

Thermophilic and hyperthermophilic microorganisms in 3–30°C hydrothermal fluids following a deep-sea volcanic eruption

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Abstract

Thermophilic and hyperthermophilic microorganisms were cultured from 18°C diffuse hydrothermal fluids at the CoAxial segment deep-sea hydrothermal vent site 3 months after an eruption resulting from an intrusion of magma into shallow crust. The abundances of these organisms decreased over a 3-year period as the shallow magma cooled. The presence of these organisms at the site suggests that these organisms grew in response to nutrient input from hydrothermal fluid circulation and then were flushed to the surface following the eruption. Thermophiles and hyperthermophiles were also found in low-temperature (3–30°C) fluids at three other chronic, highly active deep-sea vent sites. The origin of these organisms is not known but may include the overlying seawater or a shallow to deep seafloor habitat. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

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1. Introduction

Thermophilic and hyperthermophilic microorganisms (growth at temperatures above 55 and 90°C, respectively) are well established as inhabitants of terrestrial and marine hydrothermal systems [1]. However, little is known about the diversity of high-temperature microbial communities, the spatial dimensions of their habitat, or the geochemical impact these organisms may have on their environment. Likewise, little is known about how hydrothermal systems influence the growth and activity of thermophiles and hyperthermophiles. Recently, Gold [2] suggested that these organisms may occupy

extensive regions of the earth's crust and comprise a biomass possibly exceeding all surface-associated biomass. The claim has not been substantiated; however, there have been numerous reports which support the theory of high-temperature microorganisms occupying broad and diverse regions of the subsurface.

Baross and Hoffman [3] proposed that thermophiles and hyperthermophiles, as well as other hydrothermal microbes, exploit seafloor thermal and chemical gradients associated with deep-sea vents for growth. A sample of hydrothermal sulfide with temperatures estimated to be greater than 100°C contained primarily archaeal lipids, indicative of high-temperature microbial communities [4]. These sulfide structures may contain thermal and chemical gradients analogous to those below the sea-

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floor. Hyperthermophilic microorganisms have also recently been isolated from oil reservoirs in the North Sea, in the north slope of Alaska, and in continental reservoirs in France [5–7]. The lack of exogenous seawater in the French reservoirs, which could act as a source of hyperthermophile contamination, suggests that these hyperthermophiles are native inhabitants of this environment as well. Furthermore, viable thermophilic bacteria have been found in drill core samples collected more than 2000 m below the surface within the Taylorsville Triassic Basin site in Virginia, USA [8,9] and in deep-sea marine sediments more than 500 m below the seafloor in the Pacific Ocean [10].

Each of these reports suggests that thermophiles and hyperthermophiles live deep below the surface, though little is known about the microbial ecology of these environments. The 1993 deep-sea volcanic eruption along the CoAxial segment of the Juan de Fuca Ridge in the northeastern Pacific Ocean provided an opportunity to test for the presence of high-temperature microorganisms in the subseafloor. Thermophile and hyperthermophile abundances were measured over a 3-year period to determine whether they changed with the temperature and chemistry of the system. The exiting CoAxial fluids were all low temperature ($< 36^{\circ}\text{C}$) and were formed by the subseafloor mixture of high-temperature hydrothermal fluid and cold seawater. The exit temperatures of these fluids were generally below the minimum growth temperatures of the microorganisms selected for growth enrichments, namely *Thermococcus* spp. and hyperthermophilic *Methanococcus* spp. Similar fluids from two other deep-sea vent sites recently impacted by volcanic eruptions as well as two chronic hydrothermal systems were also sampled.

