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Diversity and distribution of subseafloor Thermococcales populations in diffuse hydrothermal vents at an active deep-sea volcano in the northeast Pacific Ocean

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[1] The presence, diversity, and distribution of a key group of subseafloor archaea, the Thermococcales, was examined in multiple diffuse flow hydrothermal vents at Axial Seamount, an active deep-sea volcano located in the northeast Pacific Ocean. A polymerase chain reaction (PCR) approach was used to determine if this group of subseafloor indicator organisms showed any phylogenetic distribution that may indicate distinct subseafloor communities at vents with different physical and chemical characteristics. Targeted primers for the Thermococcales 16S rRNA (small subunit ribosomal RNA) gene and intergenic transcribed spacer (ITS) region were designed and applied to organisms filtered directly from a variety of diffuse flow vents. Thermococcales were amplified from 9 of 11 samples examined, and it was determined that the ITS region is a better phylogenetic marker than the 16S rRNA in defining consistent groups of closely related sequences. Results show a relationship between environmental clone distribution and source vent chemistry. The most highly diluted vents with elevated iron and alkalinity contained a distinct group of Thermococcales as defined by the ITS region, suggesting separate subseafloor Thermococcales populations at diffuse vents within the Axial caldera.

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1. Introduction

[2] The active circulation of hydrothermal fluids and seawater within the upper 500 m of porous oceanic crust provides a rich environment for microbial growth in the subseafloor at mid-ocean ridges. However, because of the difficulties in sampling the subseafloor environment directly, the microbial and chemical properties of venting fluids can serve as indicators of the processes and habitats beneath the seafloor. Since diffuse flow vents were first discovered, the potential relationship between their chemistry and microbial populations was recognized [*Edmond et al.*, 1979b; *Jannasch and Wirsen*, 1979]. Diffuse fluids, approximately 5–100°C, are ubiquitous at mid-ocean ridge vents and volcanoes. Water-rock reaction is the primary variable controlling vent fluid chemistry, and much of the observed chemistry in diffuse flow vents can be explained by subseafloor mixing of phase-separated hydrothermal fluids with seawater [*Butterfield et al.*, 2004; *Von Damm and Lilley*, 2004]. There is also evidence of other subseafloor processes that can modify the chemical signature of the exiting fluids, such as mineral precipitation, conductive heating of crustal seawater, conductive cooling of hydrothermal vent fluid, and biologically mediated reactions [*Butterfield et al.*, 2004; *Edmond et al.*, 1979b; *Jannasch and Mottl*, 1985; *Lilley et al.*, 1984; *Von Damm and Lilley*, 2004]. Possible microbiological signatures in vent fluids include the consumption of oxygen, sulfide, nitrate, sulfate, hydrogen, and oxygen, as well as the production of ammonia and methane [*Butterfield et al.*, 2004; *Edmond et al.*, 1979a; *Jannasch and Mottl*, 1985; *Lilley et al.*, 1984; *Von Damm and Lilley*, 2004]. In addition, thermophilic and hyperthermophilic methanogens and heterotrophs cultured from low-temperature diffuse fluids are viewed as indicator organisms of hot subseafloor habitats because they usually have a minimum growth temperature that is higher than the temperature of the fluids sampled [*Holden et al.*, 1998; *Holland et al.*, 2004; *Summit and Baross*, 1998, 2001].

[3] Owing to their ability to grow well in artificial media, the Thermococcales (*Pyrococcus*, *Thermococcus*, and *Palaeococcus* spp.) is one of the most thoroughly studied groups of cultured organisms from high-temperature environments, including marine hydrothermal vents, solfatara, oil reservoirs, and freshwater thermal pools [*Zillig and Reysenbach*, 2001]. Most have a fermentative metabolism, require multiple amino acids for growth, and are anaerobic

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thermophiles or hyperthermophiles [*Zillig and Reysenbach*, 2001]. However, the Thermococcales also exhibit tremendous metabolic versatility, such as the ability to grow lithotrophically on CO coupled with H_2 production [*Sokolova et al.*, 2004], form biofilms [*Reysenbach et al.*, 2000; *Rinker and Kelly*, 2000], and grow over wide temperature and organic carbon concentration ranges [*Holden et al.*, 2001; *Summit and Baross*, 2001]. Syntrophic relationships with subseafloor autotrophic primary producers, such as methanogens and the Aquificales, may also help support Thermococcales populations [*Bonch-Osmolovskaya and Stetter*, 1991; *Rinker and Kelly*, 2000]. Additionally, members of the Thermococcales have a high tolerance to oxygen exposure and show long-term survival at cold temperatures, characteristics thought to be important for their dispersal and wide distribution, as well as their dominance in culture collections from deep-sea hydrothermal vents [*Jannasch et al.*, 1992; *Stetter*, 1989].

[4] Using cultured isolates, the distribution, and genetic and phenotypic diversity of Thermococcales populations have been studied from different biotopes at deep-sea hydrothermal vents [*Holden et al.*, 2001; *Lepage et al.*, 2004; *Marteinsson et al.*, 1995; *Summit and Baross*, 2001; *Wery et al.*, 2002]. Furthermore, using PCR-based assessments with the 16S rRNA gene, a highly conserved gene found in all living prokaryotes, *Thermococcus* spp. have been detected on enrichment surfaces in deep-sea vent fluids (in situ growth chambers) [*Nakagawa et al.*, 2005; *Nercessian et al.*, 2003; *Reysenbach et al.*, 2000; *Takai et al.*, 2004a] and black smoker chimneys and plumes from Manus Basin, the Juan de Fuca Ridge, Central Indian Ridge, and the Okinawa Trough [*Hoek et al.*, 2003; *Nakagawa et al.*, 2005; *Schrenk et al.*, 2003; *Takai et al.*, 2001; *Takai et al.*, 2004b]. However, the few microbial diversity studies of deep-sea hydrothermal vent fluids without any enrichment on surfaces have not detected the Thermococcales [*Huber et al.*, 2002; *Takai and Horikoshi*, 1999]. Another genetic marker, the 16S–23S rDNA intergenic transcribed spacer (ITS) region, has also proved useful in examining microbial diversity and distribution in marine environments. Owing to its high sequence and length variability, the ITS region is often used in phylogenetic studies to identify closely related species and strains [*García-Martínez et al.*, 1999; *Gürtler and Stanisich*, 1996]. Currently, the ITS region is being used to examine ecotypes and environmental niche adaptation in marine prokaryotic population studies [*Garcia-Martinez et al.*, 2002; *García-Martínez and Rodríguez-Valera*, 2000; *Holden et al.*, 2001; *Rocap et al.*, 2002; *Summit and Baross*, 2001].

[5] The purpose of this study was to apply PCR-based environmental sequence analysis to one of the key group of vent hyperthermophiles, the Thermococcales, to assess their presence, diversity, and distribution in low-temperature hydrothermal vent fluids at an active deep-sea volcano, Axial Seamount. We used both the 16S rRNA gene and ITS region to determine if the Thermococcales showed any phylogenetic distribution that may indicate distinct subseafloor communities at vents with different chemical characteristics. The results show that Thermococcales are widespread in diffuse vents at Axial Seamount, and that environmental sequences could be clustered to specific vents, suggesting that distinct subseafloor communities may exist at individual diffuse vents.

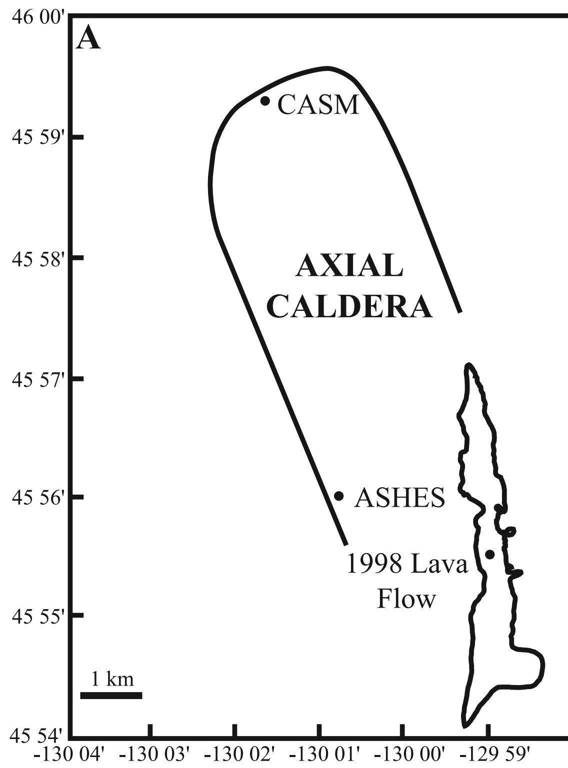
2. Study Site and Methods

[6] Located 480 km off the coast of Oregon at a depth of 1520 m on the Juan de Fuca Ridge, Axial Seamount (45° 58′ N; 130° 00′ W) is an active submarine volcano (Figure 1). The caldera of the volcano (3 × 8 km) lies between two rift zones to the north and south and is bordered on three sides by a boundary fault, and active long-term venting is located along these rift zones and near the boundary fault [*Johnson and Embley*, 1990]. Three distinct hydrothermal fields exist at Axial in the caldera; at the CASM site to the north [*Canadian American Seamount Expedition*, 1985], at the ASHES site to the southwest [*Embley et al.*, 1990], and within the 1998 lava flow along the southeast portion of the caldera [*Baker et al.*, 1999] (Figure 1). Other vents exist in the southern portion of the caldera, including a region just west of the 1998 lava flow, termed pre-1987 lava flow.

