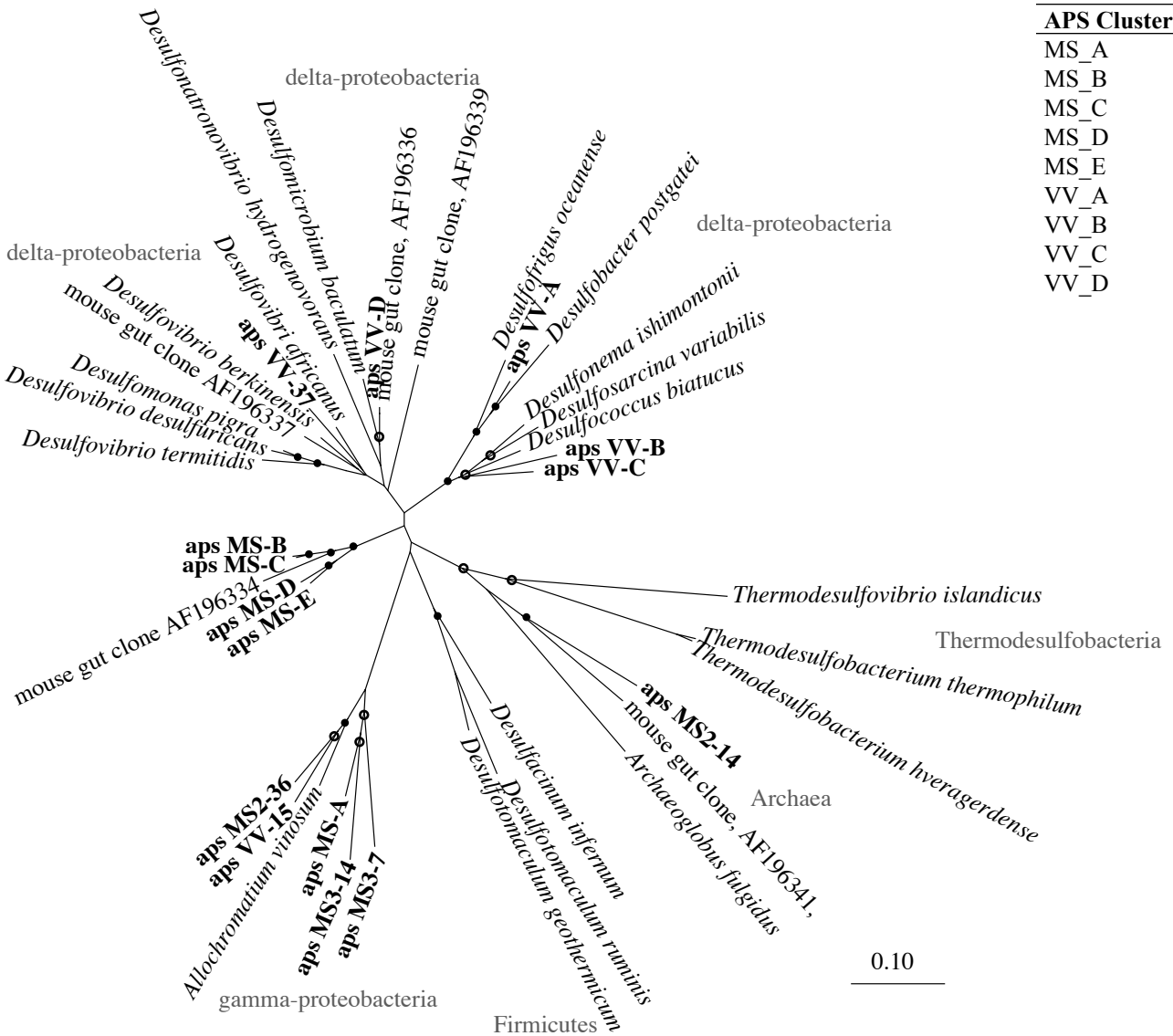


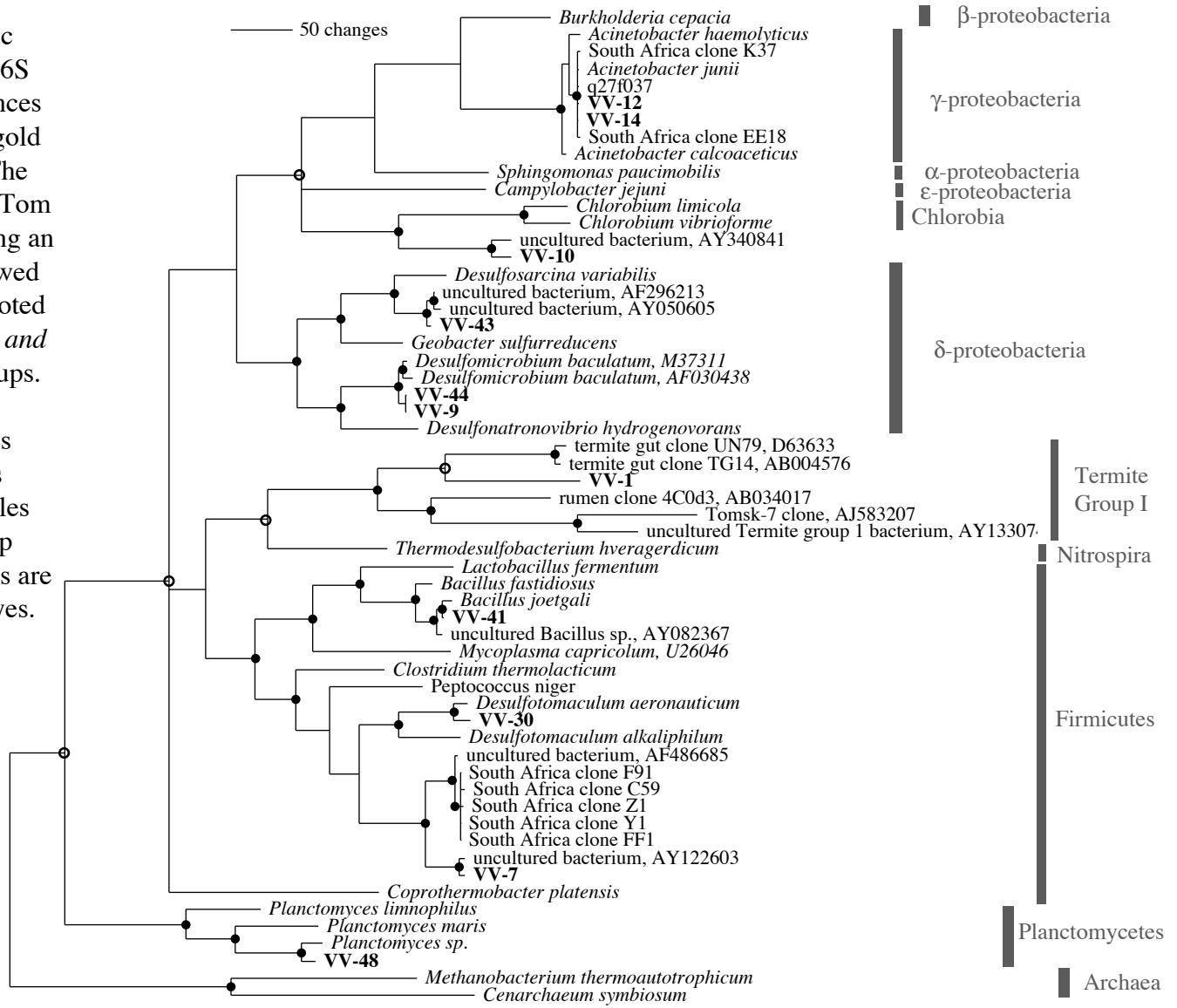
Figure 7b: Phylogenetic tree based on archaeal 16S rDNA nucleotide sequences from Merriespruit (MS) gold mine in South Africa. The tree was constructed by Leah Morgan in MrBayes using an ARB alignment and viewed in PAUP. Tree is rooted with *Thermodesulfovibrium* and *Aquifex* as bacterial outgroups. Likelihood values are indicated by filled circles (>90%) and open circles (<90%, >60%). No circles indicates <60% bootstrap value. Likelihood values are as determined by MrBayes.