2. Materials and methods

2.1. Field sampling

The June 1993 magma intrusion along the CoAxial segment of the Juan de Fuca Ridge in the northeastern Pacific Ocean (Fig. 1) produced two distinct zones of venting which were named 'Floc' and 'Flow' [11]. They were each diffuse vent fields

created by the eruption, were separated by 20 km, and emitted only low-temperature vent fluids (18 and 36°C , respectively, 3 months after the eruption, Fig. 2). The Floc site was named for the copious flocculent material being emitted from below the seafloor. The CoAxial site was sampled annually for thermophiles and hyperthermophiles for 3 years after the eruption. Samples were also collected from the Endeavour and Cleft segments as well as Axial Seamount, all on the Juan de Fuca Ridge, and from $9^{\circ}50' \text{N}$ along the East Pacific Rise (EPR, Fig. 1). The Cleft site erupted between 1983 and 1990 [12] and was sampled in 1994. The 9°N EPR site erupted in April 1991 [13] and was sampled in 1993. The other two sites sampled (Endeavour and Axial Seamount) are chronic systems which have remained active since their discovery in 1984 [14,15].

Diffuse hydrothermal fluids were sampled from the seafloor using the Deep-Submergence Vehicle *Alvin* and the Remote-Operated Vehicle *ROPOS* at each site with titanium water samplers [16] either attached to a manifold water sampler [17] or as discrete samples. The same vents were sampled annually at the Floc site. The sampling arm of the manifold sampler was placed directly into seafloor cracks and the exiting subseafloor hydrothermal fluids and flushed with the fluid until the temperature inside the sampler had stabilized. This minimized the degree of seawater contamination into the sample. Furthermore, the sites sampled lacked appreciable sediments thus minimizing the possibility of sampling sedimentary microorganisms. Background seawater samples were collected from 1 to 40 m above the seafloor using the titanium samplers.

2.2. Quantitative enrichments for thermophiles and hyperthermophiles

The number of culturable anaerobic thermophiles and hyperthermophiles was determined using the three-tube most-probable-number (MPN) technique [18,19] and various anaerobic media incubated at 55 and 90°C . The media contained Sea Salts B, trace elements, and the oxygen indicator resazurin [20] and either 0.1% (w/v) yeast extract (YE), 0.3% (w/v) yeast extract and peptone with elemental sulfur (YPS), or 0.03% (w/v) sodium formate, sodium acetate, and sodium propionate with 0.01% yeast extract

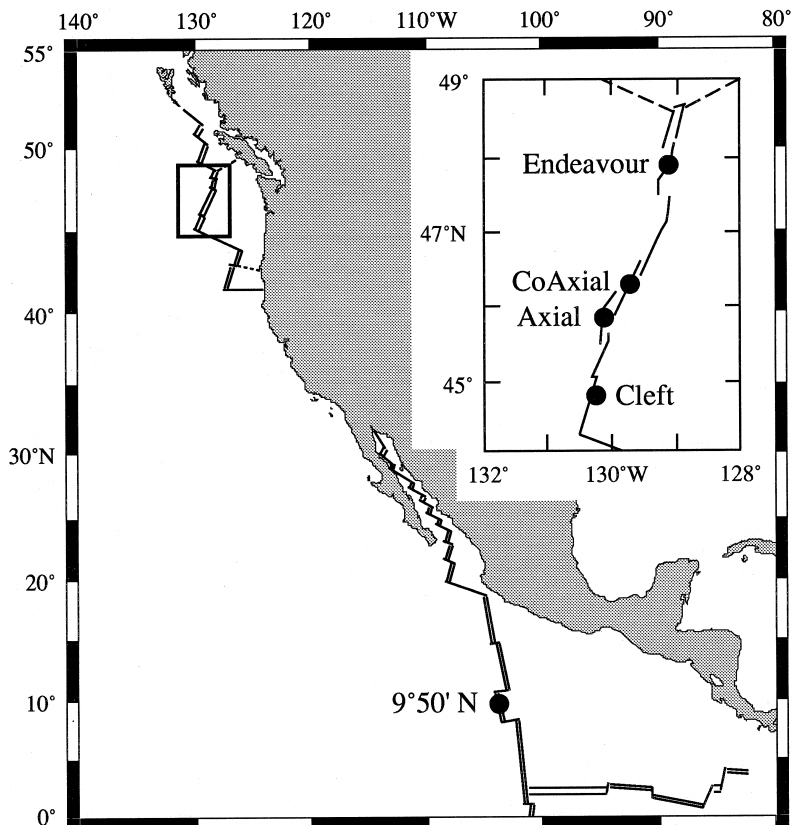


Fig. 1. Map of the eastern North Pacific Ocean showing the Juan de Fuca Ridge and the 9°50'N hydrothermal vent site along the East Pacific Rise. The inset is an enlargement of the Juan de Fuca Ridge (region within the box) which shows the locations of the Endeavour, CoAxial, Axial Seamount, and Cleft hydrothermal vent fields.

