A novel microbial habitat in the mid-ocean ridge subseafloor

Melanie Summit*† and John A. Baross‡

*Department of Earth and Planetary Sciences, Campus box 1169, Washington University, St. Louis, MO 63130; and ‡Department of Oceanography, Box 357940, University of Washington, Seattle, WA 98195

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The subseafloor at the mid-ocean ridge is predicted to be an excellent microbial habitat, because there is abundant space, fluid flow, and geochemical energy in the porous, hydrothermally influenced oceanic crust. These characteristics also make it a good analog for potential subsurface extraterrestrial habitats. Subseafloor environments created by the mixing of hot hydrothermal fluids and seawater are predicted to be particularly energy-rich, and hyperthermophilic microorganisms that broadly reflect such predictions are ejected from these systems in low-temperature (≈15°C), basalt-hosted diffuse effluents. Seven hyperthermophilic heterotrophs isolated from low-temperature diffuse fluids exiting the basaltic crust in and near two hydrothermal vent fields on the Endeavour Segment, Juan de Fuca Ridge, were compared phylogenetically and physiologically to six similarly enriched hyperthermophiles from samples associated with seafloor metal sulfide structures. The 13 organisms fell into four distinct groups: one group of two organisms corresponding to the genus *Pyrococcus* and three groups corresponding to the genus *Thermococcus*. Of these three groups, one was composed solely of sulfide-derived organisms, and the other two related groups were composed of subseafloor organisms. There was no evidence of restricted exchange of organisms between sulfide and subseafloor habitats, and therefore this phylogenetic distinction indicates a selective force operating between the two habitats. Hypotheses regarding the habitat differences were generated through comparison of the physiology of the two groups of hyperthermophiles; some potential differences between these habitats include fluid flow stability, metal ion concentrations, and sources of complex organic matter.

Extraterrestrial environments considered capable of supporting life contain liquid water and carbon and energy sources. Life as we know it can use energy in two forms: light energy, which, as solar radiation, drives most of the life and other energetic processes on this planet; and chemical energy, resident in the volcanically induced redox disequilibria generated as the interior of the Earth dumps its waste heat to space. Solar energy is best used when liquid water is present at the planetary surface, a situation that tends to be rare; however, geochemical energy might be supplied to an environment containing liquid water that lies below a planet's surface, such as are postulated on Europa (1) and Mars (2).

There are environments on Earth analogous to such a wet, geochemically fueled subsurface habitat. Although there is little evidence for a global deep biosphere such as envisioned by Gold (3), within Earth's heterogeneous crust there are locations where water, carbon and energy sources, porosity, fluid flow, and temperature conspire to create highly favorable conditions for life. One such environment is present along the spine of the mid-ocean ridge, where the linear volcano of the ridge crest drives circulation of seawater through the porous ocean crust. The geochemical energy and vigorous fluid flow that results in such prolific life at seafloor hydrothermal vents is also predicted to support life within the ocean crust. Elevated levels of DNA in black smoker fluids (4, 5) and expulsion of sulfur flocs from venting sites after dike injections (6–8) are two pieces of evidence that microbes do inhabit this subsurface environment, but perhaps the most direct evidence for microbes within the ocean crust is the culturing of microbial ''tracers'' in hydrothermal fluids (9).

Approach: Microbial Tracers of a Subseafloor Ecosystem

Fluids exiting the seafloor carry with them putative subseafloor residents, flushed from their habitats. Identifying subseafloor microorganisms requires distinguishing them from seawater-derived microorganisms entrained while sampling or mixed in by other processes. Thermal classes of microorganisms are easily distinguished and can be used as microbial tracers.

Hyperthermophilic microorganisms (organisms with growth optima over 80°C) found in low-temperature, basalt-hosted diffuse fluids make excellent indicators of a subseafloor ecosystem. If fluids that exit at low temperatures (5–30°C) contain elevated levels of microorganisms that live only at high temperatures (50–120°C), those microorganisms must have been entrained in fluids originating below the surface. Hyperthermophiles in the heterotrophic order Thermococcales are particularly good target organisms, because they are routinely cultured from near-vent sites (10), can survive in low-temperature oxic conditions (e.g., ref. 11), and grow very well in artificial media.