APS Cluster	APS Clones
MS_A	MS3-10, MS3-22
MS_B	MS3-25, MS3-30, MS3-41
MS_C	MS2-10, MS2-2, MS2-1, MS3-4
MS_D	MS2-29, MS3-37, MS3-1
MS_E	MS2-3, MS3-39, MS3-11, MS3-23
VV_A	VV-11, VV-20
VV_B	VV-9, VV-19
VV_C	VV-10, VV-29, VV-30
VV_D	VV-1, VV-16

Figure 7c: Phylogenetic tree based on APS gene nucleotide sequences from Merriespruit (MS) and Driefontein (VV) gold mines in South Africa. This unrooted tree was constructed by Tom Gihring using the neighbor joining method in ARB and viewed in PAUP. Bootstrap values are indicated by filled circles (>90%) and open circles (<90%, >60%). No circles indicates <60% bootstrap value. Bootstrap values were calculated by 1000 replicates in neighbor joining.

Figure 7d: Phylogenetic tree based on bacterial 16S rDNA nucleotide sequences from Driefontein (VV) gold mine in South Africa. The tree was constructed by Tom Gihring in MrBayes using an ARB alignment and viewed in PAUP. The tree is rooted with *Methanobacterium and Cenarchaeum* as outgroups. Likelihood values are indicated by filled circles (>90%) and open circles (<90%, >60%). No circles indicates <60% bootstrap value. Likelihood values are as determined by MrBayes.



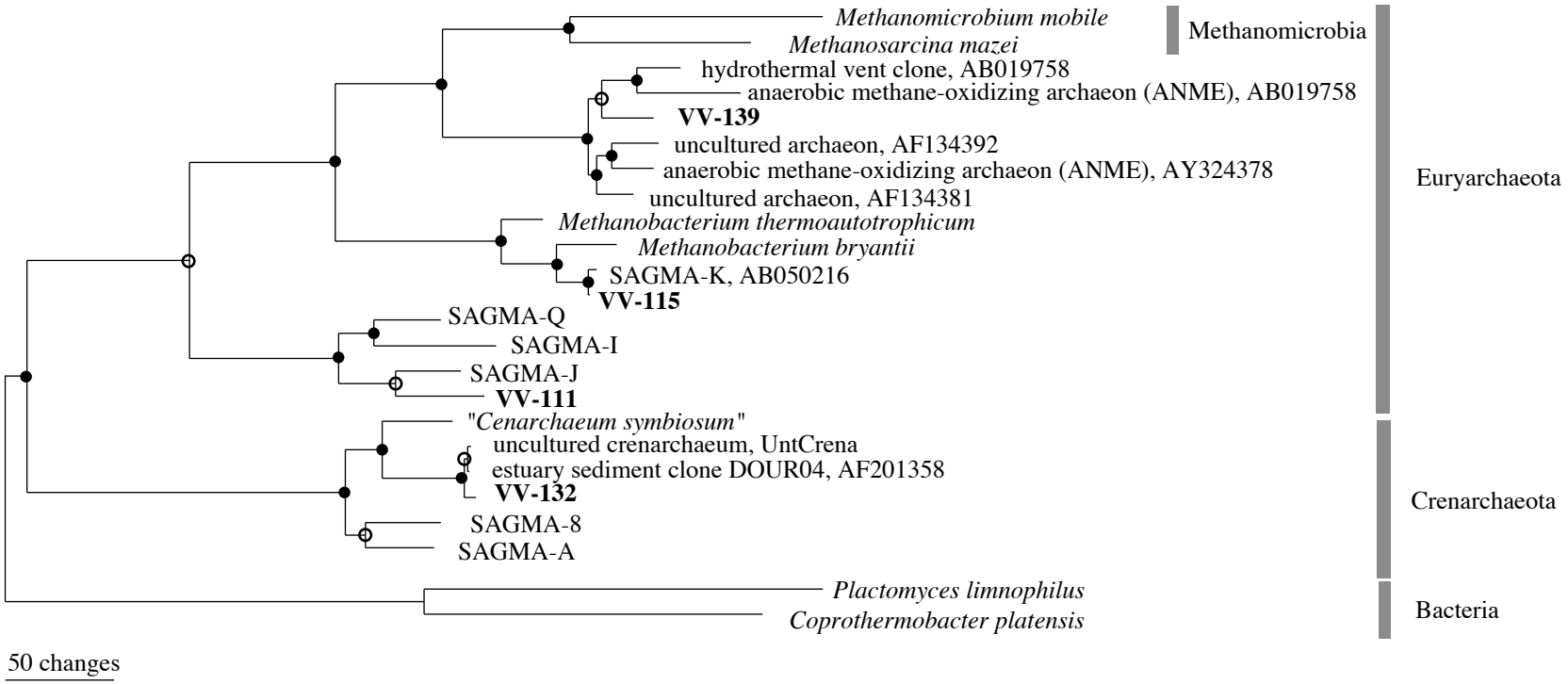


Figure 7e: Phylogenetic tree based on archaeal 16S rDNA nucleotide sequences from Driefontein (VV) gold mine in South Africa. The tree was constructed by Tom Gihring in MrBayes using an ARB alignment and viewed in PAUP. Likelihood values are indicated by filled circles (>90%) and open circles (<90%, >60%). No circles indicates <60% bootstrap value. Likelihood values are as determined by MrBayes.

represented. Over 40% of the Archaeal clone library consisted of clones identical to MS2-34 and matched closely to a clone from a contaminated aquifer; an additional 31% were most closely related to *Methanobacterium curvum*. Merriespruit APS gene sequences (Table 2a; Fig. 7c) resembled those from SOP (*Allochromatium vinosum*), and one Archaeal SRP (*Archaeoglobus fulgidis*). Fifty percent of APS gene clones from the Merriespruit sample matched closely with a sequence isolated from the mouse gastrointestinal tract and did not match closely with any known APS gene sequence from an isolated organism (Fig. 7a).

Driefontein 16S rDNA clones (Table 2b; Fig. 7d,e) were closely related to SRP (*Desulfotomaculum, Desulfomicrobium, Desulfosarcina*), a heterotroph (*Bacillus joetgali*), methane producers (*Methanomicrobium, Methanobacterium*), an obligately anaerobic photosynthesizer (*Chlorobium limicola*), a symbiont (*Cenarchaeum symbiosum*), and *Planctomycetes*. Fifty percent of clones in the Archaeal library were closely related to a methane-oxidizing Archaea (*Methanomicrobium mazei*) and are likely within the ANME (ANaerobic MEthanotroph) clone group of anaerobic methane-oxidizing archaea which live symbiotically with sulfate-reducing bacteria. One sequence fit within the Termite Group I lineage. Driefontein APS sequences (Table 2b; Fig. 7c) were closely related to SRP *(Desulfomicrobium, Desulfovibrio, Desulfonema, Desulfofrigus, Desulfobacter)* and one SOP (*Allochromatium vinosum*). Over 50% of clones from both bacterial and APS libraries (Table 2b; Fig. 7c,d) match closely with *Desulfomicrobium baculatum*.