[7] The systematic sampling and analyses of a variety of diffuse flow vents at Axial since the eruption show that these vents are chemically and geologically diverse and support a microbial subseafloor community, including hyperthermophilic methanogens and heterotrophs and potential nitrogen fixers [*Butterfield et al.*, 2004; *Huber et al.*, 2002, 2003; *Mehta et al.*, 2003]. Because of the nearshore location, the presence of well-characterized hydrothermal systems, and the abundance of diffuse venting, Axial Seamount is a readily accessible and excellent site for studying the subseafloor in young oceanic crust and examining linkages between the geochemical environment and subseafloor microbial communities.

2.1. Sample Collection

[8] Using the *ROV* ROPOS, basalt hosted diffuse fluids were collected in 1998, 1999, 2000, and 2001 from vents throughout the caldera, as indicated in Tables 1 and 2 and Figure 1. Fluids were sampled using the Hydrothermal Fluid and Particle Sampler (HFPS), which pumps vent fluids through a titanium intake nozzle and measures the temperature of the fluid throughout the sampling period. This sampler allows for the collection of both whole fluids for chemical analyses, cell counts, and culturing, as well as in situ filtered fluids for DNA-based and chemical analyses. Filtered and unfiltered fluids were sampled at a vent after a stable temperature was reached on the intake probe, and fluids were pumped at a known rate until the desired volume was collected. Temperature and volume of fluid collected was monitored throughout the 10–15 minute sampling time required to obtain approximately one liter of fluid. For filtered samples, fluid was pumped through either a 47 mm Millipore (3 μm pore size) cellulose ester filter, followed by a Sterivex-GP (0.22 μm pore size) filter, or only the >0.22 μm fraction was isolated with a Sterivex-GP filter unit. Different size filter units were used owing to both space constraints on the sampler and multiple experimental needs for each sample. A background (no detectable hydrothermal plume) seawater sample from 2540 m depth (47°42′N, 127°38′W) was collected using a 10 liter Niskin Bottle mounted on a CTD (conductivity, temperature, depth) and filtered through a sterile 47 mm 0.22 μm pore



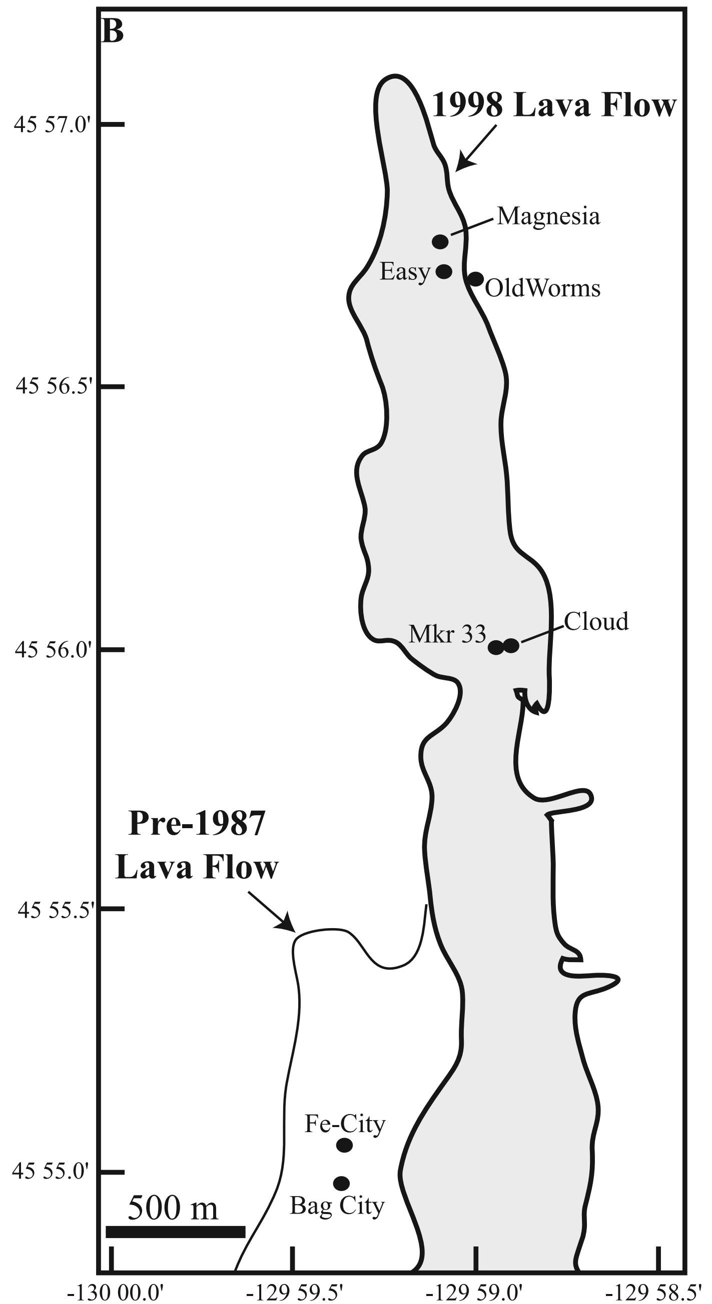


Figure 1. Map of Axial Seamount showing (a) caldera and three main vents fields and (b) individual vents from the southeast portion of the caldera. Figures were modified from Bill Chadwick.

Table 1. Chemical Characteristics of Fluid Samples Taken in Parallel With DNA Samples

Sample	Vent	Avg Tmax, °C	Avg Tavg, °C	Avg pH	Avg Alk, meq/kg	Avg Si, mmol/kg	Avg H_2S, mmol/kg	End-Member Chloride, mmol/kg	Avg SO_4, mmol/kg	Max Fe, μmol/kg	Avg Mn, μmol/kg	Avg Li, μmol/kg	Avg Cs, nmol/kg	Avg Mg, mmol/kg	Avg U, nmol/kg
FS011	Marker 33	45.7	33.0	5.03	2.42	1.21	1.79	86	25.92	1.68	17.75	39.6	7.13	49.08	7.96
FS016	Marker 33	55.0	nd	5.01	2.21	2.31	1.78	250	23.45	2.15	23.60	49.6	12.76	43.82	9.70
FS047	Marker 33	29.0	26.8	5.80	2.29	1.29	0.12	415	25.41	1.78	nd	43.6	9.71	46.19	8.36
FS009	Easy	10.2	10.2	5.82	3.33	0.37	0.21	533	27.75	56.40	7.08	27.8	2.50	52.18	12.03
FS024	Magnesia	4.5	4.4	6.30	2.50	0.19	0.01	534	27.44	3.06	0.27	25.4	2.42	52.25	12.67
FS062	Old Worms	12.2	12.0	5.75	2.73	0.54	0.28	531	27.29	7.63	1.85	36.1	2.68	51.72	6.49
FS068	Marshmallow	208.2	188.7	4.55	1.18	10.40	3.68	no data	7.65	4.42	95.70	151.0	44.59	12.27	3.29
FS075	Fe-City	8.5	7.5	7.03	2.84	0.39	0.00	534	27.33	21.60	2.91	32.6	2.70	51.80	11.42
FS076	Bag City	19.3	18.7	6.56	2.46	0.84	0.18	530	26.53	0.37	4.84	49.4	5.24	50.34	9.97
FS045	Cloud	15.9	15.8	5.95	2.31	0.61	0.12	no data	27.17	15.50	8.09	47.3	4.39	46.07	12.87
FS067	Gollum	17.8	16.0	5.82	2.54	0.53	0.16	533	27.44	0.20	5.38	34.7	4.14	52.35	11.75
CTD005	Seawater	2.0	2.0	7.80	2.50	0.17	0	542	28.20	0.01	0.01	26.6	2.10	52.90	13.00

size filter shipboard. All filters for DNA extraction were placed in 50 mL sterile Falcon tubes and stored at −80°C.

2.2. Fluid Analyses: Chemistry, Counts, and Culturing

[9] Vent fluids were analyzed as described by *Butterfield et al.* [2004]. Fluids collected with the HFPS were analyzed shipboard for H_2S, pH, and dissolved silica. On shore, fluids were analyzed for major, minor, and trace elements. Collected fluids were inoculated into various anaerobic enrichment media for most-probable-number semiquantitative enrichments of heterotrophic thermophiles and hyperthermophiles [*Greenberg et al.*, 1992]. Media formulations and enrichment methods were as previously described [*Holden et al.*, 1998]. Cultures were incubated at 90 and 70°C until visibly turbid or for a week. An 18-mL aliquot of each fluid sample was preserved in formaldehyde (3.7% final concentration) in duplicate, stored at 2°C, and counted by epifluorescence microscopy with DAPI (4′, 6-diamidino-2-phenylindole, Sigma) [*Porter and Feig*, 1980].

