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Evidence for hydrothermal Archaea within the basaltic flanks of the East Pacific Rise

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Summary

Little is known about the fluids or the microbial communities present within potentially vast hydrothermal reservoirs contained in still-hot volcanic ocean crust beneath the flanks of the mid-ocean ridge. During *Alvin* dives in 2002, organic material attached to basalt was collected at low, near-ambient temperatures from an abyssal hill fault scarp in 0.5 Ma lithosphere on the western ridge flank of the East Pacific Rise. Mineral analysis by X-ray diffractometry and scanning electron microscopy revealed high-temperature (> 110°C) phases chalcopyrite (Cu_5FeS_4) and 1C pyrrhotite ($Fe_{1-x}S$) within the fault scarp materials. A molecular survey of archaeal genes encoding 16S rRNA identified a diverse hyperthermophilic community, including groups within *Crenarchaeota*, *Euryarchaeota*, and *Korarchaeota*. We propose that the sulfide, metals and archaeal communities originated within a basalt-hosted subseafloor hydrothermal habitat beneath the East Pacific Rise ridge flank and were transported to the seafloor during a recent episode of hydrothermal venting from the abyssal hill fault. Additionally, inferred metabolisms from the fault scarp community suggest that an ecologically unique high-temperature archaeal biosphere may thrive beneath the young East Pacific Rise ridge flank and that abyssal hill fault scarps may present new opportunities for sampling for this largely unexplored microbial habitat.

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Introduction

Hydrothermal fluids circulating within basaltic ocean crust on the flanks of mid-ocean ridges are potentially important and globally widespread marine microbial habitats that rarely are sampled. These large hydrothermal fluid reservoirs contain 2% of the world ocean volume (Johnson and Pruis, 2003) and constitute a global subseafloor microbial ecosystem potentially producing 1×10^{12} gC $year^{-1}$ (Bach and Edwards, 2003). Because marine sediment quickly accumulates on the volcanic seabed as it spreads away from the ridge axis, direct sampling of hydrothermal fluid reservoirs and microbial communities within the ridge flank subseafloor is difficult, and normally requires deep sea drilling. While there have been recent advances in borehole drilling methods (Cowen *et al*., 2003; Cowen, 2004), sampling the subseafloor biosphere within the volcanic ridge flank in this way continues to be a challenge. Microbiological descriptions of this environment have, thus far, been restricted to a few sites located on the Juan de Fuca Ridge (Cowen *et al*., 2003; Huber *et al*., 2006) and the faulted flank of the Mid-Atlantic Ridge (Kelley *et al*., 2005). As a result, the characteristics and potential significance of the global ridge flank subseafloor biosphere remain controversial (Edwards *et al*., 2005).

Recent research (Haymon *et al*., 2005; Benjamin and Haymon, 2006) has identified two new sites [Mounds and Microbes (MM) and Tevnia] on the flanks of the East Pacific Rise (EPR) where hydrothermal fluids are episodically expelled from young (~0.5 Ma) volcanic crust. The MM site (Fig. 1) is located on a seafloor abyssal hill approximately 26 km west of the EPR ridge crest at 9°27′N. Abyssal hills are upthrown blocks of oceanic crust that form during extensional faulting of the seafloor as it spreads away from the mid-ocean ridge axis. Because abyssal hills are bounded by active normal faults, they typically have steep cliffs or escarpments (Fig. 1B and C) that expose cross sections of subseafloor basalt normally hidden beneath marine sediment. These faults also penetrate ridge flank basement crust and may be natural conduits connecting ridge flank hydrothermal reservoirs to the ocean floor (Yang, 2002; Haymon *et al*., 2005).

Here we present some of the first microbiological evidence for the existence of a high-temperature subseafloor biosphere beneath ridge flank abyssal hills. Using mineral and molecular analysis of organic-rich material collected