The only environmental clones closely matching APS gene sequences from either site were from SRP in the mouse gastrointestinal tract, sequenced by Deplancke, et al. (2000), who designed the APS primers used in this study (Table 2; Fig. 7a).

Alternate treeing algorithms were used for each clone library to assess the reliability of the alignments and trees. All trees showed relatively good congruence with different algorithms and are shown in Fig. A1 (Appendix 1).

Accumulation curves (Fig. 8) for Merriespruit and Driefontein showed thoroughness of sampling and how completely the sequences obtained in this study represent the diversity of the microbial community. The proportion of species observed generally increases faster than the proportion of clones sampled for all clone libraries but only the archaeal libraries reach an asymptote.

Geochemistry

Geochemical data are summarized in Table 3. These data indicated the presence of both sulfate and sulfide at Merriespruit and Driefontein, although at higher concentrations in the Driefontein borehole. A low O_2 concentration was found in the Merriespruit borehole while O_2 was not detected in the Driefontein borehole. Acetate ($C_2H_3O_2^-$) was found at low levels in both boreholes. Nitrate (NO_3) was also present in both samples, and ammonia (NH_3) was detected in Merriespruit. Merriespruit displayed higher values than Driefontein for all aqueous ions except sulfide (HS^-), sulfate (SO_4^{2-}), nitrate (NO_3), ammonia (NH_3), magnesium (Mg), and silica (Si). Gas chemistry for Driefontein indicated the presence of hydrogen (H_2), carbon dioxide (CO_2), and methane (CH_4) in fissure water.

Discussion

Genetic signatures

Phylogenetic analyses show two distinct genetic signatures in the Merriespruit (MS) and Driefontein (VV) boreholes. Although 16S rDNA data (Table 2; Fig. 7a,b,d,e) suggest that microorganisms with various metabolic pathways are present, this study focuses on biological sulfur cycling and will not address other pathways in detail. Sequences from both MS and VV samples that show close relation to SRP or SOP are likely from organisms with the ability to reduce or oxidize sulfur compounds, respectively (Table 2; Fig. 7). As mentioned previously, APS gene sequences are more reliable than 16S rDNA sequences for identifying the presence of microorganisms with metabolic pathways of the

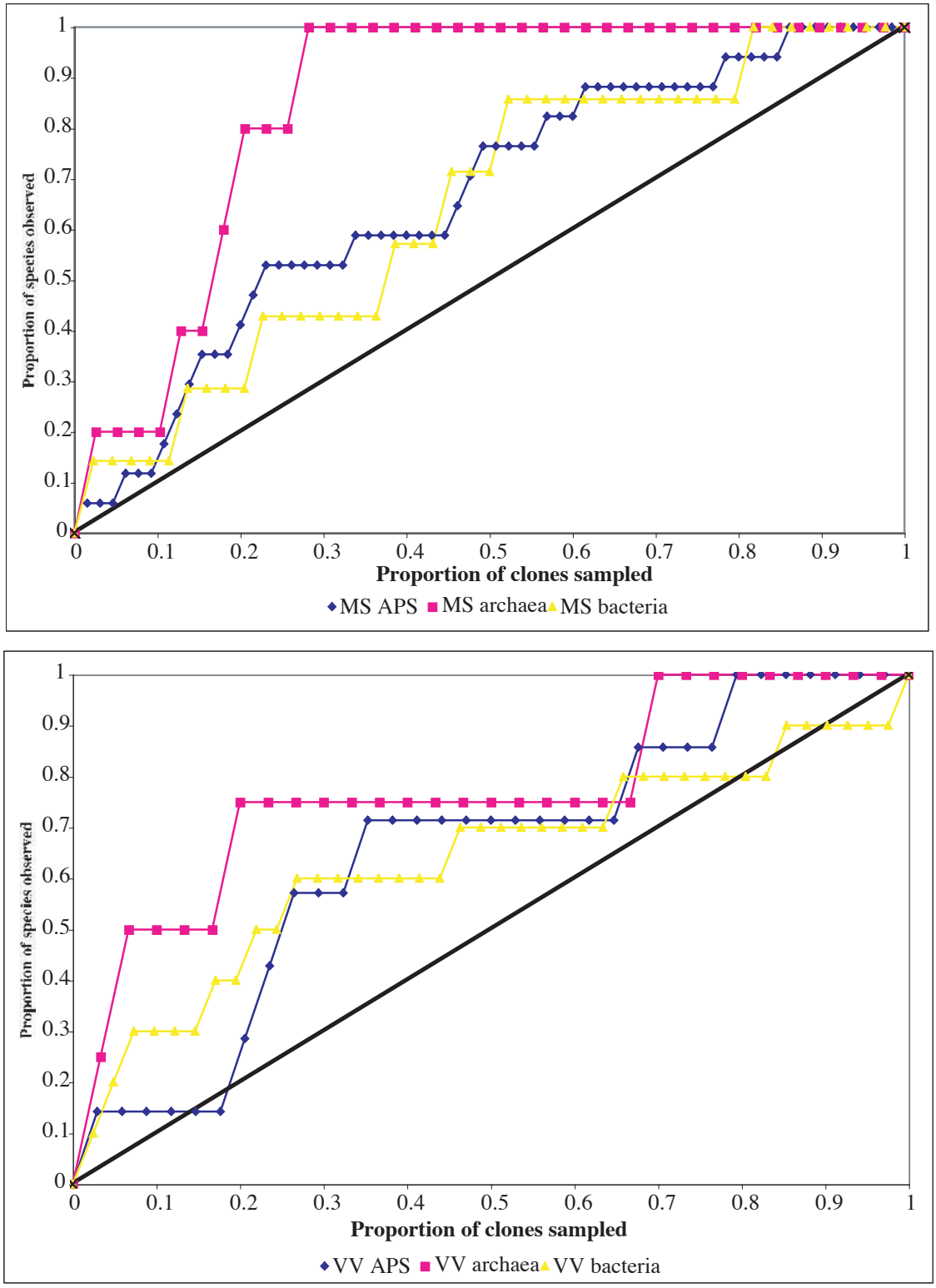


Figure 8: Accumulation curves for Merriespruit (MS) and Driefontein (VV) clone libraries to determine thoroughness of sampling. A straight, y = x line would represent a clone library that did not fully show the diversity of the sample, because every new sequence obtained would represent a new species. When most organisms from a sample are represented in the library, the plot will reach an asymptote as fewer unique species remain unsampled.

a.)	Merriespruit	Driefontein
depth (km)	1.998	0.896
lithology	quartzite	dolomite
T°C	36.3	26
pH	7.76	8
Eh (mV)	-294	-176
O_2	1.5	--
Cl	1433	35.19
Br	9.3	0.31
SO4	6.13	35.41
HS^-	2.5	40.71
S	4.19	--
$S_2O_3^{--}$	--	0.005
NO_3	0.01	0.35
NH_3	0.35	--
NO_2	<d.l.	--
acetate	0.14	0.09
Al	0.027	--
As	0.015	0.032
Ba	0.52	0.054
Ca	188	36.5
Cu	0.014	0.011
Fe	0.035	0.013
K	21.7	1.21
Li	0.99	0.021
Mg	2.32	5.73
Mn	0.072	0.005
Na	1340	26.2
Si	7.8	10
Sr	6.18	0.355
U	0.022	0.012
Zn	0.183	--
CH_4 (g)	--	208.81
CO_2 (g)	--	277.33
CO (g)	--	0.52
H_2 (g)	--	1.89

b.)	Detection limit (ppm)
Na	0.10
K	0.50
Mg	0.05
Ca	0.05
Fe	0.05
S	1.00
Li	5.00
Sr	0.05
Ba	0.05
Al	0.10
Si	0.10
Mn	0.05
Cu	0.05
Zn	0.05
As	0.10
U	0.10

Table 3: **(a)** Geochemical data from Merriespruit and Driefontein boreholes. Unless otherwise indicated, all aqueous values in ppm, gas values in uM. <d.l. = below detection limit. -- = no data. **(b)** Detection limits for geochemical species.

sulfur cycle. While potential SRP were detected in both borehole samples, only the Merriespruit sample analyses suggested the presence of SOP. Driefontein APS gene clone VV-15 is an exception, as it appears closely related to the SOP *Allochromatium vinosum*. Regardless, 16S rDNA and APS sequences indicate that the Merriespruit borehole has a greater diversity of SOP, while the Driefontein borehole has a greater diversity of SRB (Table 2).