2.3. Environmental DNA Extraction and Purification

[10] DNA was extracted and purified from environmental fluids as previously described [*Huber et al.*, 2002].

2.4. Primer Design and Testing

[11] A forward PCR primer for the 16S rRNA gene was designed to amplify selected members of the order Thermococcales, including *Pyrococcus*, *Thermococcus*, and *Palaeococcus* spp. A segment of the gene with near consensus was targeted on the basis of an oligonulceotide probe designed for fluorescence in situ hybridization analyses [*Schrenk et al.*, 2003]. Approximately 20 16S rRNA sequences for the Thermococcales were compiled from the GenBank database via the National Center for Biotechnology Information (NCBI) website and aligned with 60 lab isolates of the Thermococcales from a variety of hydrothermal vent environments in the BioEdit sequence alignment editor [*Hall*, 1999]. The previous probe, Tc589F, was modified with the insertion of a Y (serving as C or T), which expanded its matches to include *Palaeococcus* spp. The modified primer was submitted to the Probe Match function of the Ribosomal Database Project II [*Cole et al.*, 2003] to check for self-complementarities and matches in the database. The new primer only matched with members of the Thermococcales and was designated Tc589FII. The sequence for the primer is 5′-GCC YGT AAG TCC CTG

Table 2. Source of DNA Samples and Summary of Results From Microscopic, Culturing, and Molecular Analyses

Sample	Vent	Dive	Year	Size Fraction, μm	Vent Field	Volume (L)[a]	Fluid, cells/mL	±95% Conf, cells/mL	Cultures[b]	MPN Results,[c] cells/L	Percent[d]	PCR[e]	Number of Clones Sequenced
FS011	Marker 33	R473	1998	>3.0	1998 Lava Flow	1.5	2.24E+05	1.61E+04	+	≧48,000	≥0.021	+	72
FS016	Marker 33	R485	1999	>3.0	1998 Lava Flow	1	1.12E+05	3.82E+03	+	3000–96,000	≤0.086	+	64
FS047	Marker 33	R551	2000	>3.0	1998 Lava Flow	1	1.23E+05	1.59E+04	+	≥48,000	≥0.004	+	54
FS009	Easy	R473	1998	>3.0	1998 Lava Flow	1	1.19E+05	2.56E+04	+	no data	no data	+	72
FS024	Magnesia	R488	1999	>3.0	1998 Lava Flow	1	5.02E+05	3.04E+04	+	80–2400	≤0.0005	+	63
FS062	Old Worms	R622	2001	>0.2	E of 1998 Lava Flow	1	2.38E+05	2.03E+04	+	no data	no data	+	55
FS068	Marshmallow	R624	2001	>0.2	ASHES	1	3.00E+04	8.02E+03	+	80–2400	≤0.008	+	40
FS075	Fe-City	R627	2001	>0.2	Pre-1987 Lava Flow	1	1.59E+05	1.83E+04	+	60–880	≤0.0006	+	55
FS076	Bag City	R627	2001	>0.2	Pre-1987 Lava Flow	1	3.26E+05	2.45E+04	+	no data	no data	+	51
FS045	Cloud	R547	2000	>3.0	1998 Lava Flow	1	1.19E+05	1.74E+04	+	720–26,000	≤0.002	-	no data
FS067	Gollum	R624	2001	>0.2	ASHES	1	2.01E+05	2.20E+04	+	60–880	≤0.0004	-	no data
CTD005	Seawater	CTD	2002	>0.2	47°42′N, 127°38′W	3	3.97E+04	5.97E+03	-	ND	ND	-	no data

[a]Volume of fluid filtered.
[b]Positive enrichment of thermophiles or hyperthermophiles from indicated vent.
[c]For 0.3% yeast extract and peptone with (YPS) or without (YP) elemental sulfur; Ar headspace; at 70 or 90°C; ND, none detected.
[d]Percent MPN cultured microbes represent of total microbial population.
[e]Positive PCR product with Tc589FII and 64Ra primer set.

GCG-3′. A similar but longer primer designed to be used in the reverse direction was recently published for identifying hyperthermophilic archaea of the family *Thermococcaceae* [*Slobodkina et al.*, 2004].

[12] Primer Tc589FII was paired with primer 64Ra (5′-GCC NRG GCT TAT CGC AGC TT-3′) targeting the 23S rRNA [*Summit and Baross*, 2001]. This combination allows for amplification of approximately 1000 bases from the 16S rRNA gene and the complete ITS region. The primer set was then tested on 30 different members of the Thermococcales, including 25 isolates from Axial Seamount and other Juan de Fuca Ridge hydrothermal environments, as well as *T. littoralis*, *T. peptonophilus*, *T. celer*, *and T. barophilus*. Successful amplification revealed a band of approximately 1250 base pairs. The primers were also applied to DNA from bacterial species, including *Escherichia coli*, *Halomonas* spp., *Thermosipho* spp., and *Desulfuorobacterium* spp., as well as two *Methanococcus* species. No amplification product was observed. A variety of PCR conditions were tested with different annealing temperatures and magnesium chloride concentrations to find the optimal set of PCR conditions for amplification of isolate DNA. Each PCR reaction (25 μl) contained 5% acetamide, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates, 50 ng (each) primer, 1X PCR Buffer (Promega), and 1 U of *Taq* DNA Polymerase (Promega). The profile was an initial denaturation step of 2 min at 94 °C, followed by 30–36 cycles of 94°C for 30 s, 59°C for 45s, and 72°C for 2 min. The final extension step was 72°C for 10 min.

2.5. Environmental PCR and Clone Library Construction

[13] The primers were applied to the environmental DNA samples listed in Tables 1 and 2. If amplification was observed, replicate positive amplification products were reconditioned to minimize heteroduplexes [*Thompson et al.*, 2002]. To recondition, 1 μL of positive PCR product was placed into a fresh cocktail as template and put through 3 more cycles of amplification, including both the hot start and final extension. The separately reconditioned PCR products were then consolidated and cleaned using the Qiaquick PCR purification columns (Qiagen) according to the manufacturers instructions, and visualized on 1% (w/v) agarose gels stained with SYBR green (Molecular Probes). PCR bands of the correct size were gel extracted using the Qiaquick Gel extraction columns (Qiagen) according to the manufacturer's instructions. Final PCR products were cloned according to *Huber et al.* [2003]. In some cases, if no product was observed with the Tc589FII and 64Ra primer set, a nested PCR approach was applied. PCR was performed on DNA with the universal archaea specific primer 21F (5′-TTC CGG TTG ATC CYG CCG GA-3′) [*DeLong*, 1992] targeting the 16S rRNA and the 23S rRNA primer 64Ra. Each PCR reaction (25 μl) contained 5% acetamide, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates, 50 ng (each) primer, 1X PCR Buffer (Promega), and 1 U of *Taq* DNA Polymerase (Promega). An initial denaturation step of 2 min at 94 °C was followed by 15, 20, 25, and 30 cycles of 94°C for 30 s, 62°C for 45s, and 72°C for 3 min. The final extension step was 72°C for 10 min. The bands were visualized and quantified on 1% (w/v) agarose gels stained with SYBR green (Molecular Probes). The lowest possible cycle number for which a band was visible was then chosen as the template (1 μL) for the second round of PCR with Tc589FII and 64Ra using the profile as previously described. Again, the lowest number of cycles (15–25 cycles) for successful amplification was used for cloning as visualized on 1% (w/v) agarose gels. PCR products were reconditioned, pooled, gel extracted, and subsequently cloned according to *Huber et al.* [2003].

2.6. Sequencing

[14] Clones were randomly chosen for complete sequencing of the insert with PCR primers and 958R (5′-YCC GGC GTT GAM TCC AAT T-3′) [*DeLong*, 1992], 956Fa (5′-TTA ATT GGA KTC AAC GCC GG-3′), 1492Ra (5′-GGY TAC CTT GTT ACG ACT T-3′), and 1492Fa (5′-AAG TCG TAA CAA GGT ARC CGT-3′) [*Summit and Baross*, 2001]. The DYEnamic ET Dye Terminator Kit (Amersham Pharmacia Biotech Inc.) with subsequent analysis on a MegaBACE 1000 (Molecular Dynamics) was used for sequencing.

2.7. Phylogenetic and Cluster Analysis

[15] Sequencher was used to assemble sequences, and the contiguous sequences were manually aligned and manipulated in the BioEdit v4.7.8 Program Sequences [*Hall*, 1999]. Sequences that stood out in the alignment as possible chimeras or members of a different order were noted. Each clone was then divided into the 16S rRNA and ITS region, and treated separately for the remainder of the analyses. To find closely related sequences for phylogenetic analysis, all sequences were submitted to the Advanced BLAST search program (available through the National Center for Biotechnology Information), and closest matches added to the alignment. All 16S rRNA gene sequences were submitted to the Bellerophon server for chimera detection [*Hugenholtz and Huber*, 2003]. Maximum-likelihood phylogenetic trees were constructed using the Hasegawa-Kishino-Yano evolutionary model [*Hasegawa et al.*, 1985] with 50,000 puzzling steps and with mutation rates estimated from the data sets using TREE-PUZZLE version 5.0 [*Schmidt et al.*, 2002]. Branches were collapsed for quartet-puzzling support values of <50% and graphed using TreeView version 1.6 [*Page*, 1996].