(FAP). The FAP and YE media were buffered at pH 6.5 using PIPES (Sigma) and used an 80% H₂-20% CO₂ headspace, while the YPS medium was buffered at pH 6.0 with MES (Sigma) and used an argon headspace. All media were reduced with 0.05% (w/v) sodium sulfide. The FAP medium enriched for heterotrophs, the YE medium for hyperthermophilic methanogens and heterotrophs, and the YPS medium for hyperthermophilic sulfur reducers and other heterotrophs [20]. In 1993 at CoAxial and 9°N EPR, the abundances of thermophiles and hyperthermophiles were based on triplicate enrichments performed in YE and YPS media. The 1996 CoAxial abundances were based on triplicate enrichments performed in all four media. For all other sampling periods, one set each of YPS and YE tubes was incubated at 90°C, while one set each of YPS and FAP tubes was incubated at 55°C. All of the tubes were

incubated in oven-heated sandbaths with strict temperature control ($\pm 2^\circ\text{C}$). Each turbid YE enrichment was checked for methanogens by examining the organisms for autofluorescence, using a blue-violet 05 excitation filter set in a fluorescence microscope (Zeiss), and by analyzing the headspace for methane with a gas chromatograph equipped with a flame-ionization detector (Shimadzu) and a 1-m Carbosphere 60/80 column (Alltech) at room temperature.

2.3. Counts of total bacteria

A 20-ml aliquot of each seawater and diffuse-fluid sample was preserved in glutaraldehyde (type II, 1–2% final concentration) and stored at 2°C. Each sample was filtered onto a 0.2 μm pore size filter prestained with Irgalan black (Nucleopore), stained

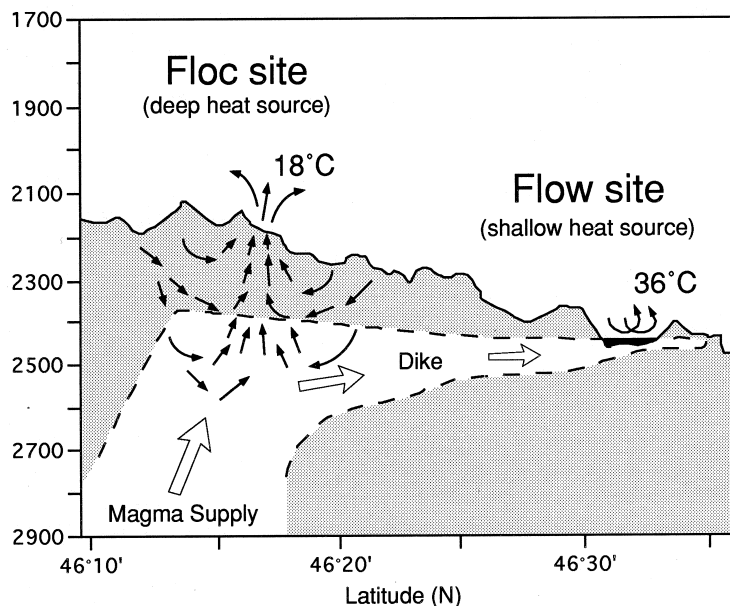


Fig. 2. Longitudinal cross-section of the CoAxial segment seafloor and the magmatic dike which intruded the segment in June 1993. The two sites of diffuse low-temperature hydrothermal discharge are shown along with the temperature of the exiting fluids at each site in October 1993. Solid arrows depict hydrothermal fluid flow and the open arrows represent magma flow (modified from [23]). The actual depth and vertical thickness of the magma intrusion are unknown; therefore, the dike boundary is drawn with dashed lines to represent this uncertainty.

with DAPI (4',6-diamidino-2-phenylindole, Sigma), and counted by epifluorescence microscopy [21].