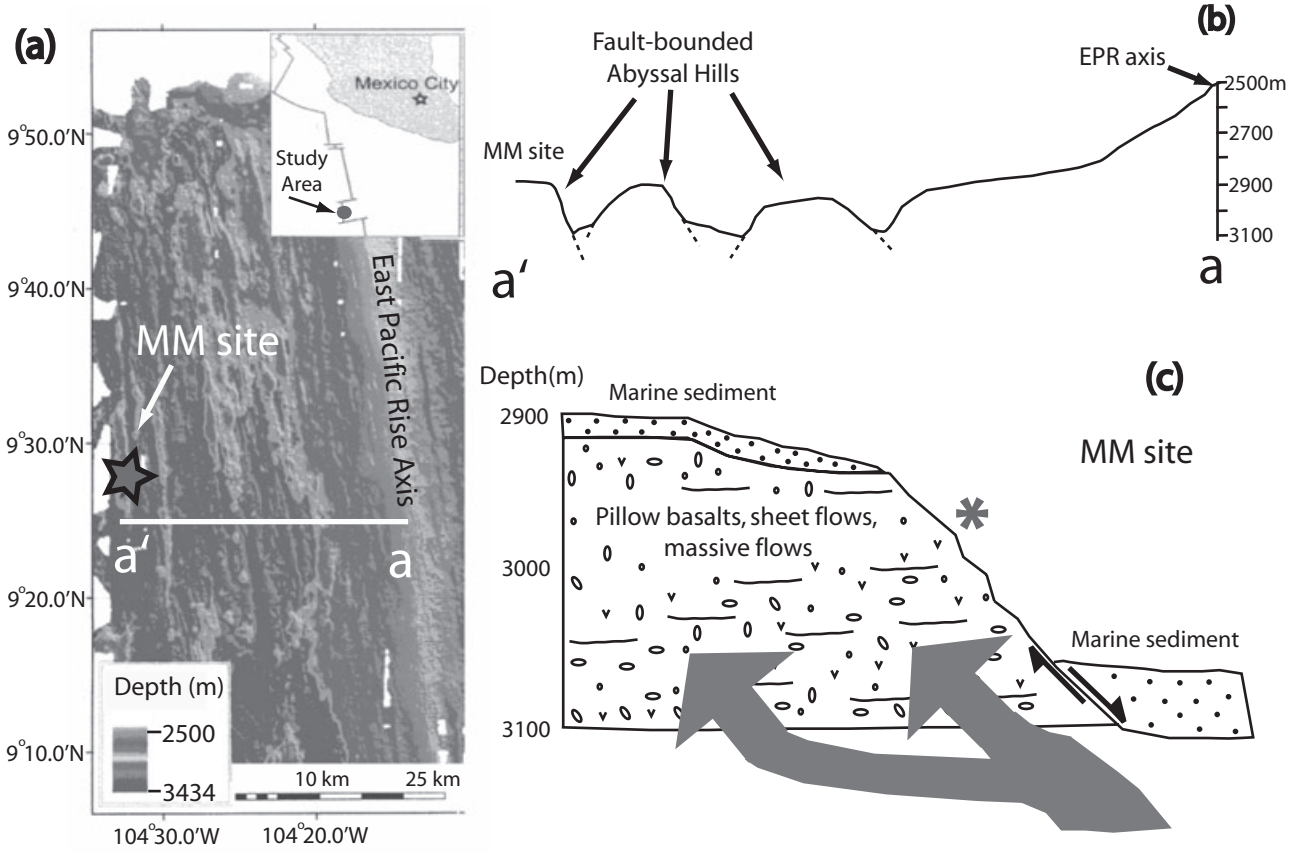

Fig. 1. Location and geologic setting of sampling site.
A. Bathymetric map showing location of MM Site (star) on an abyssal hill ~26 km west of the EPR axis at 9°27′N.
B. Depth profile along a′–a transect, from bathymetry in the study area by Wilcock and colleagues (1993).
C. Schematic cross-section of MM site abyssal hill; orange-brown floc attached to volcanic rock exposed in the fault scarp (Fig. 2) was sampled by submersible at the depth indicated by the asterisk (~50 vertical metres below the top of the abyssal hill); small arrows indicate sense of fault motion along the fault scarp, large arrows illustrate episodic earthquake-triggered hydrothermal fluid flow from deeper crustal reservoirs as proposed by Haymon and colleagues (2005).

from the fault escarpment (scarp) at MM site, we conclude that high-temperature (> 110°C), oxygen-depleted hydrothermal reservoirs support hyperthermophilic archaeal communities in basaltic volcanic crust beneath the EPR ridge flank. Further, we suggest that H_2 metabolism via sulfur reduction or methanogenesis may be an important process in this subseafloor environment. Because abyssal hills are the most abundant structural feature on the seafloor (Macdonald *et al.*, 1996), the observed community beneath the MM site could represent a globally extensive archaeal ecosystem in cooling, basaltic seafloor of ridge flanks.

Results

Fault scarp minerals

The organic-rich material collected from the MM site fault scarp consists of a mossy orange-brown flocculent mat attached to the volcanic surfaces of the scarp (Fig. 2A and B). Environmental scanning electron microscopy (ESEM) imaging shows that the flocculent attached to the fault scarp is composed primarily of tubular/cylindrical structures measuring up to 1 cm in length and 60 μm– 1 mm in width (Fig. 2C and D). Flocculent structures typically are attached to mineral/rock substrate only at one terminus (Fig. 2C). This is consistent with macroscopic observations of the fault scarp made during *Alvin* sample collection dives (Haymon *et al.*, 2005). These tubular structures are predominantly covered in Ca- and Si-bearing shell fragments (Fig. 2C and D). Small Fe-sulfide crystals (Fig. 2E and F) are found inside the tubular structures along with organic films lining the interior of the structures. Other mineral phases also are found with the flocculent structures in the bulk slurp samples, including Fe- and Mn-oxides, elemental sulfur, Cu-Fe-sulfide, and both hexagonal and cubic Fe-sulfide (Fig. 3).

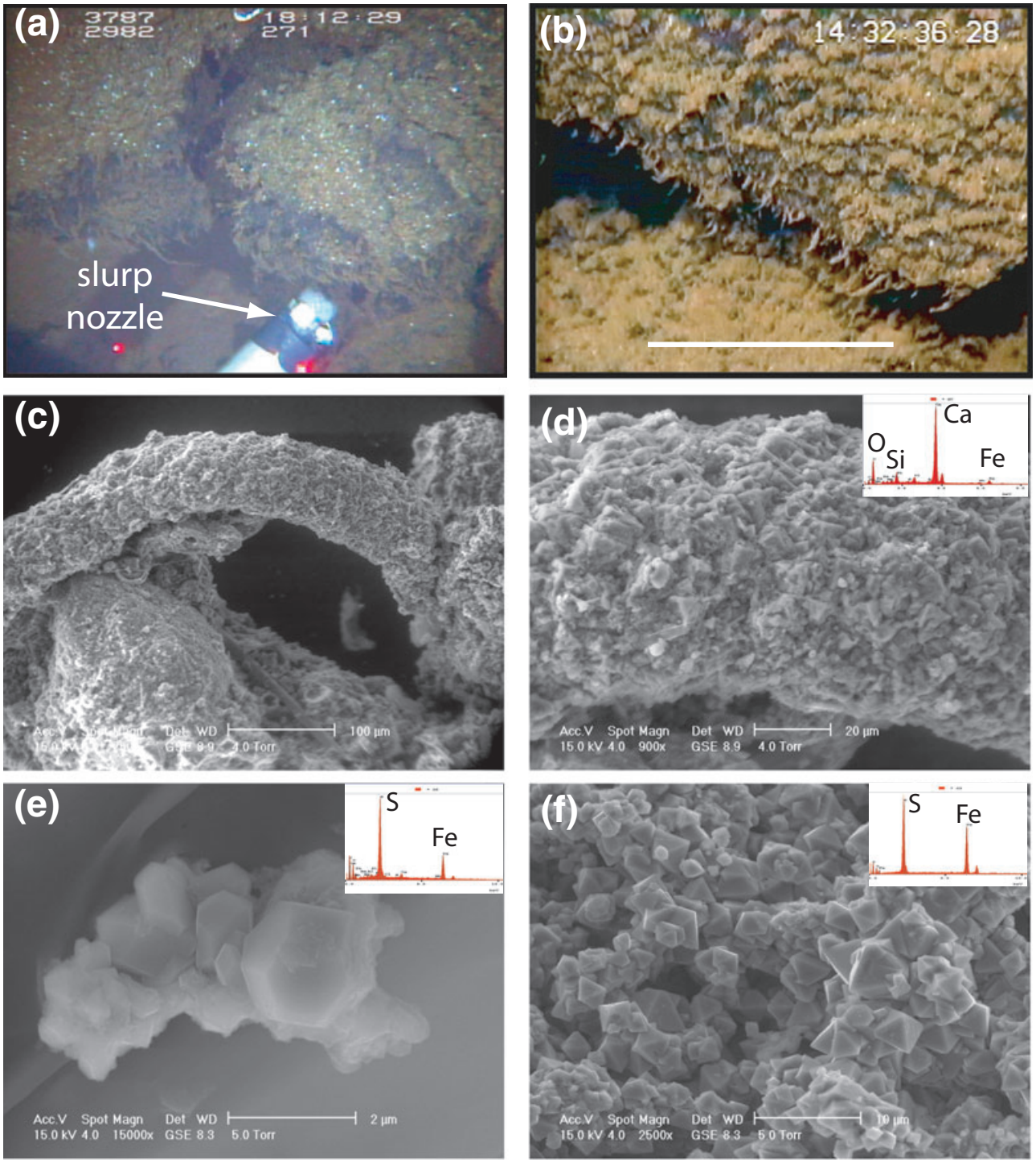

Fig. 2. MM site fault scarp flocculent.
A. *Alvin* digital video image showing strings of orange brown floc being suctioned through the nozzle of a slurp gun mounted in the basket of the submersible; scale is provided by red laser spots that are separated by 10 cm.
B. *Alvin* high-8 video image of orange-brown floc on the MM site abyssal hill fault scarp (scale bar 10 cm).
C. ESEM image of floc material (scale bar 100 µm).
D. Magnified image with inset EDS spectra showing Ca- and Si-bearing minerals covering the exterior of the floc structures (scale bar 20 µm).
E and F. Iron sulfide crystals found associated with floc structures (scale bars 2 µm and 10 µm respectively).