[16] The ITS region and 16S rRNA gene were treated separately in phylogenetic analyses owing to their different levels of sequence variability. For the ITS, sequence identity matrices were made for each sample, and clones with exactly 100% sequence match were identified. A representative clone for each 100% identity group was then chosen and individual trees for each vent constructed. Repetitive groups with the same culture outgroups were identified from all 9 sample trees, and a final consensus tree was constructed to depict the major groups found, with a representative clone from each sample and each group included (Figure 2). For the 16S rRNA gene, the same 100% identity procedure was carried out; however, because all clones were 94–100% identical, it was determined that the 100% sequence identity cutoff did not allow for enough resolution in the individual sample trees for comparison between samples. Therefore a $\geq$99% similarity index was applied to group together clones within each sample, and a final tree constructed with a representative of each $\geq$99%

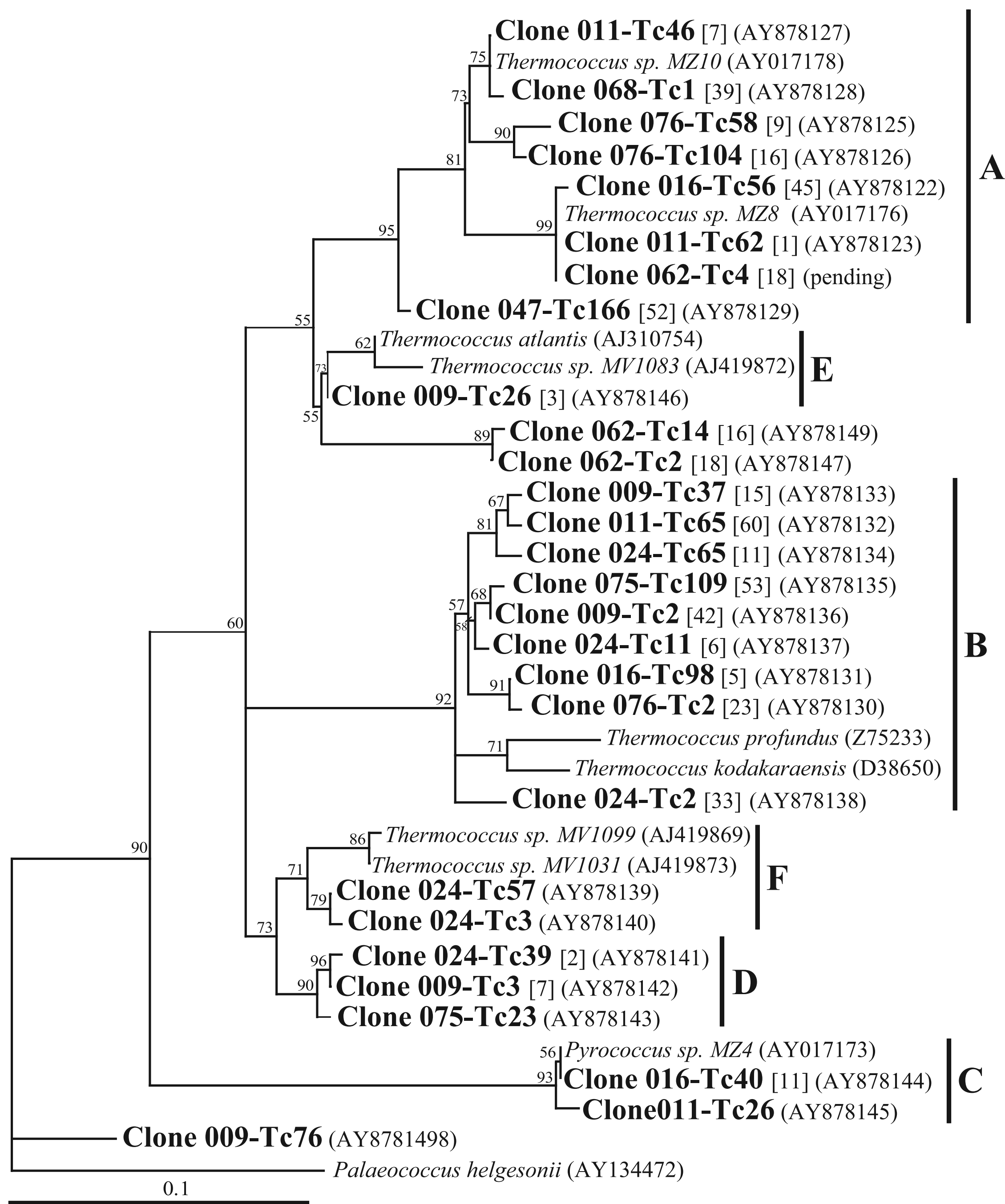


Figure 2. Maximum-likelihood quartet puzzling phylogenetic tree of cloned ITS regions for the Thermococcales. Tree shows representatives from each sample for 100% sequence identity groups, with the number of clones from that library that fall into the same group in brackets. Clones from this study are noted in bold type face and refer to specific samples and clone number. Accession numbers for all cultures and clones are indicated in parentheses. The complete ITS region (241–261 bp) was used in the alignment. Quartet puzzling support values are shown at each internal branch. The scale bar represents the expected number of changes per nucleotide position.

similarity group from each sample included [*Acinas et al.*, 2004a]. From this tree, groups were identified and their distribution among samples noted (Figure 3).

[17] A cluster analysis was performed on the microbial groups (A–F for ITS, Figure 2, and C–I for 16S, Figure 3) found at each vent using Jaccard's coefficient. A matrix was constructed where designated groups were scored for presence or absence in each vent. Jaccard's coefficient was calculated, and a cluster diagram of the samples was constructed using the unweighted pair group method using

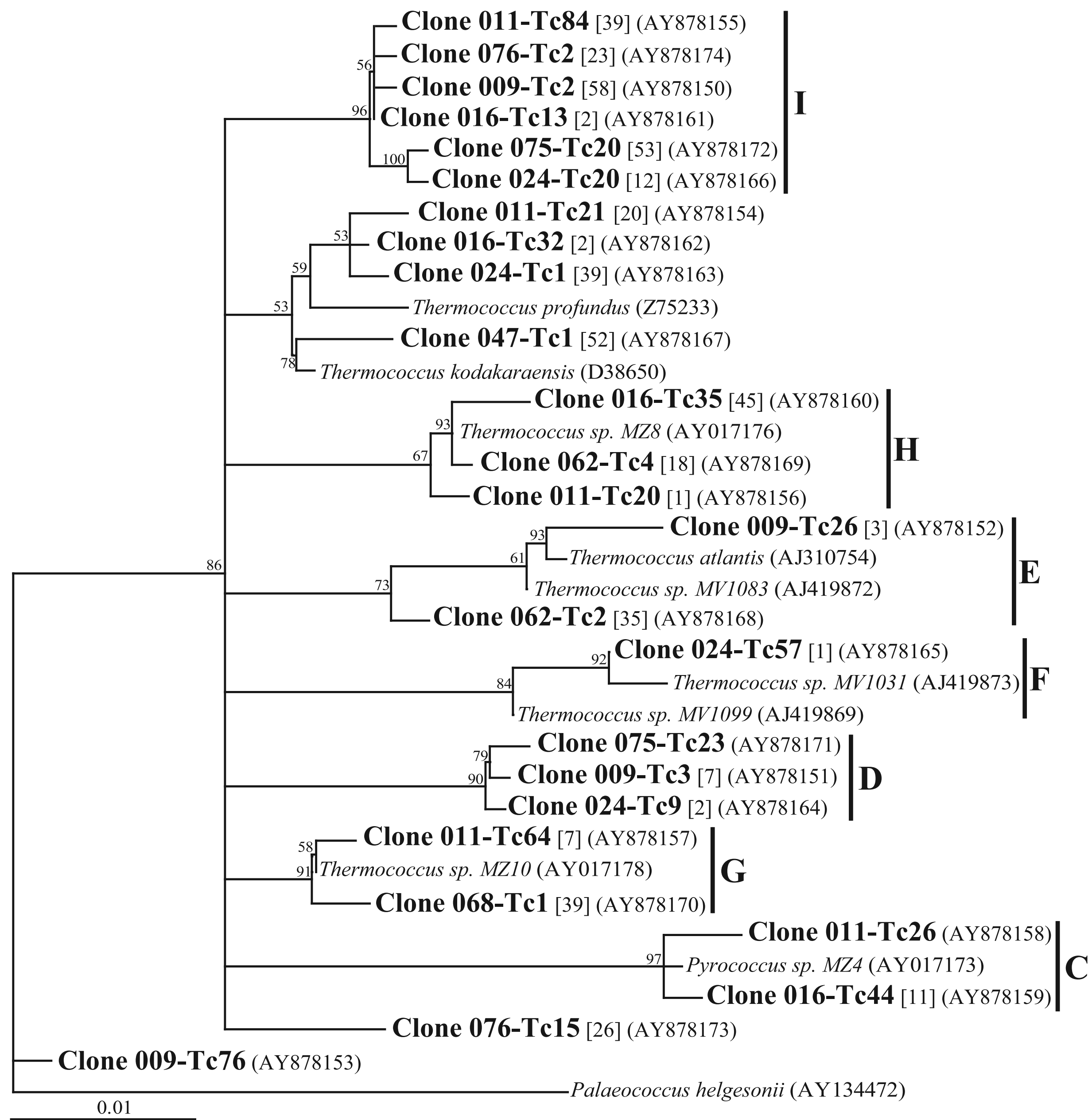


Figure 3. Maximum-likelihood quartet puzzling phylogenetic tree of cloned 16S rRNA genes for the Thermococcales. Tree shows one representative from each sample for ≥99% sequence identity groups. Clones from this study are denoted in bold type face and refer to specific samples and clone number, with the number of clones they represent from that library in brackets. Accession numbers for all cultures and clones are indicated in parentheses. The complete amplified 16S rRNA (931–943 bp) was used in the alignment. Quartet puzzling support values are shown at each internal branch. The scale bar represents the expected number of changes per nucleotide position.

