

# Abundances of Hyperthermophilic Autotrophic Fe(III) Oxide Reducers and Heterotrophs in Hydrothermal Sulfide Chimneys of the Northeastern Pacific Ocean<sup>∇†</sup>

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**The abundances of hyperthermophilic heterotrophs, methanogens, and autotrophic reducers of amorphous Fe(III) oxide in 18 samples of deep-sea hydrothermal vent sulfide chimneys of the Endeavour Segment were measured. The results indicate that conditions favor the growth of iron reducers toward the interiors of these deposits and that of heterotrophs toward the outer surfaces near high-temperature polychaete worms (*Paralvinella sulfincola*).**

Hyperthermophiles that inhabit deep-sea geothermal environments serve as tracers of the in situ chemical and physical conditions in these systems since they are generally not found in surrounding seawater and their metabolisms are reflections of the chemistry and temperature of their environment (12). Hydrothermal sulfide chimneys form at deep-sea vents when upwelling high-temperature hydrothermal fluids mix with cold seawater, resulting in the precipitation of metal sulfides (26). The resultant deposits host diverse microbial communities that thrive within their warm, porous interiors (23).

One of the best-studied deep-sea hydrothermal systems is the Endeavour Segment of the Juan de Fuca Ridge in the northeastern Pacific Ocean (see Fig. S1 in the supplemental material), where massive sulfide chimneys currently form (3, 7, 21). In one of these chimneys, approximately 65% of the total microbial population in the interior wall was archaeal on the basis of fluorescent in situ hybridization (22). The proportions of *Methanocaldococcaceae* and *Thermococcaceae* (both hyperthermophilic families within the *Euryarchaeota*) were very low ( $\leq 4\%$ ) in numerous samples taken across the structure, and 16S rRNA phylogenetic analyses indicate that the majority of the *Archaea* are unknown crenarchaeota (22). A novel hyperthermophilic member of the *Crenarchaeota* was isolated from the same deposit that is an obligately autotrophic reducer of amorphous Fe(III) oxide with an optimum growth temperature of 106°C (14). In 2004, our efforts to culture hyperthermophilic archaea at 95°C on eight types of media [NO<sub>3</sub><sup>-</sup>, S<sup>0</sup>, Fe(III) reduction, and methanogen media for autotrophs and NO<sub>3</sub><sup>-</sup>, S<sup>0</sup>, SO<sub>4</sub><sup>2-</sup>, and Fe(III) reduction media for heterotrophs] from six sulfide deposits collected from Endeavour resulted only in the growth of autotrophic dissimilatory iron reducers (four samples) and heterotrophic sulfur reducers (five samples). There are few other reports of hyperthermophilic

reducers of amorphous Fe(III) oxide from deep-sea hydrothermal environments (15, 16); therefore, determining their potential relevance at Endeavour was a goal of this study.

While molecular assessments of microbial diversity in submarine hydrothermal sulfide deposits are common (see reference 23 for a review), our understanding of the distribution of metabolic activities and microbial abundances of indicator organisms in submarine vents is in a nascent state. The purpose of this study was to determine the relative abundances of hyperthermophilic autotrophic Fe(III) oxide reducers, methanogens, and heterotrophs in the interiors of seven actively venting sulfide chimneys (Fig. 1A and B), three low-temperature (5 to 140°C) hydrothermal fluid samples that were a mixture of end member hydrothermal fluid and seawater (Fig. 1C), and two collections of 25 *Paralvinella sulfincola* polychaete worms from the exteriors of two active deposits (Fig. 1D). The samples were collected in 2006 and 2008 from the Main, Mothra, and High Rise vent fields along the Endeavour Segment by using the deep-sea research submarine *Alvin*.