The nearest taxon to 16S rDNA clone VV-10 was the phototroph *Chlorobium limicola* (Fig. 7e; Table 2b). However, these species may have very different physiologies because no light is available for photosynthesis in the fissure and groundwater environment, and the phylogenetic distance between VV-10 and *Chlorobium* (Fig. 7d) is rather high. Several APS gene sequences from Merriespruit (MS clones 2-36, 3-7, 3-10, 3-14, 3-24) were most closely related to the APS gene sequence from the sulfur-oxidizing phototroph *Allochromatium vinosum* (Table 2a; Fig. 7c). However, the APS gene developed and diverged into oxidative and reductive forms prior to the development of the photosynthetic pathway (Hipp et al., 1997). The APS gene sequences detected in the Merriespruit sample are therefore likely grouped with *Allochromatium vinosum* due to its capacity as a sulfur-oxidizer rather than as a phototroph. The cluster of Merriespruit clones that show no close relation to an APS sequence from a cultured organism (Table 2a; Fig. 7c) is closely related to a sequence isolated from the mouse gastrointestinal tract (Deplancke et al., 2000). It is unclear whether these gene sequences are from an oxidative or reductive pathway.

Driefontein Archaeal clone VV-139 is likely from the anaerobic methanotroph ANME group (Fig. 7e). All known organisms in this group are believed to be anaerobic methane-oxidizing Archaea that live symbiotically with SRP such as *Desulfosarcina variabilis* (Orphan et al., 2001), which is also likely present in the Driefontein sample (Fig. 7d). These organisms perform a reaction which is energetically unfavorable in the absence of H_2-consuming symbionts. However, Orphan, et.al. (2001) have shown that methanotrophs within the ANME group live in close proximity to SRP, which use H_2 to

reduce SO_4, allowing for continued anaerobic CH_4 oxidation. Evidence for this symbiotic relationship has been detected in seep sediments of the Eel River and Santa Barbara Basins (Orphan et al., 2001). Current data presented in this thesis (Fig. 7d,e) suggest that these processes may also be occurring in the subsurface of the Witwatersrand Basin.

Previous work by Baker et al. (2003) using dsr gene sequencing strongly suggests the presence of SRP from the *Firmicutes* lineage in the South African deep subsurface fissure water environment. These sequences were closely related to dsr sequences from *Desulfotomaculum* species, which have the ability to form spores to protect them from extreme or inhospitable conditions. To date, *Desulfotomaculum* is the most common SRP found in the South African deep subsurface and is the only SRP present in many fissure water samples (Gihring, 2004). Their ability to produce spores likely aided *Desulfotomaculum* in becoming widespread in the deep subsurface. The Driefontein sample used in this study, which was obtained from a dolomite aquifer with a residence time of ~13 kyr rather than fissure water with a residence time of 1-123 My, shows much higher diversity of SRP, including *δ-Proteobacteria* along with the more common *Firmicutes*. The relatively short residence time may explain why *δ-Proteobacteria* and other non-spore forming SRP could survive in the groundwater at Driefontein but could not reach the deeper depths and longer residence times of fissure waters.

Accumulation plots (Fig. 8) indicate that the MS and VV Archaeal clone libraries are well-sampled and likely represent a high percentage of Archaeal diversity in the boreholes. Plots for bacterial and APS libraries do not reach an asymptote, indicating that these libraries are not as comprehensive. These libraries thus contain only a portion of the total diversity of the sample. Several caveats must be considered in the interpretation of genetic data collected from environmental clone libraries. Under some conditions, PCR can amplify some sequences more frequently than others (Poltz and Cavanaugh, 1998). Chimeras and copying errors during PCR can modify sequences (Speksnijder et al., 2001). The error rate for the *ExTaq* DNA polymerase used in this study is 1 error/4000 bases, which can create a

slight apparent increase in the diversity of closely related sequences but likely does not affects data presented here. Sequences in this study were searched carefully for chimeras; mutations introduced during PCR would be undetectable but create only minute changes. Additionally, microorganisms may persist for long periods of time (potentially hundreds of millions of years) in a dormant state, where metabolic and reproductive cycles slow nearly to a halt. Therefore, the molecular evidence presented in this study can only confirm the presence of APS genes in microbial communities of the subsurface. Further work using reverse-transcriptase PCR could detect APS gene expression.

Geochemistry

The presence of sulfide (HS^-) and sulfate (SO_4^{2-}) in both samples (Table 3) generally supports the possibility of modern sulfur cycling in the fissure water. The presence of methane (CH_4) and carbon dioxide (CO_2) gases are consistent with the possible occurrence of anaerobic methanotrophs in Driefontein. Oxygen (O_2) in the Merriespruit fissure water suggests contamination of the borehole by mine air.

In considering a gene such as APS that is used in both oxidative and reductive pathways, a critical factor becomes the identification of potential electron donors and acceptors. The oxidative sulfur pathway which is likely to be prevalent in the Merriespruit sample requires the synchronous reduction of an electron acceptor. Oxygen was detected in this environment and is likely functioning as an electron acceptor. Therefore, the sulfur-oxidizing community is probably not native to the fissure water, but rather was introduced by mining activities. This contamination is apparent in molecular evidence for SOP in Merriespruit (Table 2a; Fig. 7b,c), which would likely not be present in an indigenous anaerobic community (Onstott et al., 2003). An alternative electron acceptor involves the reduction of NO_3 to NH_3 or other reduced nitrogen compounds coupled to the oxidation of sulfide to sulfate.

An electron donor must be present in order for a sulfate reduction pathway to be currently active in the Driefontein environment. Potential electron donors for sulfate reduction include simple compounds such as organic acids, which are products of the anaerobic degradation of organic compounds by fermentation (Madigan et al., 2003). Hydrogen (H_2) is also very likely to be used as an electron donor. Both H_2 and acetate ($C_2H_3O_2^-$) were present at both Merriespruit and Driefontein sites and are potential electron sources for sulfur cycling. The geochemistry of Merriespruit and Driefontein samples is consistent with and provides potential electron donors and acceptors for microbially-mediated sulfur cycling in the subsurface.

Conclusion

DNA sequencing and phylogenetic analyses of APS and 16S rRNA genes from the Witwatersrand Basin provide molecular evidence for microbial sulfur cycling in the deep subsurface. This evidence is consistent with geochemical data and indicates that sulfur cycling may be currently active in the deep subsurface. Evidence for indigenous sulfate reduction was detected in both the Merriespruit fissure water and Driefontein groundwater samples. Evidence for sulfur oxidation was found in the Merriespruit mine fissure water, likely a result of mining contamination. Sequences closely related to ANME-group methanotrophs were found in the Driefontein sample along with the potential ANME symbiont *Desulfosarcina variabilis,* suggesting that anaerobic methane oxidation may be occurring in the deepest regions of the dolomite aquifer. Evidence for sulfate-reducing prokaryotes from the *δ-Proteobacterial* lineage from the Driefontein dolomite aquifer greatly increase the known diversity of subsurface sulfate-reducing prokaryotes.