arithmetic averages in Phylip 3.65 (obtained from J. Felsenstein, University of Washington, Seattle) (Figure 4). Clone sequences are deposited in GenBank under accession numbers AY878122–AY878174.

3. Results

[18] Vent fluids were collected from 11 different hydrothermal vents located throughout the Axial caldera (Figure 1). Some chemical and microbiological characteristics of the vent sites are given in Tables 1 and 2. The samples analyzed for this study provide a cross section of the type of diffuse vents seen at Axial. These included: Marshmallow vent (FS068), an anhydrite-dominated high-temperature vent in the ASHES field with a hot fluid source just below the seafloor; Gollum vent (FS067), a stable low-temperature vent in the ASHES field; Bag City, Old Worms, and Fe-City (FS076, FS062, and FS075), stable diffuse vents outside of

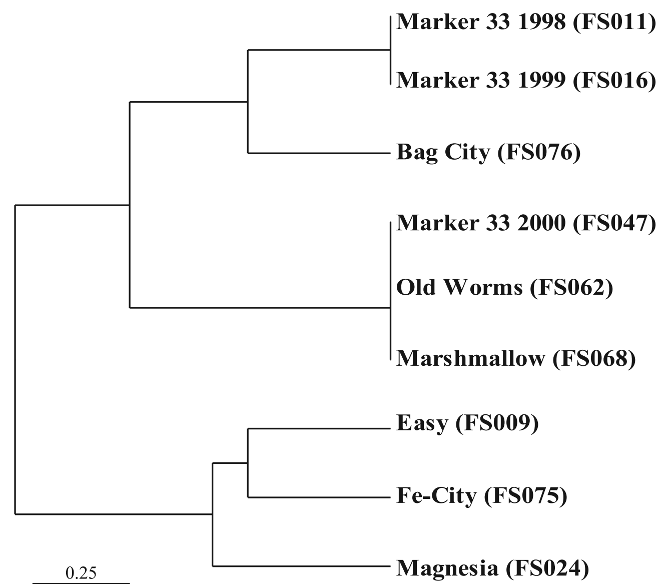


Figure 4. Cluster diagram showing distribution of Thermococcales groups at individual vents based on ITS region.

the 1998 eruption zone; Marker 33 and Cloud (FS045), stable vents within the boundary of the 1998 lava flow; and Magnesia (FS024) and Easy vent (FS009), short-lived low-temperature "snow-blower" vents that died out within two years of the 1998 eruption. Details of the geological setting, chemical composition, and chemical indications of microbial activity in and near the eruption zone have been presented previously [*Butterfield et al.*, 2004]. Prior results indicate the following microbial processes in the subseafloor: oxidation of sulfide and hydrogen; reduction of nitrate, sulfur, and iron; and methanogenesis [*Butterfield et al.*, 2004]. In addition, in some diffuse vent fluids more methane is present than can be produced through methanogenesis by reaction of primary hydrogen (<1 mmol/kg) in representative high-temperature source fluids with the abundant carbon dioxide (>100 mmol/kg) in those same fluids, suggesting fermentation of organic matter, the primary metabolism of the Thermococcales (data not shown) [*Butterfield et al.*, 2004]. The three lowest-temperature vents examined in this study, Easy, Magnesia, and Fe-City, show distinct chemical characteristics. These vents have the highest average Mg content (Table 1 and Figure 5a), indicating that they contain the least amount of high-temperature hydrothermal end-member. This is supported by all of the indicators of mixing. Easy, Magnesia and Fe-City have the highest seawater components (Mg, SO_4, U) and the lowest hydrothermal components (Si, Li, Cs) (Table 1). The higher degree of subseafloor mixing with seawater at these vents is one factor that can help explain their differences compared to other vents, but in addition, there are significant chemical differences that cannot be explained simply by the degree of mixing. Although Easy, Magnesia, and Fe-City vents are the coolest and most highly diluted, they have among the highest iron concentrations of any of the diffuse vents (Table 1) and by far the highest iron content when normalized to heat content (Figure 5). In addition, these three vents are distinguished by alkalinities higher than ambient seawater, while all of the other vent fluids in this study have alkalinities less than seawater (Figure 5b). The high dissolved iron and increased alkalinity is consistent with mobilization of iron at relatively low temperature, probably owing to reduction of the ubiquitous ferric iron precipitates that exist around the margins of diffuse hydrothermal areas on Axial Volcano [*Butterfield et al.*, 2004; *Kennedy et al.*, 2003].

[19] Positive enrichment cultures were obtained for heterotrophic thermophiles or hyperthermophiles from all hydrothermal fluids sampled at Axial Seamount (Table 2). Most-probable-number estimates of thermophilic and hyperthermophilic heterotrophs indicate that these cultured organisms make up less than 1% of the total DAPI- stained population (Table 2), which is consistent with MPN estimates of Thermococcales from other vent environments [*Holden et al.*, 1998; *Holland et al.*, 2004].

[20] Clone libraries from in situ filtered diffuse fluids were constructed using primers targeting the Thermococcales. As summarized in Table 2, 9 of the 12 environmental samples yielded a positive PCR product of approximately 1250 base pairs. In two vents, Cloud and Gollum, Thermococcales were successfully cultured although no positive PCR product was obtained. This is likely due to the low recovery of DNA from these samples or the low abundance of Thermococcales at these sites. While no PCR product was obtained from Cloud regardless of primer set used, universal archaeal primers did result in positive amplification from Gollum even though the Thermococcales-specific primers were unable to produce a product. Out of 526 clones sequenced, 5 were *Methanococcus* spp., and one was a member of the Marine Group I Crenarchaeota. Those 6 clones were eliminated from further analysis. All of the remaining 520 clones were members of the Thermococcales and appeared to be nonchimeric. All cloned ITS regions (241 bp to 261 bp) contained a tRNA-alanine as observed in other Euryarchaeota [*Achenbach-Richter and Woese*, 1988].