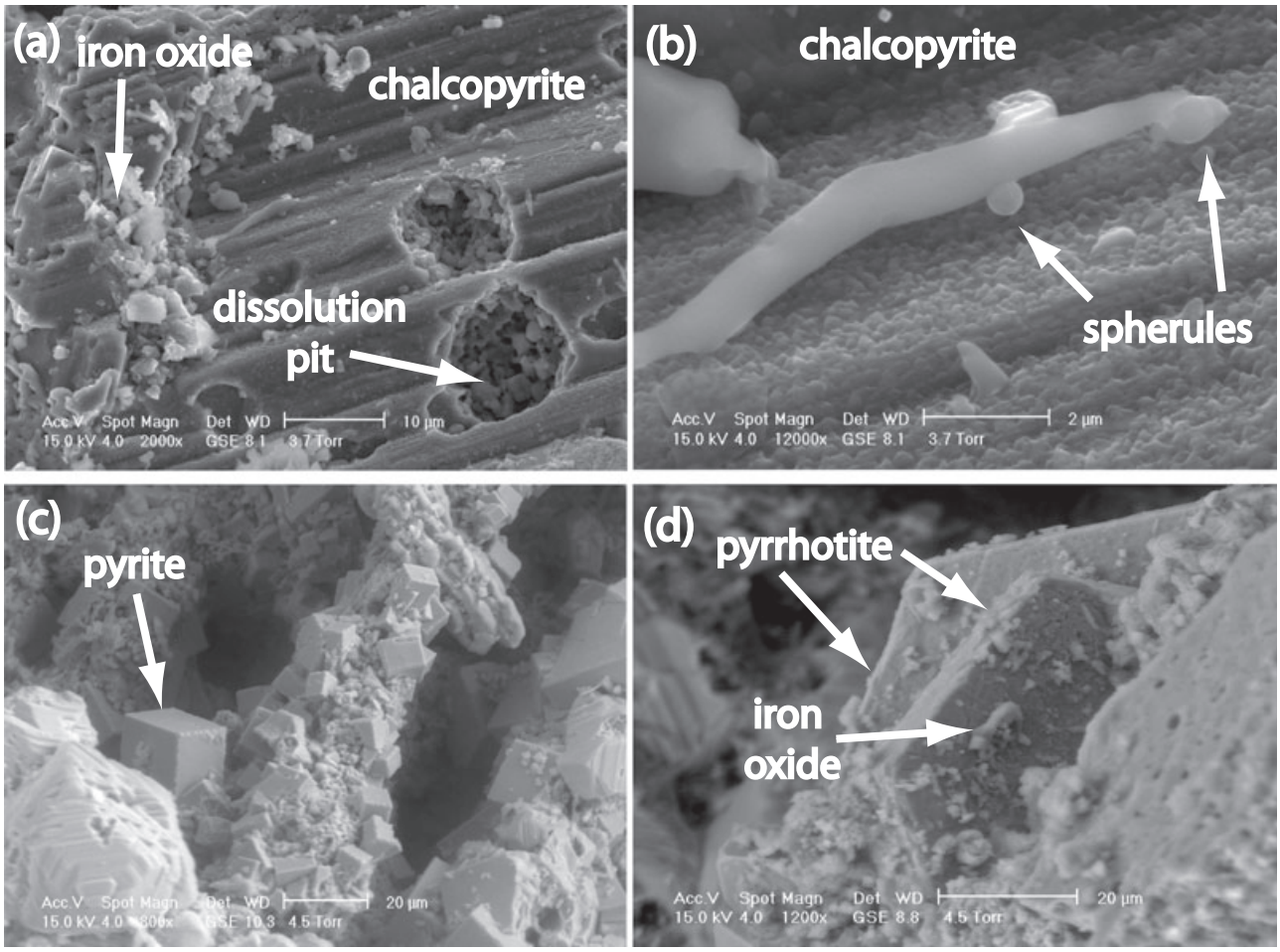

Fig. 3. Environmental scanning electron microscope photos of mineral particles and microbes associated with flocculent in the slurp samples from the MM site abyssal hill fault scarp. A high-temperature, reduced sulfide mineral assemblage is shown, including chalcopyrite (A and B), pyrite (C) and pyrrhotite (D). Identities of the sulfide phases were determined using X-ray energy dispersive analyses and X-ray diffractometry. Dissolution and iron oxide formation shown in A and D are indicative of chalcopyrite and pyrrhotite instability at ambient oxic conditions after waning of hydrothermal fluid flow. Rod-shaped organisms similar in size and morphology to organisms within the order *Thermoproteales* are found attached to high-temperature sulfide minerals. Scale bars: (A) 10 μm, (B) 2 μm, (C) 20 μm, (D) 20 μm.

Many of the larger Fe-sulfide and Cu-Fe-sulfide crystals show extensive alteration to secondary Fe-oxide phases. These secondary phases occur together with dissolution pits (Fig. 3A and D). Individual rod-shaped features 5–10 μm in length are found scattered on mineral surfaces of Cu-Fe-sulfides and Fe-Mn-oxides (Fig. 3B). These features are unstable under the accelerating voltage of the electron beam (15 KeV) and are most likely cells attached to the mineral surfaces. In some cases, these cells are observed with small (< 1 μm) spherules of undetermined composition along the length and at the termini of the cells (Fig. 3B).

X-ray diffractometry (XRD) was used to identify individual mineral phases present in the fault scarp samples. Diffraction angles (referred to as '2θ') corresponding to the atomic structure unique to each mineral were measured, and are shown in Table 1. The analysis shows 2θ peaks for crystalline chalcopyrite (Cu_5FeS_4), pyrite (FeS_2), pyrrhotite ($Fe_{1-x}S$), elemental sulfur (S^0), as well as calcite ($CaCO_3$) and halite (NaCl). The diffraction peaks for fault scarp pyrrhotite most closely match the 1C lattice superstructure. The relationship between Fe-content and the d-spacing of (102) planes in hexagonal 1C pyrrhotite has been described previously [where d_{102} corresponds to the diffraction peak detected at 2θ ~43.5° (Morimoto *et al.*, 1975)]. Using this relationship, we calculate a stoichiometry of $Fe_{0.963}S$ for the pyrrhotite present in the EPR fault scarp slurp material. Failure to detect diffraction peaks for the Fe- and Mn-oxide phases observed with the ESEM-EDS suggests either that these phases are Cu Kα X-ray-amorphous, or that these phases were present in abundances below the detection limit of powder X-ray diffraction.