2.4. Transmission electron microscopy

Electron micrographs were made of ultrathin sections of primary enrichments of thermophiles and pure cultures of hyperthermophiles. Each sample was preserved with glutaraldehyde (type I, 1–2% final concentration, Sigma) and OsO_4 , embedded with Spurr's low-viscosity medium, mounted onto formvar-coated grids, and stained for 10 min with 6% uranyl acetate and lead citrate. The samples were viewed with a JEM 1200 EXII (JEOL) transmission electron microscope.

3. Results

No thermophiles or hyperthermophiles were detected by enrichment culture from the 32°C fluids collected from the Flow site at CoAxial in October 1993 (Table 1) or from any 2°C background sea-

water samples collected from the CoAxial site. However, thermophilic heterotrophs and hyperthermophilic methanogens and sulfur reducers were readily isolated from 18°C 'Floc' vent fluids in 1993 (Table 1). Hyperthermophiles were not detected in 'Floc' fluids sampled between 1994 and 1996 after venting had subsided significantly. Moderate thermophiles were detected in 'Floc' fluids each year through 1996 (Table 1). Enrichments at 55°C in FAP medium often contained large (up to 10 cm long) white flocculent material identical in appearance to the flocculent particles ejected from the Floc site. All positive enrichments in YE medium contained coccoid-shaped cells which autofluoresced at the wavelength indicative of methanogens, and saturating levels of methane. No methane was measured in tubes scored negative for growth or in uninoculated controls.

Hyperthermophilic sulfur-reducing coccoids and autofluorescent methanogenic coccoids were cultured from diffuse fluids collected at 9°N EPR 21 months after the eruption at that site. Thermophiles and sulfidogenic and methanogenic hyperthermophiles were observed in primary enrichments of diffuse fluids col-

Table 1

Concentration (cells l⁻¹) of culturable thermophiles (55°C) and hyperthermophiles (90°C) using various media based on three-tube most-probable-number (MPN) estimates, and of total bacteria (cells l⁻¹), using epifluorescence microscopy, in diffuse hydrothermal vent fluids

Location	Date	Temp. (°C)	FAP ^a (55°C)	YPS ^b (55°C)	YE ^c (90°C)	YPS (90°C)	Total bacteria (×10 ⁷)
CoAxial Segment:							
Flow	Oct. 1993	36	– ^d	–	–	ND ^e	–
Flow	Oct. 1993	32	–	ND	ND	ND	1.9
Floc	Oct. 1993	18	–	≥120	–	ND	5.9
Floc	Oct. 1993	18	–	–	≥80	≥460	11.7
Floc	July 1994	8	400	700	ND	ND	8.9
Floc	July 1994	7	400	400	ND	ND	6.7
Floc	July 1995	8	ND	ND	ND	ND	5.5
Floc	July 1995	17	80	460	ND	ND	6.8
Floc	Aug. 1996	2.5	≥180	≥180	ND	ND	4.6
9°N EPR:							
Biomarker 141	Dec. 1993	30	–	–	≥460	≥460	–
Cleft Segment:							
Pipe Organ	June 1994	5	ND	ND	ND	ND	2.3
Vent 1	June 1994	27	ND	ND	ND	ND	3.3
Endeavour Segment:							
Salty Dog	June 1995	30	–	1860	180	1860	1.8
High Rise	June 1995	14	460	860	1500	4800	1.3
Quebec	Sept. 1995	12	80	420	80	460	5.4
Axial Seamount:							
ASHES	July 1995	30	80	ND	ND	80	4.6

^a0.03% (w/v) formate, acetate, and propionate+0.001% yeast extract; H₂-CO₂ headspace.

^b0.3% yeast extract and peptone with elemental sulfur; Ar headspace.