Hyperthermophilic tracers were previously used to show that there was a microbial ecosystem beneath sites of low-temperature fluid venting at the CoAxial ''Floc'' site and the Endeavour Segment, Juan de Fuca Ridge, and at 9°50′N on the East Pacific Rise (9). Samples of low-temperature, basalt-hosted diffuse fluids had higher concentrations of hyperthermophiles than those found in background seawater, indicating that these diffuse vents are a source of hyperthermophiles. Other potential sources of hyperthermophiles (e.g., sulfide structures) were 10–15,000 m distant. These hyperthermophiles are tracers of 50–100°C, anaerobic, stable habitats suitable for microbial growth within the crust. As well as this habitat for anaerobic, heterotrophic hyperthermophiles, there may well be habitats created within the crust for other trophic and thermal classes of organisms. The hyperthermophilic tracers should be considered indicators of what is very probably a larger, multithermal-class subseafloor microbial community.

In this study, we have continued to use indicator hyperthermophiles to probe these subseafloor habitats. To better constrain the subseafloor habitat at mid-ocean ridges, this habitat was compared with another known microbial habitat at the

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Abbreviations: IGS, 16S/23S rRNA intergenic spacer region; MCA-leucine, 1-leucine-7-amidino-4-methylcoumarin.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY017170–AY017182).

†To whom reprint requests should be addressed. E-mail: summit@zonvark.wustl.edu.

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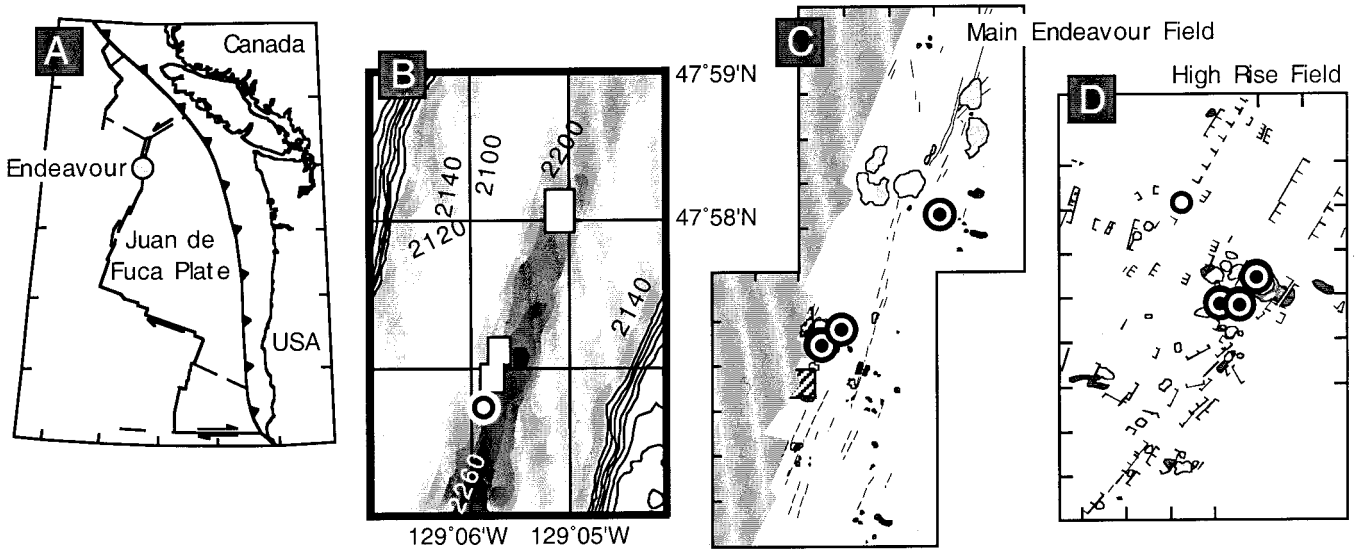

Fig. 1. (*A*) Map of northeast Pacific, showing tectonic plate boundaries and location of Endeavour Segment. (*B*) Map showing bathymetry of the Endeavour Segment, Juan de Fuca Ridge, along with the location of the Main Endeavour and High Rise fields. Contours are every 10 m; darker colors indicate deeper regions. (*C* and *D*) The two hydrothermal fields showing sampling locations where the 13 organisms in this study were isolated. Dark symbols are samples of sulfide-associated material, and light symbols are samples of diffuse fluids (one diffuse fluid sample is on map *B*). Light colors with dark borders are active sulfide structures, dark colors are inactive sulfides or sulfide talus, and hatched regions are tubeworm colonies. Tick marks on map borders are increments of 50 m. Maps modified from D. Kelley and V. Robigou.

vents: metal sulfide structures. Hyperthermophilic indicator organisms similar to those found coming from the subseafloor are also found in sulfide structures (10). Subseafloor tracer hyperthermophiles were cultured from low-temperature, basalt-hosted diffuse fluids and compared with similar hyperthermophiles from metal sulfide structures to determine whether habitats in the subseafloor are similar to those found in sulfide structures. The phylogenetic relationships between the two groups of organisms indicate that the subseafloor habitat differs significantly from that within sulfide structures.