Table 1. X-ray diffraction peaks for MM site mineral assemblage.

Diffraction peak d-spacing (Å)	2θ (degrees)	Relative peak intensity (%)	Mineral match (Bragg plane)
3.853	23.056	6.57	Sulfur (222)
3.040	29.345	100.00	Chalcopyrite (112)
2.993	29.816	10.27	Pyrrhotite (200)
2.821	31.680	10.04	Halite (200)
2.710	33.014	12.37	Pyrite (200)
2.660	33.653	13.19	Pyrrhotite (201)
2.423	37.058	17.03	Pyrite (210)
2.215	40.685	15.11	Pyrite (211)
2.074	43.587	19.55	Pyrrhotite (202)
1.916	47.391	22.30	Pyrite (220)
1.870	48.631	16.90	Chalcopyrite (220)
1.860	48.910	15.20	Chalcopyrite (204)
1.759	51.921	23.91	Sulfur (266)
1.721	53.156	7.49	Pyrrhotite (220)
1.635	56.192	15.08	Pyrite (311)
1.591	57.890	31.96	Chalcopyrite (312)

Powder X-ray diffraction patterns for several sediment cores taken from the top of the MM site abyssal hill and the adjacent sediment pond east of the fault scarp show 2θ peaks for calcite and halite. Characteristic peaks for sulfide minerals are not observed in these samples.

Molecular survey of abyssal hill archaeal community

Nearly 60% of fault scarp sequences (11 unique phylotypes) fall within well supported groups of either hyperthermophilic *Crenarchaeota* or *Euryarchaeota*. The remaining clones from the fault scarp are split between a *Korachaeota* group (~12%) and uncultured *Crenarchaeota* and *Euryarchaeota* groups. The fault scarp sequences within the uncultured *Crenarchaeota* (UC) and *Euryarchaeota* (UEI, UEII) groups are related to a number of undescribed Archaea identified from a range of environments including deep-sea hydrothermal vents, coastal marine sediments, ocean water column and terrestrial geothermal habitats (Fig. 5, Table 2).

Within the *Crenarchaeota*, fault scarp clones are closely related to organisms within the orders *Desulfurococcales* (Des group) and *Thermoproteales* (Thp group). Within the Des group, clone a87R13 shows 97% similarity to *Aeropyrum pernix* and a87Y42 shows 96% sequence identity to *Staphylothermus marinus*. Both *A. pernix* and *S. marinus* are hyperthermophiles with optimum growth temperatures at 90–95°C (Stetter, 1986; Sako *et al*., 1996). Within the Thp group, fault scarp clone group a87R27 has the highest sequence similarity to *Vulcanisaeta distributa*, an anaerobic hyperthermophile (optimum growth temperature 85–90°C) isolated from acidic terrestrial hot springs in Japan (Itoh *et al*., 2002). Additionally, clone types a87Y11 and a87R40 have high sequence similarity (98% and 95% respectively) to an uncultured *Thermoproteales* sequence isolated from a deep-sea hydrothermal vent (Page *et al*., 2004). The presence of clone types falling within the Thp group is also consistent with ESEM observations of predominantly rod-shaped organisms sometimes occurring with small spherules (Fig. 3B) at the termini (possibly S^0), which is a morphology characteristic of *Thermoproteales* (Zillig *et al*., 1981).

Within the uncultured *Crenarchaeal* group (Fig. 5), phylotypes a87R34, a87R61 and a87R57 are most closely related to sequences within the Marine Group I *Crenarchaeota* (Vetriani *et al*., 1999; Beja *et al*., 2002; Schrenk *et al*., 2004). Additionally, the G + C content of these clone types range between 51% and 52% consistent with Marine Group I Archaea indicating that these sequences represent organisms adapted for the colder environments of the open ocean. Finding open ocean archaeal sequences at MM site together with high-temperature minerals and hyperthermophilic clone types is not unexpected, because there was significant seawater entrainment in the slurp gun nozzle during sampling of the fault scarp. Other fault scarp sequences that fall within the UC group, Y5x and D1Ru (Fig. 5, Table 2), are most closely related to other sequences that have been isolated from terrestrial hot springs and marine hydrothermal environments (Takai and Horikoshi, 1999; Takai and Sako, 1999). These clone types have elevated G + C content (64% and 57% respectively), further suggesting that these organisms are adapted to hotter subseafloor environments (Galtier and Lobry, 1997).

Fault scarp clones that are phylogenetically related to cultured hyperthermophilic *Euryarchaeota* fall into two separate groups (Fig. 4). The first, a87R66, shows high sequence similarity (98%) to *Methanopyrus kandlerii*, an autotrophic hyperthermophilic (optimum growth temperature 84–110°C) methanogen typically found in high-temperature marine hydrothermal environments on the ridge crest (Kurr *et al*., 1991). The other high-temperature *Euryarchaeal* fault scarp clone group, a87R32, is closely related (99%) to a hydrothermal vent *Thermococcus*

Table 2. Summary of archaeal 16S rRNA clone sequences from MM site fault scarp.