Once onboard the ship, 12 to 24 g of the soft, porous wurtzite-sphalerite-rich material from the interiors of the chimneys (2 to 10 cm below the hard silicate-enriched outer crust) were added to 50 ml of sterile, anoxic artificial seawater composed of the salts in DSMZ medium 141 ([http://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium141.pdf](http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium141.pdf)). The serum bottle containing the sample was sealed, flushed with N<sub>2</sub>-CO<sub>2</sub> (70%:30%), and reduced with 0.025% (wt/vol) each of cysteine-HCl · H<sub>2</sub>O and Na<sub>2</sub>S · 9H<sub>2</sub>O. Two batches of 25 *P. sulfincola* worms were collected, halved, added to DSMZ medium 141 salts, and processed as described above. Low-temperature, diffuse hydrothermal fluids (50 ml) collected using 750-ml titanium syringes were transferred immediately into a sealed serum bottle that had been flushed with N<sub>2</sub>-CO<sub>2</sub> and reduced with the cysteine-sulfide solution as before.

Three-tube most-probable-number (MPN) analyses (8, 10) were performed at 90°C (Table 1). A complete description of the growth media is available in the supplemental material. Up to 99 iron reducer cells per gram (dry weight) of sulfide material in each of the seven black smoker chimneys examined were measured. In six of these, the estimated numbers of autotrophic iron reducers were

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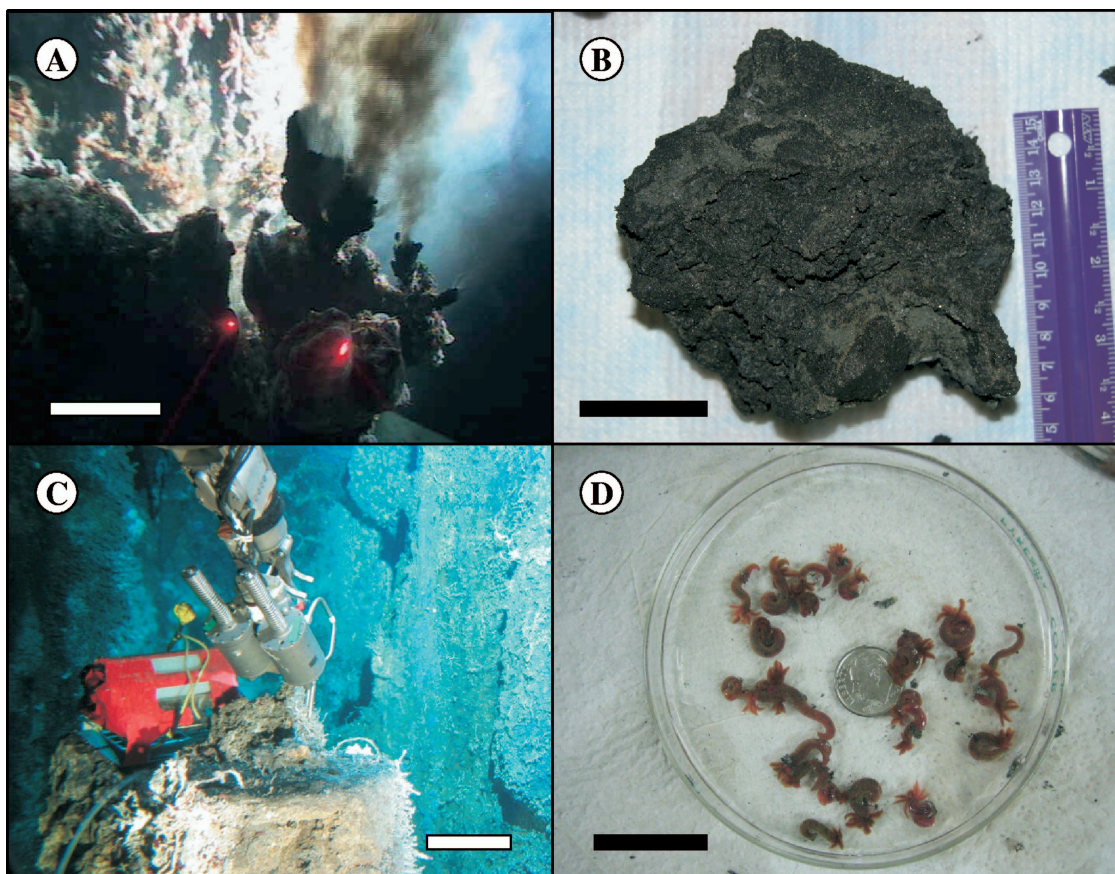