Site Description

The Endeavour segment is located near the north end of the Juan de Fuca Ridge (Fig. 1), which spreads at an intermediate rate of 50–60 mm/year (full rate; ref. 12). This segment is currently thought to be undergoing an tectonic phase of spreading (13), and the axial valley is 500-1000 m wide with valley walls 100–150 m high. The known locations of hydrothermal venting are within the axial valley on a portion of the segment characterized as a volcanic high and may be associated with tectonic features (14). The two fields of interest to this study are the Main Endeavour Field (14) and the High Rise Field (15) (Fig. 1). Both vent fields host large (10 m high) metal sulfide structures, which are typically composed of pyrite, chalcopyrite, marcasite, sphalerite, and amorphous silica (16), and which vent fluids from 330–400°C (17). The hottest fluid temperatures are in the south end of the Main Endeavour Field, where low salinities (10% of seawater) indicate that active phase separation is taking place (17). These overall gradients in fluid chemistry and temperature have been stable over the years sampled (17); the size of the sulfide structures also attests to the stability of the underlying fluid circulation (14, 15) and may be indicative of the nature of the heat source (18). Distal sites of low-temperature (5–30°C), basalt-hosted diffuse flow, such as the Quebec tubeworm field (Fig. 1), also contribute to the fluid and heat flux on the Endeavour segment.

Methods

Primary Enrichment Cultures and Isolation of Strains. Samples of fluid and rocks were added to anaerobic enrichment cultures and incubated at 90°C in medium YPS [refs. 19 and 20; 0.3% (wt/vol) each yeast extract and peptone (both from Difco) in an artificial seawater base (Sea Salts B) with additional trace elements (Trace Elements F) and elemental sulfur (Baker, sublimed, sterilized by steaming)], designed for *Thermococcus*-like heterotrophic sulfur-reducers. Growth medium was made on shore and brought to sea in anaerobic Balch tubes, ready for reduction with sodium sulfide (0.05% final concentration in media) and inoculation.

Fluid samples were introduced into the tubes via syringe and needle, and the inoculation volume was 1 ml unless otherwise stated. Solid samples required opening the sealed culture tube and restoppering; this was performed inside a disposable glove bag (Instruments for Research and Industry) under a nitrogen atmosphere. The correct head space was replaced in the tube by sparging after resealing. Samples were ready for incubation between 2 and 12 h after coming to the surface. Enrichment cultures were incubated in sand-filled baths contained in a 90°C oven until turbid or for 7 days, whichever came first. Cultures were examined on shore by using phase contrast and epifluorescence microscopy to determine whether growth had occurred.

Isolation of pure cultures of heterotrophic hyperthermophiles was performed by repeated serial dilution to extinction (19). The primary enrichment culture to be purified was serially diluted in medium YPS, and all dilutions were incubated at 90°C. The highest dilution showing positive growth was used to inoculate another serial dilution. Cultures were given strain designations after three successful serial dilutions.

Phylogenetic Analysis. The 16S rRNA gene and the 16S/23S rRNA intergenic spacer region (IGS; e.g., ref. 21) were amplified and sequenced to determine the relationships of the strains to each other. DNA was extracted from batch cultures by using the IsoQuick kit (Orca Research). The 16S rRNA gene and IGS were amplified by the PCR following the suggestions of Reysenbach *et al.* (22) using the universal 16S archaeal primer 21Fa (TTC CGG TTG ATC CYG CCG GA) and 23S primer 64R (GCC NRG GCT TAT CGC AGC TT).

All fragments were sent to the Molecular Genetics Facility at the University of Georgia, and both strands were sequenced on an Applied Biosystems sequencer using multiple internal primers. The sequences were aligned with other 16S rRNA sequences from GenBank by using tools provided by the Ribosomal Database Project (23). Alignments were finished by eye with careful attention to secondary structure and variable regions.