Phylotype[a]	Closest GenBank match[b]	% Similarity	Environment description
Thp			
a87R40 (2)	Uncultured *Thermofilaceae* (AY280443)	95	Marine hydrothermal
a87Y11 (18)	Uncultured *Thermofilaceae* (AY280443)	98	Marine hydrothermal
a87R27 (13)	*Vulcanisaeta distributa* (AB063639)	94	Terrestrial hydrothermal
Des			
a87R13 (7)	*Aeropyrum pernix* (AB078016)	97	
a87Y42 (7)	*Staphylothermus marinus* (X99560)	94	
a87R35 (10)	Uncultured crenarchaeote (AY280449)	99	Marine hydrothermal
a87Y32 (19)	Uncultured archaeon gene (AB095124)	96	Hydrothermal
Thm			
a87R32 (5)	*Thermococcus* sp. (AY099165)	99	Marine hydrothermal
Mpy			
a87R66 (4)	*Methanopyrus kandleri* (AE010349)	98	
Kor			
a87Y34 (11)	Unidentified archaeon (AB007305)	96	Marine hydrothermal
a87R58 (6)	Uncultured archaeon 20a-1 (AJ299148)	91	Marine sediments
UC			
Y5x (5)	Uncultured archaeon 20b-27 (AJ299172)	96	Marine sediment
D1Ru (2)	Uncultured crenarchaeote (AB113623)	90	Subsurface terrestrial
a87R57 (2)	Uncultured crenarchaeote (AF393466)	98	Marine water column
a87R34 (1)	Uncultured archaeon clone (AY627473)	95	Marine sediment
a87R61 (2)	Uncultured archaeon clone (AY505050)	97	Ridge flank subsurface
UE1			
A87R56 (1)	Uncultured archaeon (AB175599)	91	Marine sediment
a87R29 (2)	Uncultured euryarchaeote (AB175599)	90	Marine sediment
a87R37 (1)	Uncultured euryarchaeote (AB175599)	92	Marine sediment
a87R46 (2)	Uncultured euryarchaeote (AB175599)	91	Marine sediment
a87R1 (2)	Uncultured euryarchaeote (AB119597)	96	Estuarine sediment
a87R28 (1)	Unidentified archaeon (AJ631255)	91	Sulfidic spring
a87R50 (2)	Uncultured euryarchaeote (AB119626)	95	Estuarine sediment
a87R41 (2)	Uncultured archaeon (AY454694)	93	Estuarine sediment
a87R36 (2)	Uncultured archaeon (AJ969797)	98	Soil
a87R72 (2)	Unidentified euryarchaeote (AY396632)	92	Marine sediment
UE2			
a87R16 (1)	Unidentified archaeon (AB007303)	95	Marine hydrothermal
a87Y37 (7)	Unidentified archaeon (AB007303)	97	Marine hydrothermal

a. Number of clones for each phylotype group shown in parentheses. Thp, *Thermoproteales*; Des, *Desulfurococcales*; Thm, *Thermococcales*; Mpy, *Methanopyrales*; Kor, *Korarchaeota*; UC, uncultured *Crenarchaeota*; UE1, uncultured *Euryarchaeota* 1; UE2, uncultured *Euryarchaeota* 2.
b. Clone name (GenBank Accession Number) based upon the results of a BLAST search (NCBI).

sequence identified from the EPR (Lepage *et al.*, 2004). *Thermococcus* species are generally hyperthermophilic obligate anaerobes with a fermentative metabolism using complex carbon sources (Kobayashi, 2001).

Nearly 18% of the clone types from the MM site are most closely related to uncultured *Euryarchaeaota* isolated from hydrothermal vents or benthic marine sediments (groups UEI, UEII, Fig. 4, Table 2). Further phylogenetic analysis shows that group UEI clones are most closely related to other archaeal sequences isolated from hydrothermal vent environments (Fig. 5). However, their G + C contents range from 50% to 55% and have the highest similarity (85–96%) to environmental sequences isolated from marine and estuarine sediments (Kaku *et al.*, 2005; Kim *et al.*, 2005; Nakagawa *et al.*, 2005). This suggests that UEI Archaea are adapted to the cold (~2°C) microenvironments of deep marine sediment on the EPR ridge flank. In contrast, UEII Archaea are more likely adapted to higher-temperature subseafloor environments as evidenced by elevated G + C contents (63–65%) and sequence similarity to *Euryarchaeal* sequences collected from other high-temperature environments including black smoker chimney vent and hydrothermal sediment off the coast of Japan (Takai and Horikoshi, 1999).

Discussion

Conditions of mineral formation

The sulfide minerals at MM site are unusual for the low-temperature, oxidizing environment typical of ridge flank seafloor. Both chalcopyrite and pyrrhotite precipitate within well defined chemical and thermal conditions (Craig and Scott, 1974; Barton and Skinner, 1979; Tivey, 1995) and are commonly found in high-temperature ($\geq$ 250°C) basalt-hosted hydrothermal vent environments on the mid-ocean ridge crest (Haymon, 1983; Hannington *et al.*, 1995). Precipitation of chalcopyrite (Cu_5FeS_4) requires