FIG. 1. Samples collected from the Endeavour Segment. (A) Black smoker chimney on the Boardwalk sulfide deposit (scale bar = 10 cm). (B) Wurtzite-sphalerite-rich material from the Hulk sulfide deposit used for culturing (scale bar = 4 cm). (C) Low-temperature fluid collected with titanium syringes from the Roane sulfide deposit (scale bar = 0.5 m). (D) *Paralvinella sulfincola* polychaete worms collected from the Salut sulfide deposit (scale bar = 3 cm).

higher than those for the heterotrophs. Up to 33,000 iron reducer cells per liter in diffuse fluids emitted from the interiors of the sulfide deposits were measured. Similarly, in these fluids the estimated numbers of iron reducers were higher than those for the heterotrophs.

For the worm samples, up to 2,580 heterotroph cells per worm were measured, which greatly exceeded the numbers of iron reducers in these samples. Similar to results from 2004, methanogens were not detected in any of the samples.

TABLE 1. Abundances of hyperthermophilic autotrophic iron reducers, methanogens, and heterotrophic sulfur reducers grown at 90°C

Sample name	Sample description	MPN estimate <sup>a</sup>		
		Autotrophic Fe(III) reducers	Hydrogenotrophic methanogens	Heterotrophic sulfur reducers
Gi06	Sulfide material from Giraffe vent	91/g	ND <sup>b</sup>	91/g
Su06	Sulfide material from Sully vent; 349°C fluids <sup>c</sup>	85/g	ND	15/g
Du06	Sulfide material from Dudley vent; 327°C fluids	22/g	ND	7/g
Hu06	Sulfide material from Hulk vent; 316°C fluids	41/g	ND	ND
BW06-1	Sulfide material from Boardwalk vent; 356°C fluids	78/g	ND	2/g
BW06-2	Sulfide material from Boardwalk vent; 356°C fluids	91/g	ND	41/g
FTS06	Sulfide material from Faulty Towers vent	99/g	ND	14/g
SM06	Diffuse fluids (140°C) from S & M vent	13,800/liter	ND	840/liter
Ro06	Diffuse fluids (5°C) from Roane vent	840/liter	ND	270/liter
Ph06	Diffuse fluids (48°C) from Phang vent	33,000/liter	ND	ND
FTW06	25 <i>P. sulfincola</i> worms from Faulty Towers vent	13/worm	ND	>144/worm <sup>d</sup>
Sa08-2	25 <i>P. sulfincola</i> worms from Salut vent	1/worm	ND	2,580/worm <sup>d</sup>

<sup>a</sup> Estimates are per gram (dry weight) of sulfide material, per liter of fluid collected, or per *P. sulfincola* worm.

<sup>b</sup> ND, not detectable.

<sup>c</sup> Fluid temperatures are for hydrothermal end member fluids flowing out of the sulfide deposits at the time of collection.

<sup>d</sup> For FTW06, all nine MPN tubes showed growth indicating that the sample cell concentration exceeded the range of the MPN test. For Sa08-2, the sample was diluted 1:1,000 prior to the MPN test. The Sa08 MPN tests were run in 2008 at 85°C instead of 90°C.

Four iron reducer strains and 12 heterotroph strains were purified using three successive dilution-to-extinction transfers in liquid medium. Sequencing of their 16S rRNA genes indicates that the iron reducers are *Pyrodictium* and *Hyperthermus* species and that the heterotrophs are *Pyrococcus* and *Thermococcus* species (see Fig. S2 in the supplemental material). Among the iron reducers, only *Hyperthermus* strain BW06-2 grew on acetate and peptides without the addition of H<sub>2</sub> and CO<sub>2</sub> (see Table S1 in the supplemental material). In general, the addition of acetate or peptides to the growth medium increased neither their growth rates nor their maximum cell concentrations relative to autotrophic growth. The two *Pyrodictium* strains are obligate Fe(III) oxide reducers, while the two *Hyperthermus* strains also use nitrate as a terminal electron acceptor (see Table S2 in the supplemental material). All 12 obligate heterotroph strains grew on 0.5% (wt/vol) casein hydrolysate only, 0.5% maltose only, and 0.5% cellobiose only when elemental sulfur was present and produced H<sub>2</sub>S and H<sub>2</sub> as end products. They appear to be well adapted for growth on polypeptides and  $\alpha$ -1,4- and  $\beta$ -1,4-linked sugar polymers that likely come from vent animal secretions (13) and the biofilms of other microorganisms.