Acknowledgements

This research was supported by grants from the National Science Foundation REU program (0244130) to Dr. S.M. Pfiffner of the University of Tennessee and the LExEn program (EAR-9714214) to Dr. T.C. Onstott of Princeton University. Further support was provided by the Charles W. Potts Endowment Fund for student-faculty research in geology at Carleton and the Environmental Microbiology Group at Pacific Northwest National Laboratory in Richland, Washington. I gratefully acknowledge Gold Fields Ltd.'s Beatrix Gold Mine, Harmony's Merrispruit Mine, Driefontein Consolidated Ltd.'s West Driefontein Mine, and Anglo Gold's Tau Tona Mine for access to their gold mines and their logistical support. I am grateful to Dr. Esta vanHeerden and others at the Department of Microbial, Biochemical and Food Biotechnology of University of the Free State.

Special thanks go to Jenn Macalady and Tom Gihring for their endless advice and encouragement; Elsabe Botes and Drs. Bethuel Nthangeni, Susan Pfiffner, T.C. Onstott and Tom Kieft for their work during and after the REU; the South African and American participants in the REU program for putting up with a geologist trying to do biology; Duane Moser and Dave Cully for their help at PNL; Mike Dollhopf and David Balkwill for sequencing my clones; Mark Davidson for his help with lineage plots; Dave Auerbach for editing my drafts and listening to my crazy ideas; Ezra Lyon for his vast knowledge of all things biological; and my friends and family for their support throughout this project.

References cited

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J., 1997, Gapped BLAST and PSA-BLAST: a new generation of protein database search program., Nucleic Acids Research, p. 3389-3402.

Baker, B. J., Moser, D. P., MacGregor, B. J., Fishbain, S., Wagner, M., Fry, N. K., Jackson, B., Speolstra, N., Loos, S., Takai, K., Lollar, B. S., Fredrickson, J., Balkwill, D., Onstott, T. C., Wimpee, C. F., and Stahl, D. A., 2003, Related assemblages of sulphate-reducing bacteria associated with ultradeep gold mines of South Africa and deep basalt aquifers of Washington State: Environmental Microbiology, v. 5, no. 4, p. 267-277.

Braker, G., Ayala-del-Rio, H. L., Devol, A. H., Fesefeldt, A., and Tiedje, J. M., 2001, Community structure of denitrifiers, Bacteria, and Archaea along redox gradients in pacific northwest marine sediments by terminal restriction fragment length

polymorphism analysis of amplified nitrite reductase (nirS) and 16S rRNA genes: Applied and Environmental Microbiology, v. 67, no. 4, p. 1893-1901.
Canfield, D. E., Habicht, K. S., and Thamdrup, B., 2000, The Archean Sulfur Cycle and the Early History of Atmospheric Oxygen: Science, v. 288, p. 658-661.
Cole, J., Chai, B., Marsh, T., Farris, R., Wang, Q., Kulam, S., Chandra, S., McGarrell, D., Schmidt, T., Garrity, G., and Tiedje, J., 2003, The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy.: Nucleic Acids Research, v. 31, no. 1, p. 442-3.
Deplancke, B., Hristova, K. R., Oakley, H. A., McCracken, V. J., Aminov, R., Mackie, R. I., and Gaskins, H. R., 2000, Molecular ecological analysis of the succession and diversity of sulfate-reducing bacteria in the mouse gastrointestinal tract: Applied and Environmental Microbiology, v. 66, no. 5, p. 2166-2174.
Dhillon, A., Teske, A., Dillon, J., Stahl, D. A., and Sogin, M. L., 2003, Molecular characterization of sulfate-reducing bacteria in the Guaymas Basin: Applied and Environmental Microbiology, v. 69, no. 5, p. 2765-2772.
Drennan, G. R., Boiron, M. C., Cathelineau, M., and Robb, L. J., 1999, Characteristics of post-depositional fluids in the Witwatersrand Basin: Mineralogy and Petrology, v. 66, p. 83-109.
Friedrich, C. G., 1998, Physiology and Genetics of Sulfur-oxidizing Bacteria, *in* Poole, R. K., ed., Advances in Microbial Physiology: San Diego, Academic Press.
Friedrich, M., 2002, Phylogenetic Analysis Reveals Multiple Lateral Transfers of Adenosine-5'-Phosphosulfate Reductase Genes among Sulfate-Reducing Microorganisms: Journal of Bacteriology, v. 184, no. 1, p. 278-289.
Gihring, T. M., 2004, personal communication.
Hall, B. G., 2001a, Phylogenetic Trees Made Easy: A How-To Manual for Molecular Biologists: Sunderland, MA, Sinauer Associates.
Hall, T., 2001b, BioEdit: Raleigh, NC, Department of Microbiology, NCSU.
Hipp, W. M., Pott, A. S., ThumSchmitz, N., Faath, I., Dahl, C., and Truper, H. G., 1997, Towards the phylogeny of APS reductases and sirohaem sulfite reductases in sulfate-reducing and sulfur-oxidizing prokaryotes: Microbiology-Uk, v. 143, p. 2891-2902.
Huber, T., Faulkner, G., and Hugenholtz, P., 2004, Bellerophon; a program to detect chimeric sequences in multiple sequence alignments: Bioinformatics.
Huelsenbeck, J. P., 2000, MrBayes: Bayesian Inferences of Phylogeny: NY, University of Rochester.
Hugenholtz, P., 2002, Exploring prokaryotic diversity in the genomic era - Review: Genome Biology, v. 3, no. 2.
Lane, D. J., 1991, 16S/23S rRNA Sequencing, *in* Stackebrandt, E., and Goodfellow, M., eds., Nucleic Acid Techniques in Bacterial Systematics: Hoboken, NJ, John Wiley and Sons.
Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., and Pace, N. R., 1985, Rapid Determination of 16S Ribosomal RNA Sequences for Phylogenetic Analyses: Proceedings of the National Academy of Sciences of the United States of America, v. 82, no. 20, p. 6955-6959.
Lippmann, J., Stute, M., Torgersen, T., Moser, D. P., Hall, J. A., Lin, L., Borcsik, M., Bellamy, R. E. S., and Onstott, T. C., 2003, Dating ultra-deep mine waters with noble gases and Cl-36, Witwatersrand Basin, South Africa: Geochimica Et Cosmochimica Acta, v. 67, no. 23, p. 4597-4619.
Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A. W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüßmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A. P., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., and Schleifer, K.-H., 2002, ARB: a software environment for sequence data, submitted to Nucleic Acids Research.