Evolutionary models were tested on each data set, with a likelihood ratio test (24) performed to determine the simplest model appropriate for the data. This model was used to construct minimum-evolution distance trees with PAUP* Version 4.0b4a (D. L. Swofford, Sinauer Assoc.), and the parameters for the evolutionary models were estimated by using a maximum likelihood evaluation of the trees. This procedure was iterated three times after which a heuristic search with these final parameters and the minimum-evolution criteria including 1,000 random-addition replicates generated the final tree. One hundred to 1,000 bootstrap replicates (25) were also performed using these parameters. Trees were visualized with TREEVIEW (26).

Physiological Characterization. Approximate growth limits for the isolates were determined in medium YPS for temperature and salinity. Unless otherwise stated, temperature was 90°C, salinity was 1× Sea Salts B (ref. 19; 32.3 g/liter total salts), and pH was 6. Temperature was varied in ovens or oil baths. Salinity was varied by diluting or concentrating the Sea Salts B portion of medium YPS.

Isolates were grown on media containing a variety of carbon sources to test nutritional requirements and plasticity. Media were built on the YPS base, by replacing the complex organics (yeast extract and peptone) with single organic compounds added at 0.2% along with 0.01% yeast extract (Difco) to supply trace nutrients. Organisms were also tested for growth on a mixture of 20 amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan, and valine), each at 0.8%, supplemented with 0.01% yeast extract in the same media base as above; this solution was filter sterilized rather than autoclaved. Organisms were also grown on decreasing levels of equal parts yeast extract and peptone to test their overall carbon requirement. All of the above test media included elemental sulfur.

Cultures were grown with and without elemental sulfur to test for sulfur requirement or enhancement of growth caused by sulfur addition. Similar tests were performed with an 80% H_2/20% CO_2 head space versus an Ar headspace.

Isolates were grown on medium YPS in the presence of $CuSO_4$ and $ZnSO_4$ to test for possible differential resistance to metal ions. Maximal concentrations of these metals in the environment were approximated by adding the maximal concentrations calculated for endmember hydrothermal vent fluid (rounded up to 100 μM or 1 mM, respectively; ref. 27), adding sodium sulfide at 2 mM, and allowing natural reactions to take place.

Isolates were tested for secretion of extracellular protease after the method of Vetter and Deming (28). Cultures grown at 90°C in YPS to 108 cells/ml were filter-sterilized to form a cell-free preparation. Aliquots of this cell-free medium (3 ml) and 6 μl of MCA-leucine (1-leucine-7-amidino-4-methylcoumarin, Sigma) were mixed in plastic fluorescence cuvettes (forming a 10 μM MCA-leucine solution) and sealed with Parafilm. Cuvettes were incubated at 22°C, 55°C, or 80°C for up to 1 day. Uninoculated but reduced medium YPS was used as a control. All samples were monitored for increase in fluorescence at 440 nm when excited with 355 nm by using a Perkin-Elmer LS-5B luminescence spectrometer; fluorescence was measured on room-temperature samples. Resulting rates were compared with *Pyrococcus furiosus* as a standard.

Results

Sample Collection, Primary Enrichments, and Isolates. Samples were collected in and near the Main Endeavour Field and the High Rise Field (Fig. 1) on the Research Vessel *Atlantis II* during "Mixing Zephyrs" I (June 1995) and II (September 1995) using Deep Submergence Vehicle *Alvin*. Titanium water samplers (29) were used for collection of fluids, either individually or attached to a manifold water sampler (30). The titanium sampler or manifold intake was positioned at the location of the highest temperature reading and filled (after flushing the manifold if in use). Two sites of basalt-hosted, low-temperature diffuse fluids were sampled: a 12°C vent at the Quebec tubeworm field, located 200 m south of the southern extent of the Main Endeavour Field; and a 14°C vent located 10–20 m northeast of the Three Sisters sulfide structure in the High Rise Field (Fig. 1). Sites of diffuse flow were identified by refraction ("shimmering water") and macrofaunal colonization. Temperature measurements confirmed the upflow of warm water. Samples of sulfide chimney nozzles, sulfide flanges, and alvinellid worms were removed from sites within the Main Endeavour and High Rise Fields (Fig. 1) by using *Alvin*'s manipulator arms and brought to the surface unprotected or in a plastic box. Samples chilled to ambient temperatures (2–4°C) while coming to the surface, and some warmed to room temperature before processing. No attempt was made to keep the samples under pressure. Thirteen isolates were obtained from YPS primary enrichments of diffuse fluids (MZ6, MZ8–MZ12, and MZ14), sulfide rocks (MZ1, MZ2, MZ5, and MZ13), hot hydrothermal fluids (MZ4), and worms (MZ3).