The MPN results presented in this study indicate that hyperthermophilic heterotrophs are most abundant near the outer surfaces of the chimneys in close proximity to *P. sulfincola*. This polychaete worm was chosen as a source of hyperthermophiles in this study because it lives in the porous outer walls of black smoker chimneys at temperatures (up to 55°C) that exceed those in areas containing other metazoans at deep-sea vents (6) and because they have previously been shown to be a source of hyperthermophilic heterotrophs (11, 20). The results also indicate that there is a transition to a more autotrophic composition among hyperthermophiles toward the interiors of the deposits. Based on MPNs, iron reducers appear to be more abundant than methanogens within the hyperthermophilic autotroph community. The absence of hyperthermophilic methanogens is curious since they have been found in 90°C MPN analyses of low-temperature, basalt-hosted hydrothermal fluids from the Endeavour Segment (10) and in molecular and fluorescent in situ hybridization analyses of Endeavour sulfide chimneys (22). We cannot exclude the possibility that there is a culturing bias or other potentially limiting factors, such as O<sub>2</sub> exposure during sample recovery, or that the organisms are viable but not culturable. However, other possible explanations for the absence of hyperthermophilic methanogens include environmental conditions that limit their growth, such as H<sub>2</sub> limitation, and reduction potentials that are outside the range needed for growth.

Analysis of H<sub>2</sub> concentrations in hydrothermal fluids from the Endeavour Segment (4, 18, 19, 24) by thermodynamic modeling of hydrogenotrophic methanogenesis (9) suggests that the H<sub>2</sub> activity of the Endeavour fluids may be below the minimum needed for growth. In contrast, the minimum H<sub>2</sub> activity needed for growth of iron reducers should be significantly less than that for methanogenesis due to the higher free energy available from the reduction of amorphous Fe(III) oxide (1). In 2005, the dissolved H<sub>2</sub> concentrations in hydrothermal end member fluids from the Main Endeavour Field were 0.05 to 0.1 mmol per kg, suggesting that these fluids were also only mildly reducing (4). These conditions would also likely

favor the growth of hyperthermophilic Fe(III) oxide reducers, since they generally prefer mildly reducing conditions (5), over that of hydrogenotrophic methanogens that require reduction potentials below -330 mV (25).

Mildly reducing conditions would also occur at 90°C within sulfide deposits if there were a significant influx of oxygen-saturated seawater into the deposit (27). This would also provide the source of Fe(III) oxide needed to support the iron reducers. Evidence that seawater entrainment and Fe(III) oxide do occur in some Endeavour sulfide chimneys is provided by detailed petrographic and chemical analyses (17). For example, pockets of anhydrite (CaSO<sub>4</sub>), which forms during heating of seawater above 150°C (2), occur in both the exterior and the interior walls of some Endeavour sulfide chimneys, and poorly crystallized magnesium-rich clay has been documented to occur in the outer chimney walls (17). Petrographic relationships and isotopic analyses indicate that anhydrite precipitates directly from mixed hydrothermal fluid and seawater throughout much of the chimney's evolution as long as fluid temperatures remain high and high-angle fractures allow seawater ingress. Trace amorphous Fe(III) oxide has also been documented to occur in the inner and outer walls of an actively venting sulfide chimney at Endeavour (17). Additional evidence for seawater entrainment into the interiors of chimneys is provided by the presence of the chlorosulfate mineral gordaite (17).