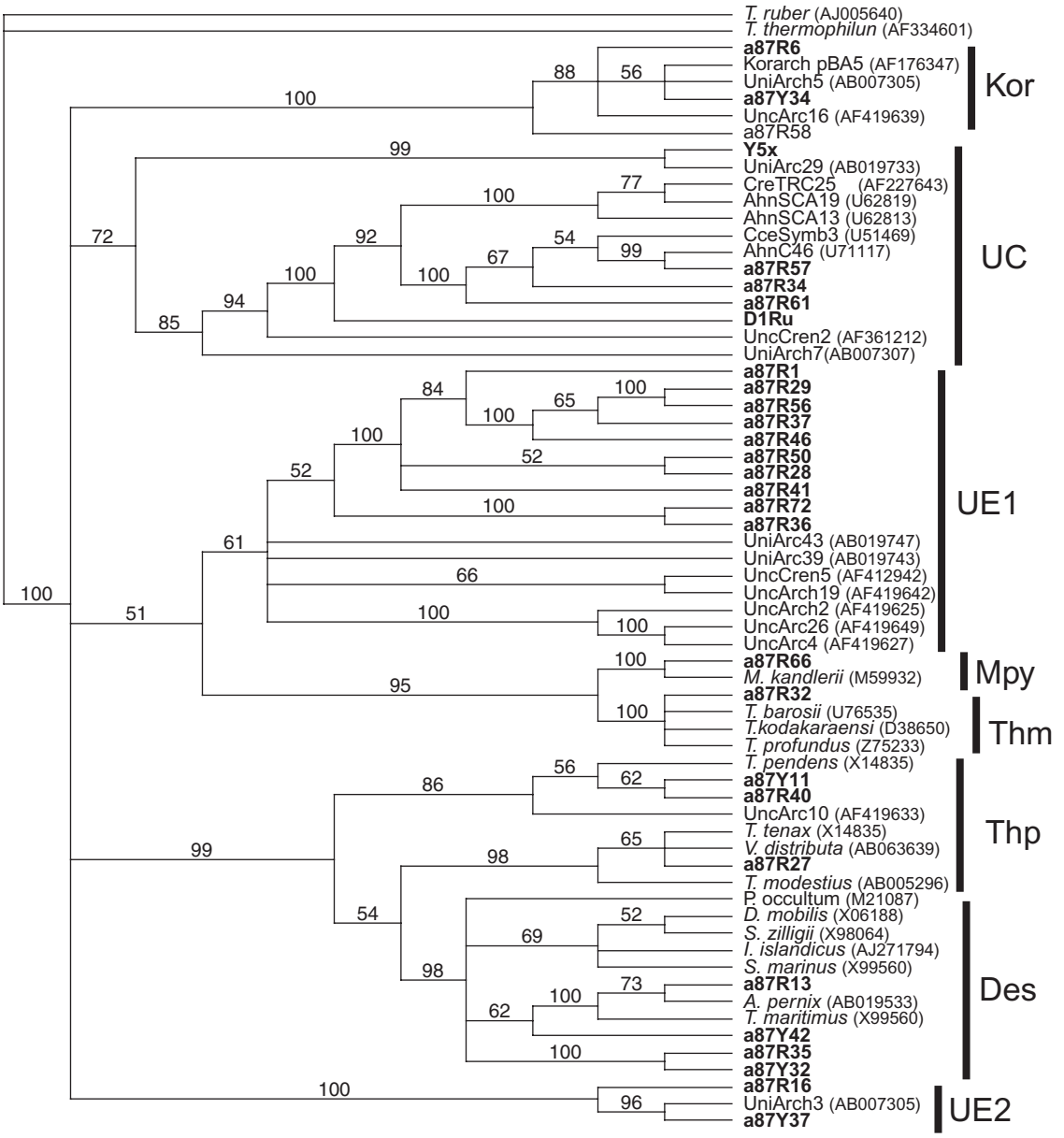

Fig. 4. Archaeal 16S rRNA phylogenetic tree. A maximum parsimony analysis with 1000 bootstrap replicates and approximately 1100 nucleotide positions was conducted. Sequence groups isolated from the fault scarp (shown in bold) cluster with eight groups: *Korarchaeota* (Kor), uncultured *Crenarchaeota* (UC), *Thermoproteales* (Thp), *Desulfurococcales* (Des), *Thermococcales* (Thm), *Methanopyrales* (Myp) and uncultured *Euryarchaeota* (UE1 and UE2). Bacterial sequences (*Thermocrinis ruber*, *Thermodesulfobacterium thermophilum*) were used as outgroups.

mobilization of Cu from subsurface basaltic rocks and transport of Cu in hydrothermal fluids as copper chloride complexes. These chloride complexes are unstable below ~250°C (Crerar and Barnes, 1976). Similarly, the 1C superstructure of pyrrhotite is unstable below 110°C (Morimoto *et al*., 1975). Precipitation of chalcopyrite, 1C pyrrhotite and pyrite also requires oxygen concentrations well below that of ambient seawater (Hannington *et al*., 1995). Taken together, these observations indicate that a hot (> 110°C), oxygen-depleted hydrothermal habitat exists within ridge flank basaltic crust beneath MM abyssal hill.

Because hydrothermal venting along the MM fault scarp was not observed during sampling, we have considered the possibility of sulfide minerals originating on the ridge crest, and either collecting on the fault scarp as sediment from hydrothermal plumes, or being on the seafloor as it spreads away from the ridge axis. These are, however, unlikely scenarios because of: (i) the direct association of Fe-sulfide particles with biological structures attached to

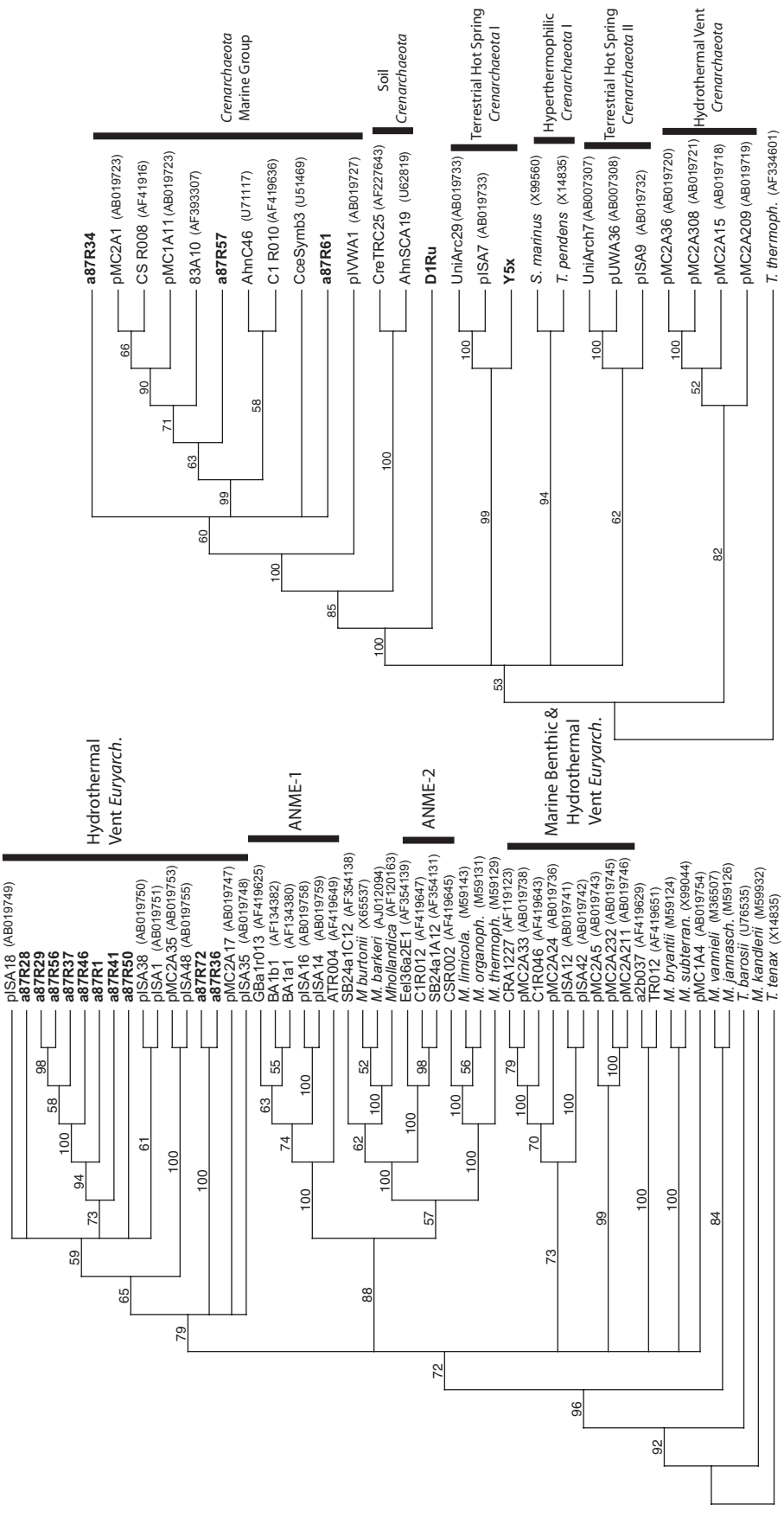

Fig. 5. Phylogenetic relationships of uncultured *Euryarchaeota* (left) and *Crenarchaeota* (right) clone groups from fault scarp materials at MM site. A maximum parsimony analysis with 1000 bootstrap replicates was used to construct both trees. Clone sequences within the UC group (right) fall within two subgroups: *Crenarchaeota* Marine Group I and terrestrial hot spring *Crenarchaetoa*. Clone sequences within UEI (left) are most closely related to a hydrothermal vent *Euryarchaeota* group composed of environmental sequences collected from hydrothermal sediments, seamounts and chimney structures off the coast of Japan (Takai and Horikoshi, 1999).

the fault scarp (Fig. 2); (ii) the absence of Fe-sulfide minerals in sediment samples taken from the top of the MM abyssal hill or the adjacent sediment pond at the base of the MM fault scarp (Fig. 1C) and; (iii) the small temperature anomalies that were measured within the MM site fault scarp crevices (+0.15–0.20°C compared with +0.05°C variation observed in ambient seawater at a control site) indicating ongoing venting of highly diffuse, warm hydrothermal fluid from the scarp at the time of sample collection (Haymon *et al*., 2005).