^c0.1% yeast extract; H₂-CO₂ headspace.

^dNot determined.

^eNot detectable (detection limit of 60 cells l⁻¹).

lected at the Endeavour segment and at the ASHES vent field on Axial Seamount. All of the turbid YE-medium tubes contained coccoid-shaped autofluorescent cells and saturating levels of methane. The turbid 90°C YPS-medium tubes contained mostly coccoids. The highest numbers of thermophiles and hyperthermophiles were measured in the Main Endeavour Field along the Endeavour segment. Neither thermophilic nor hyperthermophilic microorganisms were detected in the diffuse fluids from the Cleft segment collected in 1994, 4–11 years after the eruptions at that site.

Electron micrographs of the 55°C FAP enrichments from 'Floc' in 1994 showed that a diverse assemblage of microbial morphologies was present in these vent fluids (Fig. 3A). One common morphology observed is shown in Fig. 3B which is unique, but most closely resembles the structures found in type II methanotrophs and nitrifiers which consists of a system of paired peripheral membranes [22].

Another common morphology observed in the enrichments is shown in Fig. 3C and is suggestive of a sulfur-oxidizing bacterium [23]. The hyperthermophilic microorganisms cultured in YPS medium from CoAxial and 9°N EPR diffuse fluids were exclusively sulfidogenic coccoids (Fig. 3D) with optimal growth temperatures of 88°C. The methanogenic coccoids enriched in YE medium generally had optimal growth temperatures of 85°C.

4. Discussion

Among the hyperthermophiles cultured from CoAxial and 9°N EPR diffuse fluids were obligately anaerobic sulfur reducers of the genus *Thermococcus* whose minimum growth temperature of 55–60°C (Holden, J.F., Takai, K., Zyskowski, J.A., Mathur, E. and Baross, J.A., in preparation) significantly exceeded the 10–30°C exit temperatures of the fluids.