For submarine hydrothermal systems, there is a critical need to determine the relative abundances, distributions, and metabolic characteristics of indicator organisms since they may provide insight into the chemical conditions present in the environments that are not readily accessible for study (e.g., the subsurfaces and the interiors of deep-sea chimneys). Investigation of these indicator organisms will also provide insights into the character of microbes present even when they themselves are not necessarily the most abundant or biogeochemically important organisms (12). Some of the more fundamental questions regarding vents concern the distributions of autotrophs versus heterotrophs and various autotrophs with differing growth requirements. A long-term goal is to determine what biogeochemical impacts these and other microorganisms have on hydrothermal vent systems and how this may translate into a better understanding of life deeper within the Earth's crust.

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#### REFERENCES

1. Amend, J. P., and E. L. Shock. 2001. Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and Bacteria. *FEMS Microbiol. Rev.* **25**:175-243.
2. Bischoff, J. L., and W. E. Seyfried. 1978. Hydrothermal chemistry of seawater from 25°C to 350°C. *Am. J. Sci.* **278**:838-860.
3. Delaney, J. R., V. Robigou, R. E. McDuff, and M. K. Tivey. 1992. Geology of a vigorous hydrothermal system on the Endeavour segment, Juan de Fuca Ridge. *J. Geophys. Res.* **97**:19663-19682.
4. Ding, K., and W. E. Seyfried, Jr. 2007. In situ measurement of pH and dissolved H<sub>2</sub> in mid-ocean ridge hydrothermal fluids at elevated temperatures and pressures. *Chem. Rev.* **107**:601-622.
5. Feinberg, L. F., R. Srikanth, R. W. Vachet, and J. F. Holden. 2008. Constraints on anaerobic respiration in the hyperthermophilic archaea *Pyrobaculum*.