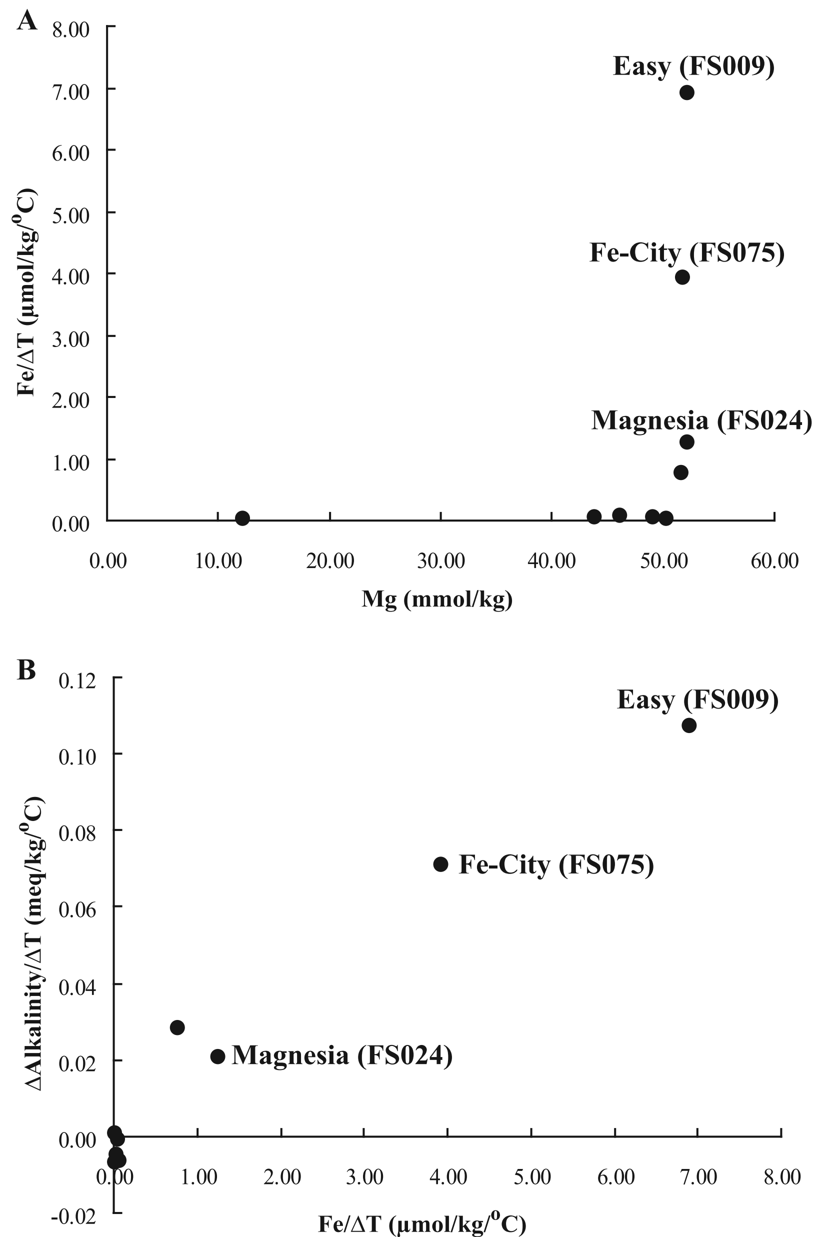


Figure 5. Trends of iron, alkalinity, and magnesium in diffuse vents of this study. Delta properties are calculated as measured property minus the ambient seawater background property. Concentration of iron in background seawater is small enough to be insignificant. Use of delta properties eliminates most of the effects of mixing hydrothermal fluid with seawater. (a) Fe/ΔT versus Mg, with three vents highlighted. An increase in iron occurs in the lowest-temperature vents. (b) ΔAlkalinity/ΔT versus Fe/ΔT shows a roughly linear correlation of the ΔAlkalinity and iron in the lowest-temperature vents, consistent with reduction of solid phase ferric iron to soluble Fe^{2+} and consumption of acid. The remaining vents have relatively low iron and negative ΔAlkalinity.

ITS clone identities ranged from 75% to 100% sequence similarity, whereas 16S rRNA clones (931 bp to 943 bp) ranged from 94% to 100% sequence similarity.

[21] The majority of clone sequences in this study grouped most closely with cultured members of the Thermococcales, including both *Pyrococcus* and *Thermococcus spp*. We included outgroups from vent locations throughout the world in both the 16S rRNA gene and ITS region analyses, and no global biogeographical trend was seen, with clones from this study grouping with cultured organisms from Axial Seamount, sulfide chimneys at the Mid-Atlantic Ridge, shallow marine springs in Vulcano, Italy, and mineral precipitates from the Okinawa Trough. This supports other studies which have shown the Thermococcales to be widespread and ubiquitous in hydrothermal environments [*Lepage et al.*, 2004].

[22] As discussed in the methods section, the 16S and ITS region were treated separately in all analyses owing to their different levels of sequence similarity. Individual phylogenetic trees were constructed for each vent sample for each gene with the same outgroups included. Consistent groups, those containing the same outgroups with strong support values, were noted and their distribution among samples recorded. We found that the ITS and 16S rRNA did not yield identical phylogenetic trees or groupings (Figures 2 and 3). While some groups (C, D, E, and F) contained the same groups on both trees, the tightly clustered groups A and B on the ITS trees were not found on the 16S rRNA trees. Instead, these groups broke into smaller subgroups (G, H, and I). For both the ITS region and the 16S rRNA, there were sequences that could not be assigned to a defined group.

[23] To determine if there was any relationship between the presence and absence of a certain groups for the ITS and 16S rRNA gene in each vent, we performed a cluster analysis (Figure 4). Microbial communities from individual vents were clustered by application of the unweighted pair-group method with arithmetic mean method (UPGMA) to the Jaccard's coefficient matrix. Despite the inconsistencies between the ITS and 16S rRNA phylogenies, UPGMA cluster analysis with both genes showed three vents consistently clustering together, Magnesia, Fe-City, and Easy (Figure 4).

4. Discussion

[24] Members of the Thermococcales isolated from low-temperature diffuse fluids with optimum growth temperature well above the temperature of sampled vents serve as indicator organisms of a warm anaerobic subseafloor habitat. While they are the most commonly isolated organisms from deep-sea hydrothermal vents due to relative ease of cultivation and selection methods employed, phylogenetic surveys of hydrothermal fluids often miss the Thermococcales, possibly owing to their low abundance in the subseafloor, their attachment to particles in the subseafloor, or the large amounts of seawater that may dilute their numbers at the point of sampling [*Huber et al.*, 2002]. The targeted environmental PCR analysis with order-specific primers used in this study attempted to minimize the problem of seawater organisms and allowed for the direct examination of the incidence, diversity, and distribution of one member of the warm anoxic subseafloor population in a variety of diffuse flow vents.

[25] It has been suggested that distinct microbial populations may exist at individual vents owing to differences in chemical properties of the vent [*Lilley et al.*, 1984], and that for microbial populations, each vent, even within a vent field like Axial, is potentially an independent habitat [*Karl*, 1995]. Vents only meters apart can have markedly different chemistries [*Butterfield et al.*, 2004; *Nakagawa et al.*, 2005; *Von Damm*, 2000; *Von Damm and Lilley*, 2004], reflecting different fluid sources, pathways, mixing ratios, segregation of fluids, precipitation/dissolution reactions and microbial processes in the subsurface. For example, while diffuse flow is often viewed as a mixture of two end-members, seawater and hydrothermal fluid, there is now evidence for a third "intermediate" fluid type consisting of warm but unreacted seawater [*Butterfield et al.*, 2004; *Cooper et al.*, 2000; *Ravizza et al.*, 2001]. Additionally, the geological context of the vent can also control how fluids move through and out of the crust, such as through linear cracks in the seafloor (Marker 33), through the interior of permeable lava pillars (Marker 113), out of drain-back caverns through partially broken lobate lava roof skylights (Roof), or through what may be a lava tube opening (Cloud) [*Butterfield et al.*, 2004]. These factors, and many others discussed in references included here, influence the sampled chemistry and microbial diversity of a single diffuse flow hydrothermal vent.

[26] For the environmental clone libraries, with both the 16S rRNA gene and in particular with the ITS region, we found sequences grouping together in robust groups of closely related sequences. The significance of this microdiversity remains unknown. Some of the variation may be due to errors in PCR, cloning, and sequencing [*Acinas et al.*, 2004a; *Wintzingerode et al.*, 1997], though this is likely not as significant for the ITS region, which exhibits considerable sequence variation. In addition, some of the microdiversity may be due to multiple copy rRNA gene sequences present in one organism, although most archaea appear to only have a single RNA (rrn) operon [*Acinas et al.*, 2004b]. *Palys et al.* [1997] have suggested that these clusters of similar sequences should correspond to ecotypes [*Cohan*, 2001]. Recent studies seem to support this interpretation and show that some clusters appear to represent closely related yet ecologically distinct groups of organisms [*Acinas et al.*, 2004a; *García-Martínez and Rodríguez-Valera*, 2000; *Moore et al.*, 1998; *Rocap et al.*, 2002]. In particular, the use of the ITS region as a high-resolution marker has revealed that closely related organisms (>97% 16S rRNA sequence similarity) can represent separate physiological populations [*Holden et al.*, 2001; *Jaspers and Overmann*, 2004; *Rocap et al.*, 2002, 2003; *Summit and Baross*, 2001]. Although our cluster analyses with both the 16S rRNA and ITS region showed similar results, we found tighter, more consistent groups using the ITS region, supporting other studies which suggest it is a better genetic marker for indicating habitat diversity [*Ferris et al.*, 2003; *Rocap et al.*, 2002].

[27] We looked for relationships to explain the clustering pattern in Figure 4 by examining the characteristics of the sample sites, as listed in Tables 1 and 2. Our analysis with the ITS region shows that three vents, Magnesia (FS024), Easy (FS009), and Fe-City (FS024) cluster together and have a different Thermococcales composition than the other vents. As discussed, these three vents have some common characteristics in their chemistry that are distinct from all other diffuse vents in this study. They are among the most highly diluted (lowest temperature, highest Mg) and show signs of iron reduction (high Fe concentrations, high alkalinity). The ITS region group A was not detected in these three vents but was found in the other five vents. Instead, these three vents contained ITS region groups D, E, and F, which were not found in the other five vents. ITS region group B appeared in all but 3 vents, while ITS region group C only appeared in Marker 33 1998 and 1999. These two vents contained the highest number of observed unique Thermococcales clones of all vents sampled and are identical in terms of Thermococcales groups identified (data not

shown). What distinguishes the Magnesia, Easy, and Fe-City chemically is the indication that they have increased iron content due to reduction of ferric iron in the subseafloor. It is possible that the genetic distinctness of *Thermococcus spp.* in these three vents results from entrainment of organisms present in the iron-rich deposits, and the presence of ITS region groups D, E, and F uniquely in the three high-iron vents can be interpreted as the addition of a subpopulation present in these deposits at Axial Volcano. The common presence of most other ITS region groups in all of the vent sites may represent subpopulations entrained from the basalt-hosted upflow zone.