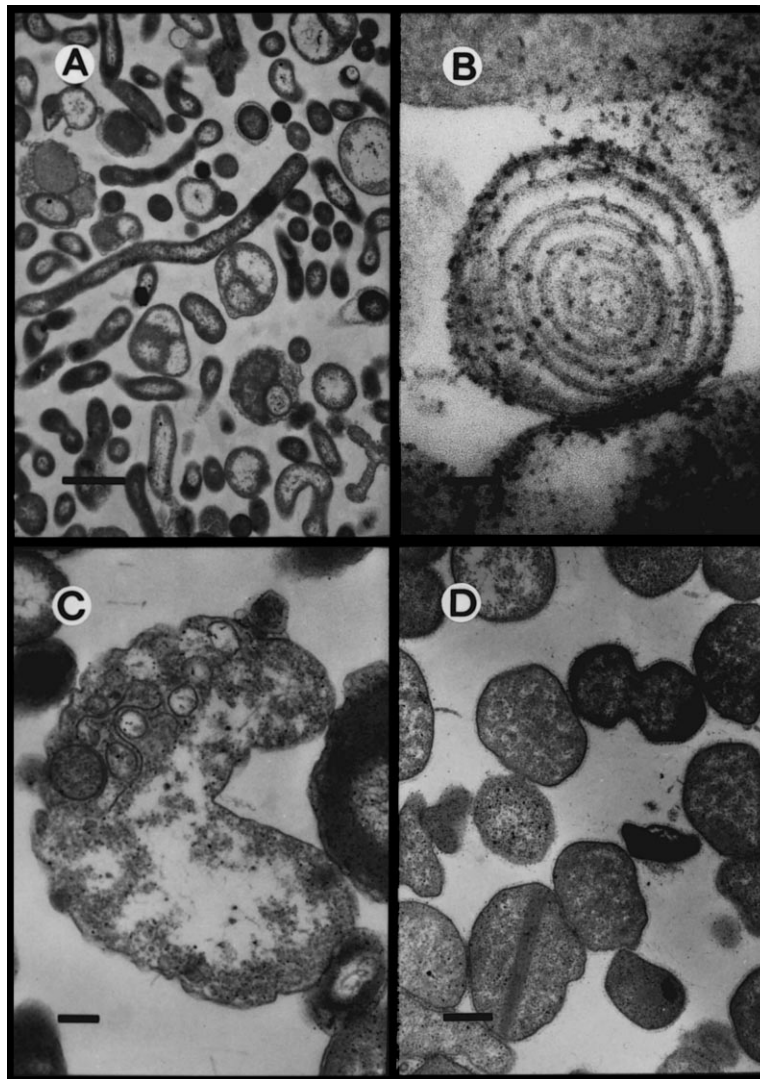


Fig. 3. Electron micrographs of ultrathin sections of enrichment cultures. A highly diverse assemblage of microorganisms was found in primary enrichments at 55°C containing anaerobic low-organic (FAP) medium and inoculated with Floc fluids collected in 1994 (A, 1 μm scale bar), including morphologies suggestive of possibly a methane- or ammonia-oxidizing bacterium (B, 50 nm scale bar) and a sulfur-oxidizing bacterium (C, 0.2 μm scale bar). Hyperthermophilic sulfur-reducing archaea were cultured from 10 to 30°C diffuse fluids at the CoAxial and Endeavour segments, Axial Seamount, and 9°N East Pacific Rise (D, representative strain 9N2 from 9°N EPR, 0.5 μm scale bar).

The presence of these hyperthermophilic microorganisms in low-temperature diffuse fluids suggests that they originated from a subsurface hydrothermal habitat where temperatures were within their growth range. The lack of sediments at the sampling sites and the manner in which the samples were collected minimized the likelihood of sampling high-

temperature microorganisms from sources other than the subsurface. The original source of the organisms is unknown, but may be overlying seawater, off-axis sediments, or a subsurface habitat. The depth and spatial extent of this habitat is also unknown. The different thermal groups of microorganisms cultured from 'Floc' and the morphological var-

iations shown in Fig. 3 suggest both a high microbial diversity and complex thermal and chemical gradients associated with this environment.

Diffuse-fluid vents are formed when seawater mixes with hydrothermal end-member fluids within the seafloor and may be found at locations distinctly removed from high-temperature black-smoker environments. The low-temperature vents at 'Floc' and 'Flow' were 15 and 35 km, respectively, from the nearest known high-temperature venting. The diffuse-fluid venting at the sites began and declined with the placement and cooling of the 1993 magma intrusion. The dike originated southwest of the Floc site and migrated northeast along the rift zone (Fig. 2) [24]. The Flow site was at the distal end of the eruption where the magma reached the seafloor. The heat source for this vent was near the surface and cooled to ambient seawater temperature within a year [25,26]. No high-temperature microorganisms were detected at the Flow site 3 months after the eruption despite its higher fluid temperatures. The absence of thermophiles and hyperthermophiles at 'Flow' may be due to the lack of hydrothermally derived nutrients at the site and the inability of dike-driven hydrothermal convection to access a putative subseafloor thermophile niche. In contrast, the heat source at the Floc site was deeper and remained hotter longer than at 'Flow' [25,26] and took approximately 3 years to cool to ambient temperature. The end-member hydrothermal fluid temperatures at 'Floc' were higher than at 'Flow' as evident by the low-chloride, high-volatile fluids found at 'Floc' which are indicative of phase-separation, or boiling, of seawater within the crust [25]. Thermophiles and hyperthermophiles were found at the Floc site 3 months after the eruption, but their abundances declined with time as fluids cooled and venting subsided.

Our findings at CoAxial prompted us to examine other diffuse-fluid vents for high-temperature microorganisms at other sites which had either recently experienced an eruption or were long-lived chronic hydrothermal systems which had remained active for more than 10 years. Thermophiles and hyperthermophiles were found at 9°N EPR (which had experienced an eruption 21 months earlier), at Axial Seamount, and at the Endeavour segment. There was extensive hydrothermal discharge at all three sites.