Macalady, J., and Banfield, J. F., 2003, Molecular geomicrobiology: genes and geochemical cycling: Earth and Planetary Science Letters, v. 209, no. 2003, p. 1-17.
Madigan, M. T., Martinko, J. M., and Parker, J., 2003, Brock Biology of Microorganisms: Upper Saddle River, NJ, Prentice Hall.
Moser, D. P., Onstott, T. C., Fredrickson, J. K., Brockman, F. J., Balkwill, D. L., Drake, G. R., Pfiffner, S. M., and White, D. C., 2003, Temporal Shifts in the Geochemistry and Microbial Community Structure of an Ultradeep Mine Borehole Following Isolation: Geomicrobiology Journal, v. 20, p. 517-548.
Moyer, C. L., Tiedje, J. M., Dobbs, F. C., and Karl, D. M., 1998, Diversity of deep-sea hydrothermal vent Archaea from Loihi Seamount, Hawaii: Deep Sea Research Part II: Topical Studies in Oceanography, v. 45, no. 1-3, p. 303-317.
Omar, G. I., Onstott, T. C., and Hoek, J., 2003, The origin of deep subsurface microbial communities in the Witwatersrand Basin, South Africa as deduced from apatite fission track analyses: Geofluids, v. 3, p. 69-80.
Onstott, T. C., 2004a, personal communication.
-, 2004b, unpublished.
Onstott, T. C., Moser, D. P., Pfiffner, S. M., Fredrickson, J. K., Brockman, F. J., Phelps, T. J., White, D. C., Peacock, A., Balkwill, D., Hoover, R., Krumholtz, L. R., Borcsik, M., Kieft, T. L., and Wilson, R., 2003, Indigenous and contaminant microbes in ultradeep mines: Environmental Microbiology, v. 5, no. 11, p. 1168-1191.
Orphan, V. J., Hinrichs, K.-U., Ussler III, W., Paull, C. K., Taylor, L. T., Sylva, S. P., Hayes, J. M., and DeLong, E. F., 2001, Comparitive Analysis of Methane-Oxidizing Archaea and Sulfate-Reducing Bacteria in Anoxic Marine Sediments: Applied and Environmental Microbiology, v. 67, no. 4, p. 1922-1934.
Pace, N. R., 1997, A molecular view of microbial diversity and the biosphere: Science, v. 276, no. 5313, p. 734-740.
Poltz, M. F., and Cavanaugh, C. M., 1998, Bias in template-to-product ratios in multitemplate PCR: Applied and Environmental Microbiology, v. 64, no. 10, p. 3724-3730.
Reysenbach, A. L., Longnecker, K., and Kirshtein, J., 2000, Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a Mid-Atlantic Ridge hydrothermal vent: Applied and Environmental Microbiology, v. 66, no. 9, p. 3798-3806.
Speksnijder, A., Kowalchuk, G. A., De Jong, S., Kline, E., Stephen, J. R., and Laanbroek, H. J., 2001, Microvariation artifacts introduced by PCR and cloning of closely related 16S rRNA gene sequences: Applied and Environmental Microbiology, v. 67, no. 1, p. 469-472.
Takai, K., Moser, D. P., DeFlaun, M., Onstott, T. C., and Fredrickson, J. K., 2001, Archaeal diversity in waters from deep South African gold mines: Applied and Environmental Microbiology, v. 67, no. 12, p. 5750-5760.
Teske, A., Dhillon, A., and Sogin, M. L., 2003, Genomic markers of ancient anaerobic microbial pathways: Sulfate reduction, methanogenesis, and methane oxidation: The Biological Bulletin, v. 204, no. 2, p. 186.
Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D., 1997, The ClustalX-Windows interface: Flexible strategies for multiple alignment aided by quality analysis tools.: Nucleic Acids Research, v. 25, p. 4876-4882.
Viljoen, M. J., and Reimold, W. U., 2002, An Introduction to South Africa's Geological and Mining Heritage: Randburg, South Africa, MINTEK, in association with the Geological Society of South Africa.
Wagner, M., Roger, A. J., Flax, J. L., Brusseau, G. A., and Stahl, D. A., 1998, Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration: Journal of Bacteriology, v. 180, no. 11, p. 2975.

Appendix 1

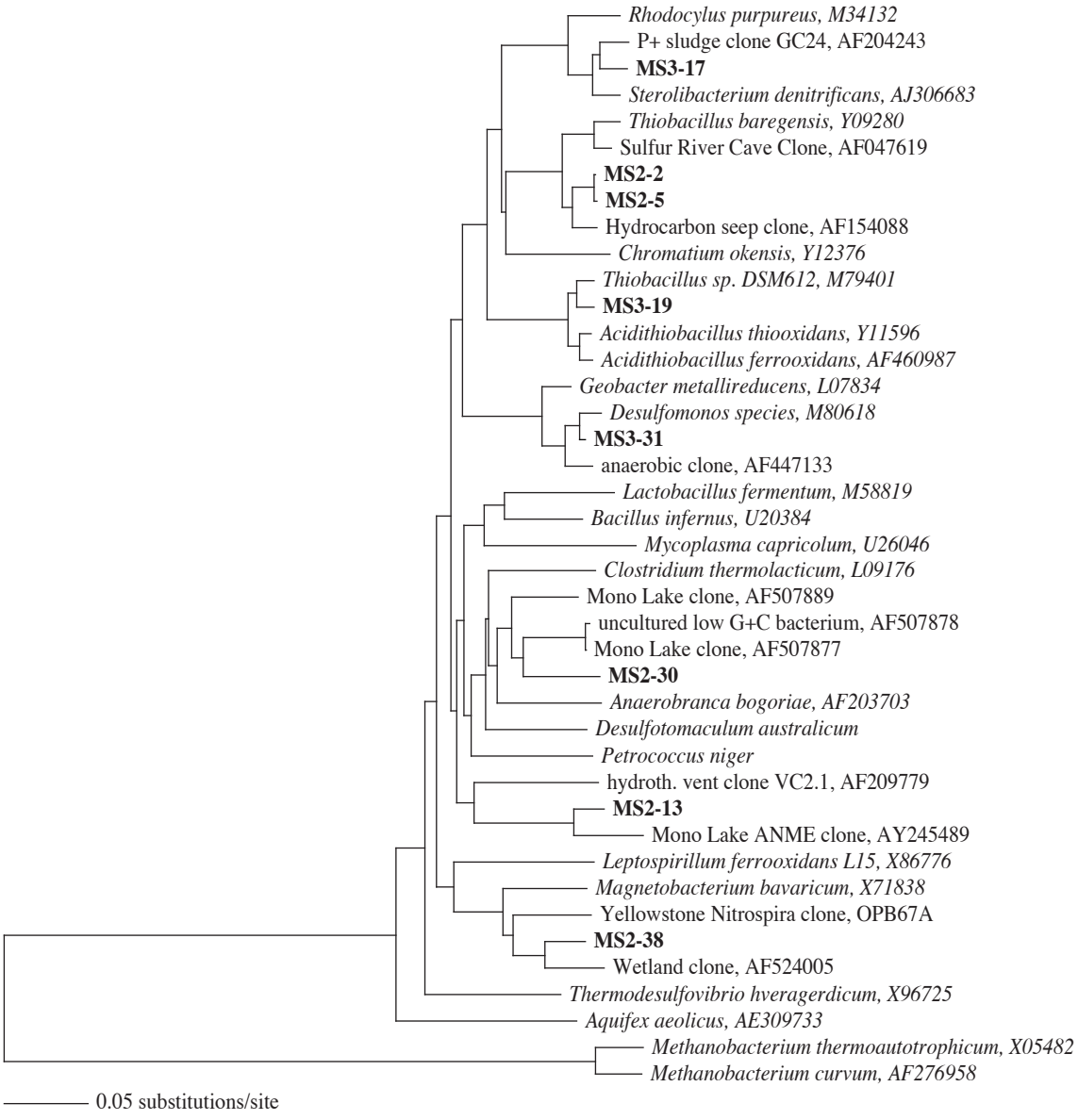


Figure A1a: Phylogenetic tree based on bacterial 16S nucleotide sequences from Merriespruit (MS) gold mine in South Africa. The tree was constructed by Leah Morgan in PAUP using neighbor joining and rooted with *Methanobacteria* as an outgroup.