- lum islandicum* and *Pyrobaculum aerophilum*. Appl. Environ. Microbiol. **74**:396–402.
6. **Girguis, P. R., and R. W. Lee.** 2006. Thermal preference and tolerance of Alvinellids. Science **312**:231.
  7. **Glickson, D. A., D. S. Kelley, and J. R. Delaney.** 2007. Geology and hydrothermal evolution of the Mothra Hydrothermal Field, Endeavour Segment, Juan de Fuca Ridge. Geochem. Geophys. Geosyst. **8**:Q06010.
  8. **Greenberg, A. E., L. S. Clescen, and A. D. Eaton.** 1992. Standard methods for the examination of water and wastewater, 18th ed. American Public Health Association, Washington, DC.
  9. **Hoehler, T. M.** 2004. Biological energy requirements as quantitative boundary conditions for life in the subsurface. Geobiology **2**:205–215.
  10. **Holden, J. F., M. Summit, and J. A. Baross.** 1998. Thermophilic and hyperthermophilic microorganisms in 3–30°C hydrothermal fluids following a deep-sea volcanic eruption. FEMS Microbiol. Ecol. **25**:33–41.
  11. **Holden, J. F., K. Takai, M. Summit, S. Bolton, J. Zyskowski, and J. A. Baross.** 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.
  12. **Holland, M. E., J. A. Baross, and J. F. Holden.** 2004. Illuminating seafloor ecosystems using microbial tracers, p. 291–303. In W. S. D. Wilcock, E. F. Delong, D. S. Kelley, J. A. Baross, and S. C. Cary (ed.), The seafloor biosphere at mid-ocean ridges. Geophysical monograph vol. 144. American Geophysical Union Press, Washington, DC.
  13. **Juniper, S. K., J. A. J. Thompson, and S. E. Calvert.** 1986. Accumulation of minerals and trace elements in biogenic mucous at hydrothermal vents. Deep-Sea Res. **33**:339–347.
  14. **Kashefi, K., and D. R. Lovley.** 2003. Extending the upper temperature limit for life. Science **301**:934.
  15. **Kashefi, K., J. M. Tor, D. E. Holmes, C. V. Gaw Van Praagh, A.-L. Reysenbach, and D. R. Lovley.** 2002. *Geoglobus ahangari* gen. nov., sp. nov., a novel hyperthermophilic archaeon capable of oxidizing organic acids and growing autotrophically on hydrogen with Fe(III) serving as the sole electron acceptor. Int. J. Syst. Evol. Microbiol. **52**:719–728.
  16. **Kashefi, K., E. S. Shelobolina, W. C. Elliott, and D. R. Lovley.** 2008. Growth of thermophilic and hyperthermophilic Fe(III)-reducing microorganisms on a ferruginous smectite as the sole electron acceptor. Appl. Environ. Microbiol. **74**:251–258.
  17. **Kristall, B., D. S. Kelley, M. D. Hannington, and J. R. Delaney.** 2006. Growth history of a diffusely venting sulfide structure from the Juan de Fuca Ridge: a petrological and geochemical study. Geochem. Geophys. Geosyst. **7**:Q07001.
  18. **Lilley, M. D., D. A. Butterfield, E. J. Olson, J. E. Lupton, S. A. Macko, and R. E. McDuff.** 1993. Anomalous CH<sub>4</sub> and NH<sub>4</sub><sup>+</sup> concentrations at an un sedimented mid-ocean-ridge hydrothermal system. Nature **364**:45–47.
  19. **Lilley, M. D., D. A. Butterfield, J. E. Lupton, and E. J. Olson.** 2003. Magmatic events can produce rapid changes in hydrothermal vent chemistry. Nature **422**:878–881.
  20. **Pledger, R. J., and J. A. Baross.** 1989. Characterization of an extremely thermophilic archaeobacterium isolated from a black smoker polychaete (*Paralvinella* sp.) at the Juan de Fuca Ridge. Syst. Appl. Microbiol. **12**:249–256.
  21. **Robigou, V., J. R. Delaney, and D. S. Stakes.** 1993. Large massive sulfide deposits in a newly discovered active hydrothermal system, the High-Rise field, Endeavour segment, Juan de Fuca Ridge. Geophys. Res. Lett. **20**:1887–1890.
  22. **Schrenk, M. O., D. S. Kelley, J. R. Delaney, and J. A. Baross.** 2003. Incidence and diversity of microorganisms within the walls of an active deep-sea sulfide chimney. Appl. Environ. Microbiol. **69**:3580–3592.
  23. **Schrenk, M. O., J. F. Holden, and J. A. Baross.** 2008. Magma-to-microbe networks in the context of sulfide hosted microbial ecosystems, p. 233–258. In R. P. Lowell, M. R. Perfit, J. Seewald, and A. Metaxas (ed.), Magma to microbe: modeling hydrothermal processes at oceanic spreading ridges. Geophysical monograph vol. 178. American Geophysical Union Press, Washington, DC.
  24. **Seewald, J., A. Cruse, and P. Saccocia.** 2003. Aqueous volatiles in hydrothermal fluids from the Main Endeavour Field, northern Juan de Fuca Ridge: temporal variability following earthquake activity. Earth Planet. Sci. Lett. **216**:575–590.
  25. **Sowers, K. R.** 1995. Methanogenic archaea: an overview, p. 3–13. In K. R. Sowers and H. J. Schreier (ed.), Archaea: a laboratory manual (methanogens). Cold Spring Harbor Press, Plainview, NY.
  26. **Tivey, M. K.** 1995. Modeling chimney growth and associated fluid flow at seafloor hydrothermal vent sites, p. 158–177. In S. E. Humphris, R. A. Zierenberg, L. S. Mullineaux, and R. E. Thomson (ed.), Seafloor hydrothermal systems: physical, chemical, biological, and geological interactions. Geophysical monograph vol. 91. American Geophysical Union Press, Washington, DC.
  27. **Tivey, M. K.** 2004. Environmental conditions within active seafloor vent structures: sensitivity to vent fluid composition and fluid flow, p. 137–152. In W. S. D. Wilcock, E. F. Delong, D. S. Kelley, J. A. Baross, and S. C. Cary (ed.), The seafloor biosphere at mid-ocean ridges. Geophysical monograph vol. 144. American Geophysical Union Press, Washington, DC.