The highest abundances of subseafloor high-temperature microbes were at the Main Endeavour Field. The Endeavour site contains massive sulfide deposits believed to be formed by prolonged high-volume hydrothermal fluid output fed by a cracking front into hot basaltic rock [14]. Assuming the subseafloor acts as a steady-state chemostat, the higher thermophile and hyperthermophile abundances at Endeavour suggest this site may harbor a subsurface habitat which is either larger or more productive than those at the other sites examined. In contrast, no high-temperature organisms were detected in 1994 at a 4–11-year-old eruption site along the Cleft segment. Diffuse-fluid venting at this site had virtually ceased by 1995 (J. Holden, personal observation).

The original source of these diffuse-fluid thermophiles and hyperthermophiles is unknown, but is either seawater, off-axis sediments, or the seafloor itself. Near-bottom currents may have carried high-temperature microorganisms from a nearby vent site by means similar to that used by hydrothermal vent macrofauna. Studies at CoAxial suggest that vent macrofauna larvae began colonizing the Floc site within months (or possibly within weeks) of the eruption [27] and the same currents may have carried hyperthermophiles to the site after the eruption. However, neither thermophiles nor hyperthermophiles were detected in background seawater at CoAxial suggesting that their numbers in seawater are low. Alternatively, high-temperature microorganisms may have resided in or on the seafloor at the CoAxial site prior to the eruption. Hyperthermophiles were found in a hydrothermal plume released from the seafloor resulting from the 1996 North Gorda Ridge seafloor eruption, but not in background seawater. This suggests that viable hyperthermophiles were present in either the seafloor or in surface sediments prior to the eruption and were entrained into this plume during its release [28].

The presence of hyperthermophiles in diffuse fluids at temperatures below their minimum temperature needed for growth suggests that the organisms were growing within the seafloor where temperatures were permissive. However, the depth and extent of any subseafloor hydrothermal habitat for thermophiles and hyperthermophiles are unknown. A subseafloor microbial population would require fluid circulation to supply nutrients and energy sources and to re-

move metabolites. Subseafloor fluid circulation is limited primarily to the top layer of ocean crust, known as the extrusive layer (500–1000 m thick), where basalt permeabilities are 10–100-fold higher than the underlying basalt layers [29,30]. Therefore, high-temperature microorganisms may potentially inhabit cracks and void spaces between the surface and the lower boundary of the extrusive layer. At CoAxial, geophysical fluid circulation models predict that circulating fluids within the temperature range necessary for hyperthermophile growth (50–100°C) are within 1 m of the dike and extend down to its bottom boundary [26]. This set of isotherms provides a maximum estimate for the extent of the subsurface hyperthermophile biotope at CoAxial while the dike was suitably warm.

In conclusion, our findings support the theory of high-temperature microorganisms living within the seafloor, and that these organisms respond to physical and chemical changes in their environment brought on by a deep-sea magma intrusion into shallow crust. The high-temperature microorganisms found at CoAxial represent one of several microbial populations which showed a significant increase in abundance following the 1993 magma intrusion including mesophilic iron-, methane-, and sulfur-oxidizing bacteria [31]. There was likely a thermal gradient within the seafloor which favors high-temperature anaerobes in the deep portion of the gradient and aerobic mesophiles near the surface. Subseafloor microbes which bloomed after the eruption may have served as an important food source for vent macrofauna which colonized 'Floc' within months of the eruption [27]. Ongoing research is focused on understanding the magnitude, diversity, and impact of thermophiles and hyperthermophiles in the subseafloor and in seafloor drill core samples using culturing techniques, 16S rDNA sequence comparisons of natural DNA, and geochemical signatures of microbial activity. There are two hypotheses concerning the original source of subsurface high-temperature microorganisms, namely a seawater source and a seafloor source. Future research will use enrichment techniques and molecular probe analyses to determine the abundances and survivability of hyperthermophiles in both environments in the absence of favorable growth conditions. Most important, newly formed vent sites and diffuse-fluid

hydrothermal vent fields may serve as a portal into the microbial assemblages and processes occurring within the ocean floor and provide a source of novel high-temperature microorganisms.

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