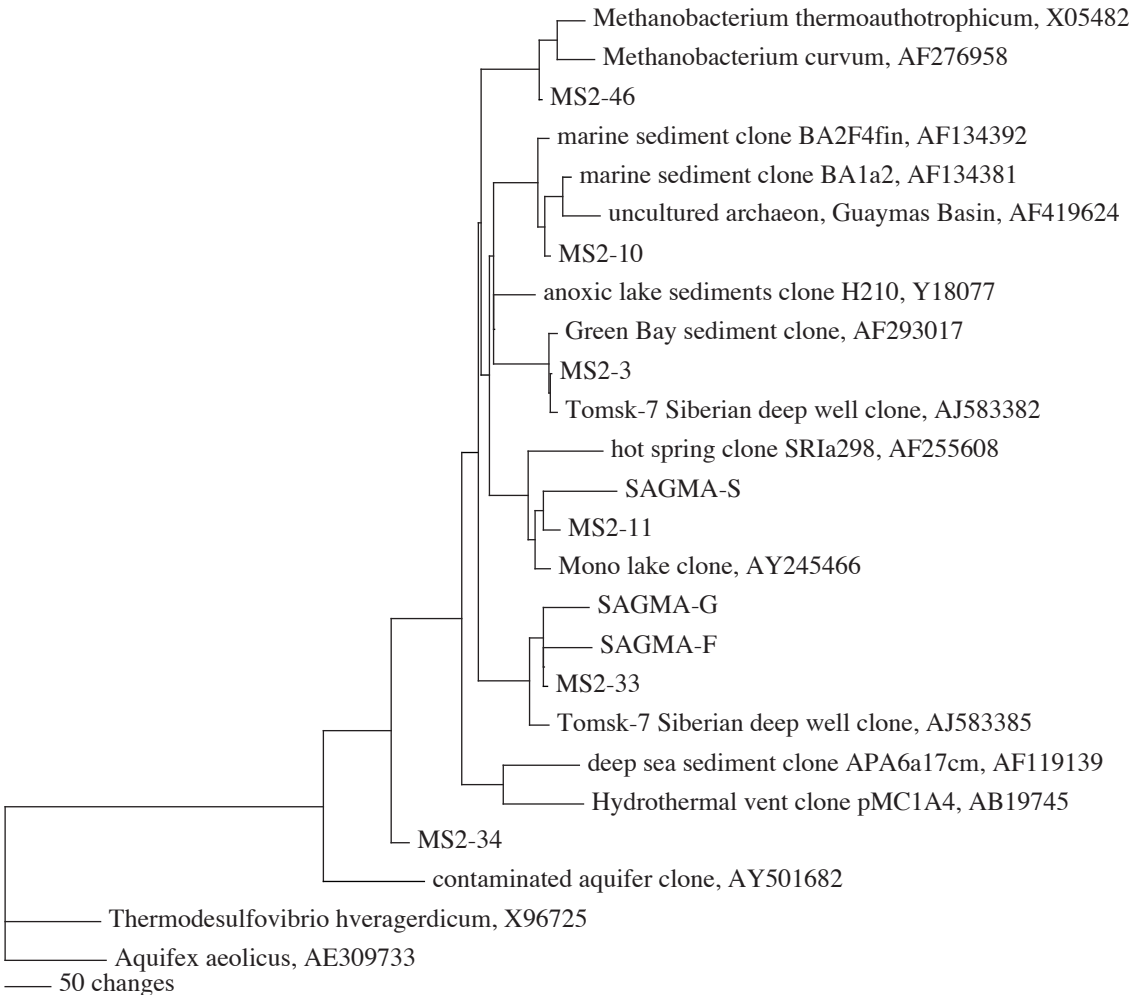


Figure A1b: Phylogenetic tree based on archaeal 16S nucleotide sequences from Merriespruit (MS) gold mine in South Africa. The tree was constructed by Tom Gihring in ARB using maximum likelihood and rooted with *Thermodesulfovibrio* and *Aquifex* as bacterial outgroups.

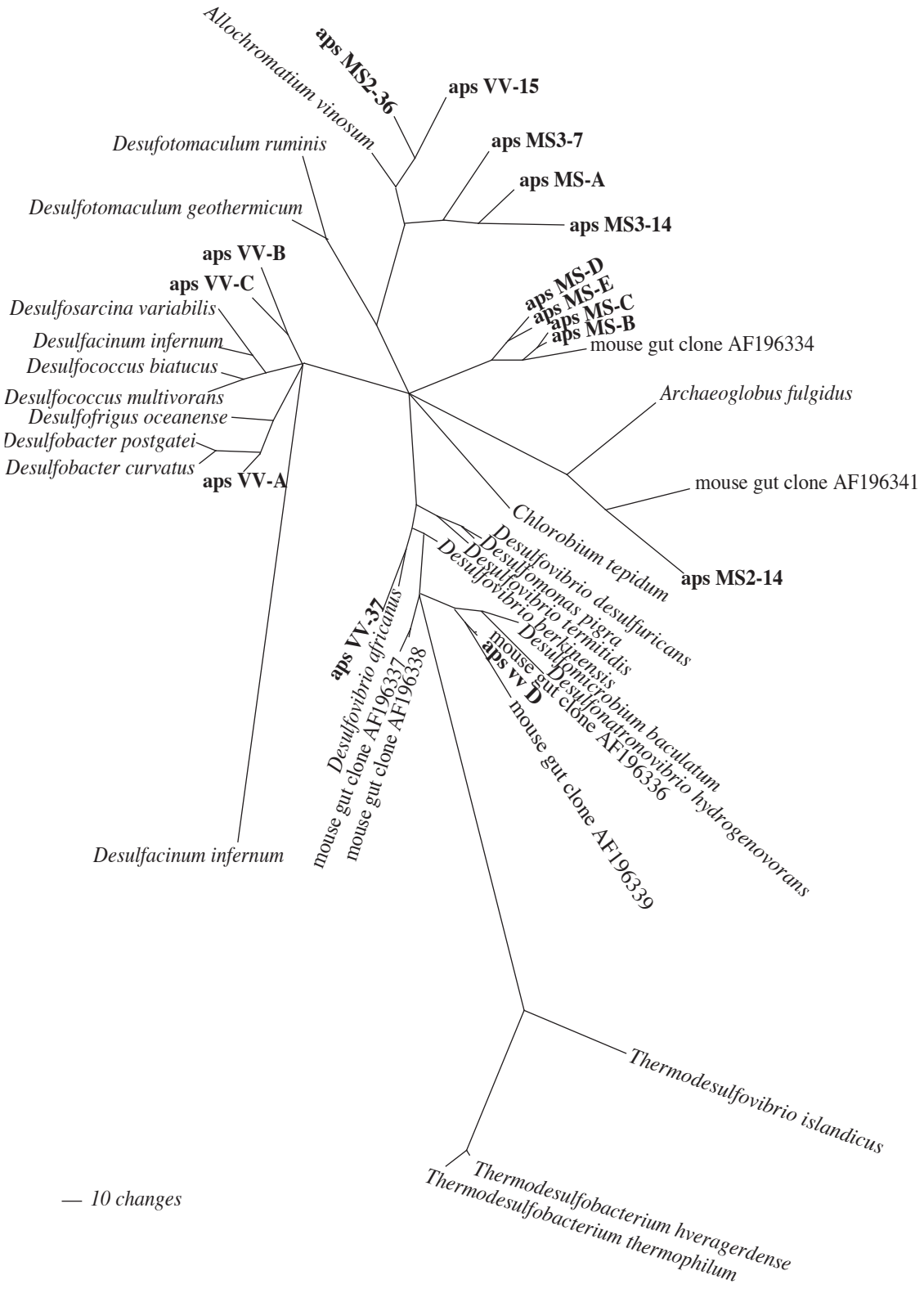


Figure A1c: Phylogenetic tree based on APS nucleotide sequences from Merriespruit (MS) and Driefontein (VV) gold mines in South Africa. This unrooted tree was constructed by Leah Morgan in MrBayes and viewed in PAUP.