Implications for archaeal biosphere beneath young abyssal hills

While physiological and metabolic traits cannot be unambiguously determined from 16S rRNA sequence data, phylogenetic relationships of fault scarp clone types can yield some insight into the environment and chemical processes within the ridge flank basement crust (Cowen *et al*., 2003). All described species of *Thermoproteales*, *Desulfurococcales*, *Methanopyrales* and *Thermococcales* have optimum growth temperatures above 75°C (Garrity and Holt, 2001) indicating that hyperthermophily is a conserved trait within these groups. Although no members of *Korarchaeota* have been cultured, these organisms so far have been detected only in high-temperature environments (Barns *et al*., 1996; Takai and Horikoshi, 1999; Nercessian *et al*., 2003; Auchtung *et al*., 2006). Also, many clone types in UC and UE groups have G + C contents that are consistent with a hyperthermophilic origin. Presence of these archaeal sequences in the low-temperature environment (~2°C) of the abyssal hill therefore suggests that the upper oceanic crust of the ridge flank at MM site can support hyperthermophilic archaeal communities.

A preliminary bacterial 16S rRNA clone library shows little evidence for thermophilic bacterial sequences in MM site samples (C.J. Ehrhardt, R.M. Haymon and P.A. Holden, in preparation). The majority of bacterial sequences are closely related to the epsilon subgroup of *Proteobacteria* that is prevalent in marine environments where hydrothermal fluids mix with ambient seawater. Unlike the archaeal community, the bacterial sequences identified most likely represent colonization communities on the fault scarp and not active communities living within the ridge flank subseafloor.

Many of the clone types identified from the fault scarp are closely related to sulfur-respiring *Crenarchaeal* and *Euryarchaeal* groups. Sulfur is a common reductant for hyperthermophilic taxa within *Crenarchaeota* and *Euryarchaeota*. Specifically, sulfur respiration coupled to H_2 oxidation ($H_2 + S^0 \rightarrow H_2S$) is widespread in the *Crenarchaeal* orders *Desulfurococcales* and *Thermoproteales* (Huber and Stetter, 1998). Sulfur is also a potential metabolite for *Thermococcus* species (Kobayashi, 2001). This suggests that sulfur (S^0) reduction to sulfide (S^{2-}) is an important metabolism in subseafloor ridge flank fluids.

Thermophilic and hyperthermophilic microorganisms have been found previously in basalt-hosted ridge flank fluids. Cowen and colleagues (2003) found sequences of thermophilic and anaerobic Bacteria and Archaea in fluids emanating from 3.5 Mya basaltic basement crust. A more recent study by Huber and colleagues (2006) collected crustal fluids from the Baby Bare Seamount on the Juan de Fuca Ridge (JdFR) flank and isolated thermophilic, anaerobic, H_2-oxidizing Bacteria as well as Archaea closely related to hyperthermophilic *Thermococcus* spp. Similarities in the archaeal community at MM site and the JdFR flank suggest that oxygen-depleted, high-temperature habitats may be common in the global ridge flank subsurface biosphere. Additionally, certain organisms such as *Thermococcus* spp. that are found in both EPR and JdFR ridge flank samples may be globally distributed beneath ridge flanks.

Another interesting similarity between the MM sites and other ridge flank sites is the presence of clone types closely related to organisms in the order *Thermoproteales* (Cowen *et al*., 2003; Cowen, 2004). With one recent exception (Page *et al*., 2004), organisms within this order have been conspicuously absent from molecular surveys of several different marine hydrothermal environments (Nercessian *et al*., 2004). In addition, nearly all cultured and described species within *Thermoproteales* have been isolated from high-temperature terrestrial and freshwater systems (Huber and Stetter, 2001) with only one noted exception (Volkl *et al*., 1993). These observations suggest that the *Thermoproteales* group may be an under-recognized and possibly significant inhabitant of subseafloor marine hydrothermal systems.

There are some interesting differences between the fluids and microbial communities at MM site and those in previous studies of ridge flank basement crust. The inferred temperature of ridge flank fluids at MM site (> 110°C) is much higher than temperatures of crustal fluids measured on the JdFR flank at Baby Bare Seamount, 19.7°C (Huber *et al*., 2006), and ODP hole 1026B, 62°C (Cowen *et al*., 2003). Elevated subseafloor temperatures at MM site may partly explain the presence of hyperthermophilic archaeal clone types (Des, Thp, Mpy) that were not detected in crustal fluids from the JdFR flank. Further investigation is needed to determine whether these community characteristics are related to geologic differences between these two sites (i.e. age of oceanic crust, seafloor spreading rate, sedimentation rate) or to the overall paucity of molecular data from subseafloor ridge flank environments.

While there remains little direct evidence of active metabolisms in the global ridge flank subseafloor bio-

sphere (Edwards *et al*., 2005), it has been suggested that oxidation of H_2 in ridge flank basement fluids could support a significant chemolithoautotrophic biosphere in ridge flank oceanic crust (Bach and Edwards, 2003). Previous molecular surveys of basalt-hosted ridge flank fluids showed little evidence for Archaea consistent with this metabolism (Cowen *et al*., 2003; Huber *et al*., 2006). Most recently, thermophilic methanogens were recovered from black rust deposits on an observatory borehole deployment on the eastern flank of the JdFR (Nakagawa *et al*., 2006). However, it is unclear whether the hydrogen in this system is derived from corrosion of the steel lining on the deployment or subseafloor processes.

At the MM site, we find several sequences that are closely related to high-temperature, H_2-oxidizing Archaea including autotrophic *Crenarchaeota* (Des, Thp) and methanogenic *Euryarchaeota* (Mpy). This is one of the first reports of methanogenic Archaea in a basalt-hosted ridge flank environment and indicates a potential role for hyperthermophilic H_2-based archaeal communities in the EPR ridge flank basement crust. While 16S clone types indicate that H_2-metabolizing microbes may be present in the EPR ridge flank, direct sampling of hydrothermal fluids coupled with other types of molecular analysis [i.e. FISH, reverse transcription polymerase chain reaction (RT-PCR)] will be needed to verify and quantify the extent of H_2 metabolism beneath abyssal hills.