[28] A relationship between environmental characteristics of the habitat and genetic diversity of the microbial population has been observed in terrestrial hot springs [*Blank et al.*, 2002; *Ferris et al.*, 2003; *Skirnisdottir et al.*, 2000], and geographical trends in distribution of hot spring microbes have also been noted, suggesting that physical isolation may be important for microbial diversification [*Fenchel*, 2003; *Papke et al.*, 2003; *Whitaker et al.*, 2003]. It is clear from our analyses that Magnesia, Easy, and Fe-City vents are distinct from other vents at Axial chemically as well as genetically on the basis of both the 16S rRNA and ITS region of environmentally cloned *Thermococcus spp*. Other studies at deep-sea hydrothermal vents support the hypothesis that microbial populations are linked to the chemical environment. For example, using 16S rRNA and culturing, one previous study found variability in the composition of deep-sea vent microbial communities from multiple chimneys and fluids in a single hydrothermal field in the Mid-Okinawa Trough, and this variability was linked to the geochemistry of the sampled vent fluids [*Nakagawa et al.*, 2005]. Another study using cultured *Thermococcus spp*. found the cultures formed groups that correlated with their sample location and source material (fluid, rock, or animal), indicating that they were adapted to the specific environment from which they were isolated [*Holden et al.*, 2001]. Additionally, *Summit and Baross* [2001] found that *Thermococcus spp*. in sulfide chimney and basaltic subseafloor habitats from one segment of the Juan de Fuca Ridge formed phylogenetically distinct groups and displayed different phenotypic characteristics. Isolates from sulfides produced more active proteases, tolerated higher zinc ion concentrations, and lived at wider temperature and salinity ranges than those isolated from the subseafloor. Because there is no evidence of restricted exchange between the two habitats, the authors suggest that this phylogenetic grouping reflects significant differences between sulfide and subseafloor habitats that result in the selection and maintenance of separate groups of Thermococcales [*Summit and Baross*, 2001].

[29] Our work indicates that there may be a link between the genetic diversity of the Thermococcales and source vent chemistry, suggesting distinct subseafloor Thermococcales populations at individual vents within Axial Seamount. Significantly, vents that have distinct subpopulations in the Thermococcales also have distinct physical and chemical properties, with differences in temperature, degree of mixing, and presence or absence of evidence for iron reduction. While it appears that different vents may select for genetically distinct subpopulations of Thermococcales, the details of this selection process and how it is manifested in physiological diversity are unknown. The key factor in the chemistry of the vents that have the distinct subpopulations of Thermococcales is the evidence that the fluids have passed through an iron-rich habitat. It is possible that there are particular Thermococcales living in this habitat that are not common to the typical mineralogy of basalt and altered basalt that characterizes most of the hydrothermal upflow zone. Thermococcales at Easy, Fe-City, and Magnesia may be adapted to more alkaline conditions, lower temperatures, and higher Fe concentrations as compared to Thermococcales from other diffuse vents. Preliminary growth experiments with Thermococcales isolates from Axial indicate wide growth temperature ranges without any correlation to vent temperature. Future experiments will focus on gathering additional physiological data from our cultures to determine the link between relevant chemical parameters and physiological traits.

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References

Achenbach-Richter, L., and C. R. Woese (1988), The ribosomal gene spacer region in Archaebacteria, *Syst. Appl. Microbiol.*, *10*, 211–214.

Acinas, S. G., et al. (2004a), Fine-scale phylogenetic architecture of a complex bacterial community, *Nature*, *430*, 551–554.

Acinas, S. G., et al. (2004b), Divergence and redundancy of 16S rRNA sequences in genomes with multiple *rrn* operons, *J. Bacteriol.*, *186*, 2629–2635.

Baker, E. T., C. G. Fox, and J. P. Cowen (1999), In situ observations of the onset of hydrothermal discharge during the 1998 submarine eruption of Axial Volcano, Juan de Fuca Ridge, *Geophys. Res. Lett.*, *26*(23), 3445–3448.

Blank, C. E., et al. (2002), Microbial composition of near-boiling silica-depositing thermal springs throughout Yellowstone National Park, *Appl. Environ. Microbiol.*, *68*, 5123–5135.

Bonch-Osmolovskaya, E. A., and K. O. Stetter (1991), Interspecies hydrogen transfer in cocultures of thermophilic Archaea, *Syst. Appl. Microbiol.*, *14*, 205–208.

Butterfield, D. A., et al. (2004), Mixing, reaction, and microbial activity in the sub-seafloor revealed by temporal and spatial variation in diffuse flow vents at Axial Volcano, in *The Subseafloor Biosphere at Mid-Ocean Ridges*, *Geophys. Monogr. Ser.*, vol. 144, edited by W. S. D. Wilcock et al., pp. 269–289, AGU, Washington, D. C.

Canadian American Seamount Expedition (1985), Hydrothermal vents on an axis seamount of the Juan de Fuca Ridge, *Nature*, *313*, 212–214.

Cohan, F. M. (2001), Bacterial species and speciation, *Syst. Biol.*, *50*, 513–524.

Cole, J. R., et al. (2003), The Ribosomal Database Project (RDP-II): Previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy, *Nucleic Acids Res.*, *31*, 442–443.

Cooper, M. J., H. Elderfield, and A. Schultz (2000), Diffuse hydrothermal fluids from Lucky Strike hydrothermal vent field: Evidence for a shallow conductively heated system, *J. Geophys. Res.*, *105*(B8), 19,369–19,376.

DeLong, E. F. (1992), Archaea in coastal marine environments, *Proc. Natl. Acad. Sci. U. S. A.*, *89*, 5685–5689.

Edmond, J. M., et al. (1979a), On the formation of metal-rich deposits at ridge crests, *Earth Planet. Sci. Lett.*, *46*, 19–30.

Edmond, J. M., et al. (1979b), Ridge crest hydrothermal activity and the balances of the major and minor elements in the ocean: The Galapagos data, *Earth Planet. Sci. Lett.*, *46*, 1–18.

Embley, R. W., K. M. Murphy, and C. G. Fox (1990), High-resolution studies of the summit of Axial Volcano, *J. Geophys. Res.*, *95*(B8), 12,785–12,812.