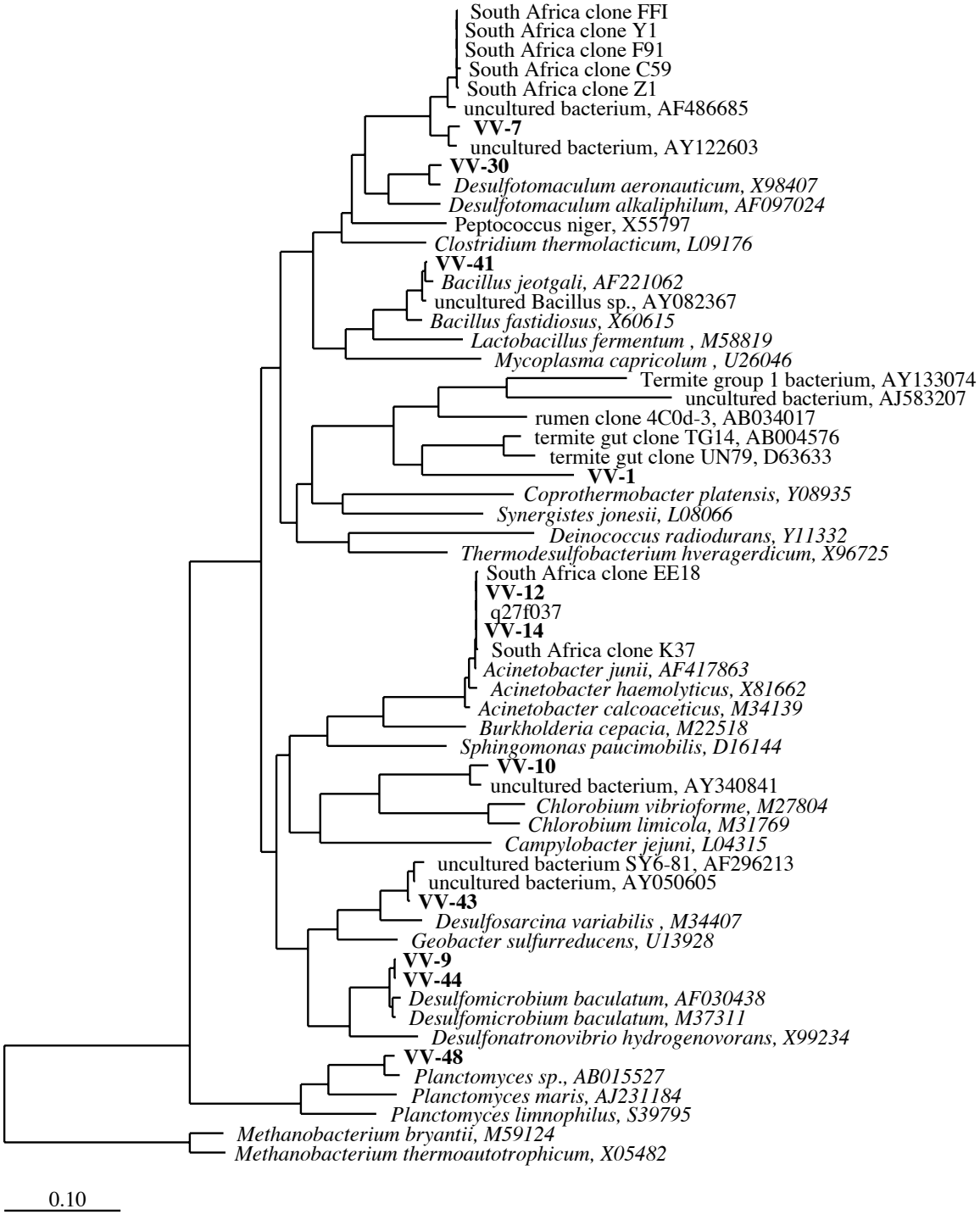


Figure A1d: Phylogenetic tree based on bacterial 16S nucleotide sequences from Driefontein (VV) gold mine in South Africa. The tree was constructed by Tom Gihring in PAUP using maximum likelihood. The tree is rooted with *Methanobacterium* as an outgroup.

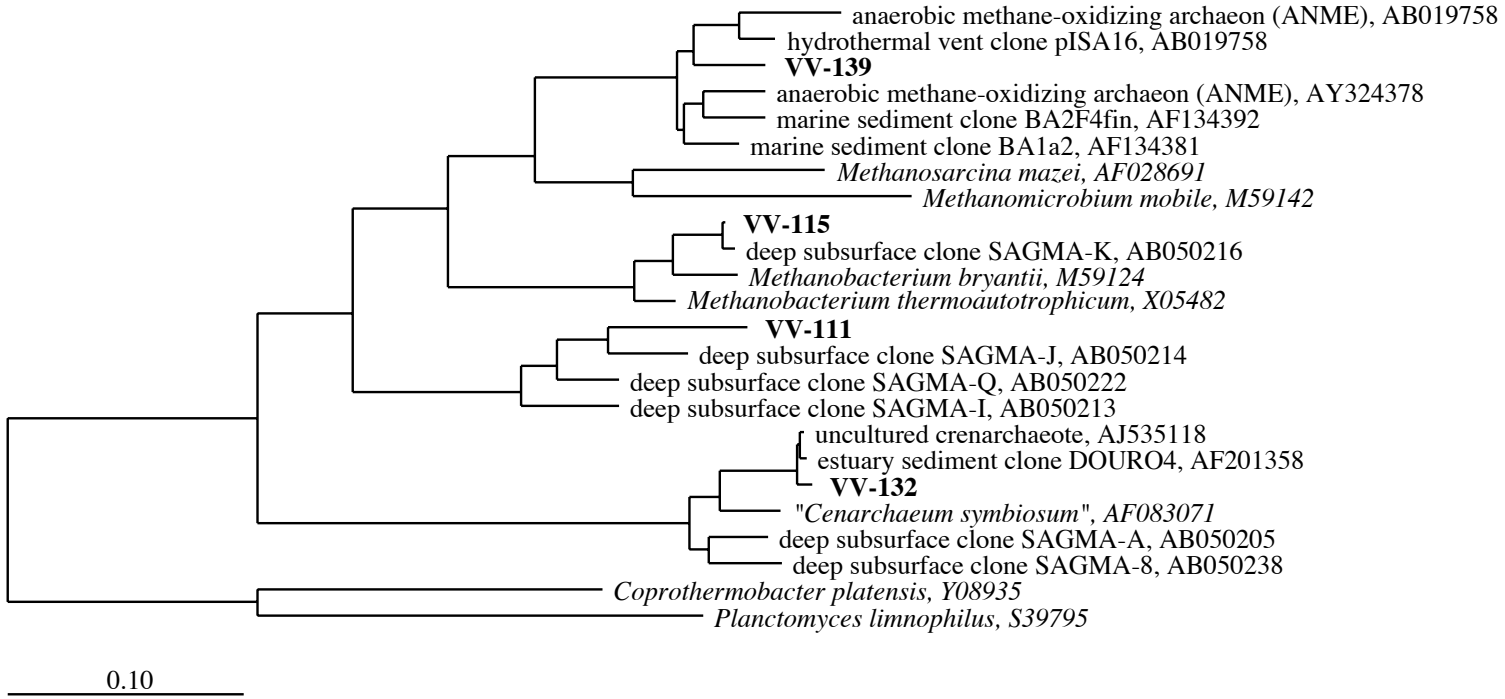


Figure A1e: Phylogenetic tree based on archaeal 16S nucleotide sequences from Driefontein (VV) gold mine in South Africa. The tree was constructed by Tom Gihring in PAUP using maximum likelihood and rooted with *Planctomyces* and *Coprothermobacter* as outgroups.