Our results also may help to explain the origins of hyperthermophilic organisms found in other low-temperature (1–4°C) deep marine subseafloor habitats. Recent molecular surveys (Kormas *et al*., 2003; Sorensen *et al*., 2004) performed on marine sediments collected from Ocean Drilling Project boreholes identified DNA sequences related to a number of hyperthermophilic archaeal lineages and uncultured organisms previously identified only in high-temperature hydrothermal environments. While this unusual finding could be an artefact of the genetic databases (Sorensen *et al*., 2004) or result from lateral fluid flow from distant hydrothermal sources (Kormas *et al*., 2003), we suggest that expulsion of hyperthermophilic archaeal communities from hydrothermal ridge flank basement reservoirs (Fig. 1C) could be contributing to the global archaeal subseafloor biosphere within marine sediments.

The results presented here offer new insights into the vast and often inaccessible microbial biosphere in ridge flank oceanic crust. The presence of high-temperature sulfide phases and hyperthermophilic archaeal phylotypes on the MM fault scarp indicates that abyssal hill fault scarps may be connected to deep, hot (> 110°C) hydrothermal reservoirs beneath the EPR ridge flank. While we are only beginning to understand the nature of basalt-hosted microbial habitats beneath the ridge flank, this work is part of a growing body of evidence that extremophilic microbial habitats persist into the vast expanses of oceanic basement crust beneath the flanks of the global mid-ocean ridge system. In the future, abyssal hill fault scarps should offer a promising new venue for ridge flank subseafloor research and a new window into this unexplored archaeal ecosystem.

Experimental procedures

Site description and sample collection

The MM site is located on the western flank of the EPR at 9°27′N, 104°32.3′W, approximately 26 km from the ridge crest (Fig. 1A). In 2002, submersible dives on *DSRV Alvin* (Dives 3785 and 3787) explored the axis-facing fault scarp of this abyssal hill and sampled orange-brown flocculent from beneath small ledges in the scarp with two slurp guns mounted on *Alvin* (Fig. 2A). The slurp samples were collected from the fault scarp at a depth of 2982 m, approximately 50 vertical metres beneath the top of the volcanic basement (Fig. 1C). Because there was no visual evidence of active hydrothermal venting along the fault scarp at the time of sampling (Haymon *et al*., 2005), we could not measure the chemical composition of ridge flank fluids. Immediately after the dive, the slurp samples were frozen and stored at –80°C until microbial community and mineral analyses could be performed at UC-Santa Barbara.

Mineral analysis

Mineral phases were identified by powder XRD using a Phillips Xpert Diffractometer. Cu Kα radiation (1.5405 Å) at 40 kV and 50 mA was used. Diffraction peaks between 2θ values of 2° and 70° were recorded and peaks identified using the peak search function of Phillips X'Pert Graphics and Identify software. Minerals were identified by comparing the observed diffraction pattern with the ICDD-PDF database for minerals.

Composition and morphology of fault scarp minerals were analysed using an FEI XL30 environmental scanning electron microscope (ESEM) with field emission gun. Because the ESEM requires no sample preparation or fixation procedures, bulk fault scarp materials and individual strings of flocculent were mounted directly on an aluminum stub fixed to a Peltier cooling stage. Accelerating voltage of 15 KeV was typically used for image collection. X-ray microanalysis was conducted using a Princeton Gamma Tech energy dispersive spectrometer (EDS) and a PRISM IG intrinsic germanium detector mounted on the ESEM.

Molecular analysis

DNA extractions were performed on 0.2–0.5 g of flocculent material using the UltraClean™ Soil DNA Kit by MoBio Laboratories with the following modifications. A heat lysis step was added prior to bead beating lysis as follows: 260 μl of bead beating solution and 39 μl of S1 solution were added to each sample. After gentle mixing, 130 μl of IRS solution was added. Two cycles of incubating samples at 70°C followed by brief vortexing were performed. This was followed by cen-

trifugation at 9300 *g* for 30 s. The lysate was then transferred to a new tube until it could be pooled with the supernatant from the bead-beating lysis step.

DNA extracts were amplified using the *Taq* PCR Core Kit (Qiagen) and primers 21F and 1100A, which targeted genes encoding archaeal 16S rRNA (Delong, 1992; Embley *et al.*, 1992). Reaction mixtures for PCR consisted of the following: 20 ng of template DNA, 25 pmol forward primer, 25 pmol reverse primer, 1 × Qiagen PCR Buffer, 1.5 mM $MgCl_2$, 200 μm dNTP, 2.5 units *Taq* DNA polymerase. Polymerase chain reactions were performed under the following conditions: 3 min denaturation at 94°C, followed by 30 cycles (45 s denaturation at 94°C, 60 s annealing at 55°C, 90 s extension at 72°C), followed by a final 7 min extension step at 72°C.

Polymerase chain reaction products were run on 0.8% agarose gel to verify the amplicon size and cleaned using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Polymerase chain reaction products were cloned and plasmid DNA was isolated using the Qiagen PCR CloningPlus Kit. Blue/white screening of cloned plasmids was carried out on LB agar plates containing 0.1 mg ml^{-1} ampicillin, 0.08 mg ml^{-1} Xgal and 50 μM IPTG. Picked colonies were grown overnight in 2 ml of LB Broth containing 0.075 mg ml^{-1} ampicillin and plasmid DNA extracted using the Qiaquick plasmid Miniprep kit (Qiagen) according to the manufacturer's instructions. Approximately 500 ng of plasmid DNA with PCR product inserts were sequenced at the UC Berkeley DNA sequencing facility. A total of 139 cloned PCR inserts were sequenced. All inserts were sequenced with the T7 sequencing primer. Representative sequences for each phylotype were sequenced in the reverse direction using the M13 forward sequencing primer. All representative phylotypes had the entire length of the PCR insert sequenced, approximately 1100 nucleotides.

Sequences were manually edited and aligned using Sequencher™ 3.0 and Bioedit v7.0.5. G + C contents also were calculated with Bioedit. Alignments of environmental sequences from the fault scarp and archaeal reference sequences were generated first using the CLUSTALW multiple alignment function in BioEdit, and alignments were then visually checked for accuracy. The entire length of the PCR insert (~1100 nucleotides) was used for alignments. Only reference sequences that covered the entire PCR insert were included in the alignments and phylogenetic trees. Sequences that were greater than 97% similar were grouped into one phylotype. Unique phylotype sequences were submitted to GenBank (accession numbers DQ417462–DQ417490). Phylogenetic analysis based on full alignments of the sequenced 16S rRNA region was constructed in PAUP v4.0 software (Swofford, 2003) using maximum parsimony criterion.

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