Fenchel, T. (2003), Biogeography for bacteria, *Science*, *301*, 925–926.
Ferris, M. J., et al. (2003), Cyanobacterial ecotypes in different optical microenvironments of a 68°C hot spring mat community revealed by 16S–23S rRNA internal transcribed spacer region variation, *Appl. Environ. Microbiol.*, *69*, 2893–2898.
García-Martínez, J., and F. Rodríguez-Valera (2000), Microdiversity of uncultured marine prokaryotes: The SAR11 cluster and the marine Archaea of Group I, *Mol. Ecol.*, *9*, 935–948.
García-Martínez, J., et al. (1999), Use of the 16S–23S ribosomal genes spacer region in studies of prokaryotic diversity, *J. Microbiol. Methods*, *36*, 55–64.
Garcia-Martinez, J., et al. (2002), Prevalence and microdiversity of *Alteromonas macleodii*-like microorganisms in different oceanic regions, *Environ. Microbiol.*, *4*, 42–50.
Greenberg, A. E., et al. (1992), *Standard Methods for the Examination of Water and Wastewater*, 18th ed., Am. Water Works Assoc., Washington, D. C.
Gürtler, V., and V. A. Stanisich (1996), New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region, *Microbiology*, *142*, 3–16.
Hall, T. A. (1999), BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT, *Nucleic Acids Symp. Ser.*, *41*, 95–98.
Hasegawa, M., et al. (1985), Dating of the human-ape splitting by a molecular clock of mitochondrial DNA, *J. Mol. Evol.*, *22*, 160–174.
Hoek, J., et al. (2003), Microbial diversity of a sulphide spire located in the Edmond deep-sea hydrothermal vent field on the Central Indian Ridge, *Geobiology*, *1*, 119–127.
Holden, J. F., et al. (1998), Thermophilic and hyperthermophilic microorganisms in 3–30°C hydrothermal fluids following a deep-sea volcanic eruption, *FEMS Microbiol. Ecol.*, *25*, 33–41.
Holden, J. F., et al. (2001), Diversity among three novel groups of hyperthermophilic deep-sea *Thermococcus* species from three sites in the northeastern Pacific Ocean, *FEMS Microbiol. Ecol.*, *36*, 51–60.
Holland, M. E., et al. (2004), Illuminating subseafloor ecosystems using microbial tracers, in *The Subseafloor Biosphere at Mid-Ocean Ridges*, *Geophys. Monogr. Ser.*, vol. 144, edited by W. S. D. Wilcock et al., pp. 291–303, AGU, Washington, D. C.
Huber, J. A., et al. (2002), Temporal changes in archaeal diversity and chemistry in a mid-ocean ridge subseafloor habitat, *Appl. Environ. Microbiol.*, *68*, 1585–1594.
Huber, J. A., et al. (2003), Bacterial diversity in a subseafloor habitat following a deep-sea volcanic eruption, *FEMS Microbiol. Ecol.*, *43*, 393–409.
Hugenholtz, P., and T. Huber (2003), Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases, *Int. J. Syst. Evol. Microbiol.*, *53*, 289–293.
Jannasch, H. W., and M. J. Mottl (1985), Geomicrobiology of deep-sea hydrothermal vents, *Science*, *229*, 717–725.
Jannasch, H. W., and C. O. Wirsen (1979), Chemosynthetic primary production at East Pacific sea floor spreading centers, *Bioscience*, *29*, 592–598.
Jannasch, H. W., et al. (1992), Comparative physiological studies on hyperthermophilic archaea isolated from deep-sea hot vents with emphasis on *Pyrococcus* strain GB-D, *Appl. Environ. Microbiol.*, *58*, 3472–3481.
Jaspers, E., and J. Overmann (2004), Ecological significance of microdiversity: Identical 16S rRNA gene sequences can be found in bacteria with highly divergent genomes and ecophysiologies, *Appl. Environ. Microbiol.*, *70*, 4831–4839.
Johnson, H. P., and R. W. Embley (1990), Axial seamount: An active ridge axis volcano on the central Juan de Fuca Ridge, *J. Geophys. Res.*, *95*(B8), 12,689–12,696.
Karl, D. M. (1995), Ecology of free-living, hydrothermal vent microbial communities, in *The Microbiology of Deep-Sea Hydrothermal Vents*, edited by D. M. Karl, pp. 35–124, CRC Press, Boca Raton, Fla.
Kennedy, C. B., et al. (2003), Ultrastructure and potential sub-seafloor evidence of bacteriogenic iron oxides from Axial Volcano, Juan de Fuca Ridge, north-east Pacific Ocean, *FEMS Microbiol. Ecol.*, *43*, 247–254.
Lepage, E., et al. (2004), Molecular diversity of new *Thermococcales* isolates from a single area of hydrothermal deep-sea vents as revealed by randomly amplified polymorphic DNA fingerprinting and 16S rRNA gene sequence analysis, *Appl. Environ. Microbiol.*, *70*, 1277–1286.
Lilley, M. D., et al. (1984), Reduced gases and bacteria in hydrothermal fluids: The Galapagos spreading center and 21 N East Pacific Rise, in *Hydrothermal Processes at Seafloor Spreading Centers*, edited by P. A. Rona et al., pp. 411–449, Springer, New York.
Marteinsson, V. T., et al. (1995), Phenotypic characterization, DNA similarities, and protein profiles of twenty sulfur-metabolizing hyperthermophilic anaerobic archaea isolated from hydrothermal vents in the Southwestern Pacific Ocean, *Int. J. Syst. Bacteriol.*, *45*, 623–632.
Mehta, M., et al. (2003), Phylogenetic diversity of nitrogenase (*nifH*) genes in deep-sea and hydrothermal vent environments of the Juan de Fuca Ridge, *Appl. Environ. Microbiol.*, *69*, 960–970.
Moore, L. R., et al. (1998), Physiology and molecular phylogeny of coexisting Prochlorococcus ecotypes, *Nature*, *393*, 464–467.
Nakagawa, S., et al. (2005), Variability in microbial community and venting chemistry in a sediment-hosted backarc hydrothermal system: Impacts of subseafloor phase-separation, *FEMS Microbiol. Ecol.*, *54*, 141.
Nercessian, O., et al. (2003), Archaeal diversity associated with in situ samplers deployed on hydrothermal vents on the East Pacific Rise (13°C), *Environ. Microbiol.*, *5*, 492–502.
Page, R. D. M. (1996), TREEVIEW: An application to display phylogenetic trees on personal computers, *Comput. Appl. Biosci.*, *12*, 357–358.
Palys, T., et al. (1997), Discovery and classification of ecological diversity in the bacterial world: The role of DNA sequence data, *Int. J. Syst. Evol. Microbiol.*, *47*, 1145–1156.
Papke, R. T., et al. (2003), Geographical isolation in hot spring cyanobacteria, *Environ. Microbiol.*, *5*, 650–659.
Porter, K. G., and Y. S. Feig (1980), The use of DAPI for identifying and counting aquatic microflora, *Limnol. Oceanogr.*, *25*, 943–948.
Ravizza, G., et al. (2001), Sr isotope variations in vent fluids from 9 degrees 46′-9 degrees 54′ N East Pacific Rise: Evidence of a non-zero-Mg fluid component, *Geochim. Cosmochim. Acta*, *65*, 729–739.
Reysenbach, A.-L., et al. (2000), Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a Mid-Atlantic Ridge hydrothermal vent, *Appl. Environ. Microbiol.*, *66*, 3798–3806.
Rinker, K. D., and R. M. Kelly (2000), Effect of carbon and nitrogen sources on growth dynamics and expolysaccharide production for the hyperthermophilic archaeon *Thermococcus litoralis* and bacterium *Thermotoga maritima*, *Biotechnol. Bioeng.*, *69*, 537–547.
Rocap, G., et al. (2002), Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S–23S ribosomal DNA internal transcribed spacer sequences, *Appl. Environ. Microbiol.*, *68*, 1180–1191.
Rocap, G., et al. (2003), Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation, *Nature*, *424*, 1042–1047.
Schmidt, H. A., et al. (2002), TREE-PUZZLE: Maximum likelihood phylogenetic analysis using quartets and parallel computing, *BMC Bioinf.*, *18*, 502–504.
Schrenk, M. O., et al. (2003), Incidence and diversity of microorganisms within the walls of an active deep-sea sulfide chimney, *Appl. Environ. Microbiol.*, *69*, 3580–3592.
Skirnisdottir, S., et al. (2000), Influence of sulfide and temperature on species composition and community structure of hot spring microbial mats, *Appl. Environ. Microbiol.*, *66*, 2835–2841.
Slobodkina, G. B., et al. (2004), PCR-based identification of hyperthermophilic archaea of the Family *Thermococcaceae*, *Appl. Environ. Microbiol.*, *70*, 5701–5703.
Sokolova, T. G., et al. (2004), The first evidence of anaerobic CO oxidation coupled with H_2 production by a hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent, *Extremophiles*, *8*, 317–323.
Stetter, K. O. (1989), Extremely thermophilic chemolithoautrophic archaebacteria, in *Autotrophic Bacteria*, edited by H. G. Schlegel and B. Bowien, pp. 167–176, Springer, New York.
Summit, M., and J. A. Baross (1998), Thermophilic subseafloor microorganisms from the 1996 North Gorda Eruption, *Deep Sea Res., Part II*, *45*, 2751–2766.
Summit, M., and J. A. Baross (2001), A novel microbial habitat in the mid-ocean ridge subseafloor, *Proc. Natl. Acad. Sci. U. S. A.*, *98*, 2158–2163.
Takai, K., and K. Horikoshi (1999), Genetic diversity of archaea in deep-sea hydrothermal vent environments, *Genetics*, *152*, 1285–1297.
Takai, K., et al. (2001), Distribution of archaea in a black smoker chimney structure, *Appl. Environ. Microbiol.*, *67*, 3618–3629.
Takai, K., et al. (2004a), Geochemical and microbiological evidence for a hydrogen-based, hyperthermophilic subsurface lithoautotrophic microbial ecosystem (HyperSLiME) beneath an active deep-sea hydrothermal field, *Extremophiles*, *8*, 269–282.
Takai, K., et al. (2004b), Spatial distribution of marine crenarchaeota Group I in the vicinity of deep-sea hydrothermal systems, *Appl. Environ. Microbiol.*, *70*, 2404–2413.
Thompson, J. R., et al. (2002), Heteroduplexes in mixed-template amplifications: Formation, consequence and elimination by ‘reconditioning PCR’, *Nucleic Acids Res.*, *30*, 2083–2088.
Von Damm, K. L. (2000), Chemistry of hydrothermal vent fluids from 9 degree-10 degrees North, East Pacific Rise: “Time zero,” the immediate post-eruptive period, *J. Geophys. Res.*, *105*(B5), 11,203–11,222.
Von Damm, K. L., and M. D. Lilley (2004), Diffuse flow hydrothermal fluids from 9° 50′ N East Pacific Rise: Origin, evolution, and biogeochemical controls, in *The Subseafloor Biosphere at Mid-Ocean Ridges*, *Geophys. Monogr. Ser.*, vol. 144, edited by W. S. D. Wilcock et al., pp. 245–268, AGU, Washington, D. C.

Wery, N., et al. (2002), Diversity of anaerobic heterotrophic thermophiles isolated from deep-sea hydrothermal vents of the Mid-Atlantic Ridge, *FEMS Microbiol. Ecol.*, *41*, 105–114.

Whitaker, R. J., et al. (2003), Geographic barriers isolate endemic populations of hyperthermophilic archaea, *Science*, *301*, 976–978.

Wintzingerode, F. V., et al. (1997), Determination of microbial diversity in environmental samples: Pitfalls of PCR-based rRNA analysis, *FEMS Microbiol. Rev.*, *21*, 213–229.

Zillig, W., and A.-L. Reysenbach (2001), The order Thermococcales, in *Bergey's Manual of Systematic Bacteriology*, edited by D. R. Boone et al., pp. 341–348, Springer, New York.

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