Phylogenetic Diversity. The 16S/IGS region of the 13 isolates was successfully amplified and sequenced. The entire 16S rRNA gene was unambiguously aligned and used in the phylogenetic analysis. Comparison trees were made from a data set with variable loops corresponding to *Escherichia coli* regions 71–98, 198–219, 403–433, and 1445–1457 removed; the same tree topology resulted so the variable regions were retained in the analyses. The IGS region data set (about 290 bases) started at the end of the 16S rRNA gene and included the first few bases of the 23S rRNA gene. All organisms in the alignment contained an alanine tRNA as in other Euryarchaeota (31). There were no difficulties in aligning the IGS region within the Thermococcales. Sequences of the 16S/IGS region for the 13 organisms in this study were deposited in GenBank and assigned accession numbers AY017170 to AY017182.

The most appropriate model of evolution for the 16S rRNA and IGS data sets, respectively, were the Tamura-Nei three-parameter model (32) and the General Time-Reversible model (33). Both data sets were best modeled with an estimated proportion of sites remaining invariant and gamma-distributed rate variations between the remaining sites (34).

The 16S rRNA tree constructed by using only the 13 isolates from this study (Fig. 2) shows that the isolates fell into four distinct clusters. The outlying group contained one diffuse fluid organism (MZ14) and one sulfide-associated organism (MZ4) and had a very high bootstrap confidence. The other three clusters contained more closely related organisms. All of the remaining organisms cultured from sulfide structures were very closely related to each other (MZ1, -2, -3, -5, and -13); this cluster had high bootstrap support. The rest of the isolates from diffuse fluids (MZ6, -8, -9, -10, -11, and -12) were all together in one group that had moderate bootstrap support, but within this is a cluster with excellent bootstrap support (MZ8, -9, and -11).

The IGS tree of the same organisms (Fig. 2) supported the same groups. Bootstrap values supported the four clusters mentioned above as well as the group comprised of MZ6, -10, and -12; values were higher than those for the 16S rRNA data set and tree in some cases and lower in others.

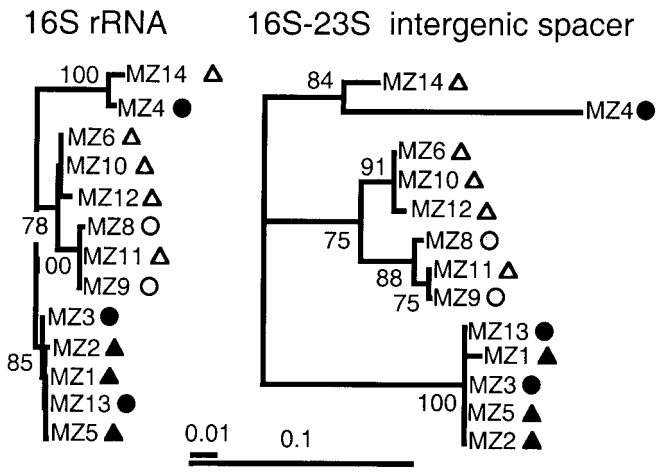

Fig. 2. Phylogenetic trees constructed from the 16S rRNA gene and the IGS region of the 13 Endeavour isolates. Percent of 1,000 bootstrap replicates supporting clusters is shown. Organisms represent samples from two vent fields, High Rise (triangles) and the Main Endeavour Field (circles) (Fig. 1) and two biotopes, sulfides (dark) and the subseafloor (light). Organisms clustered with respect to biotopes but not with respect to geographic separation. Scale for the two trees is identical; small bar represents one change per hundred nucleotides.

The phylogenetic tree constructed from a 16S rRNA data set containing the 13 isolates and all characterized members of *Thermococcus* and *Pyrococcus* (Fig. 3) shows that the two outlying organisms correspond to the genus *Pyrococcus* and the remaining isolates are members of *Thermococcus*. The group of sulfide-derived organisms and one group from diffuse fluids (MZ8, -9, and -11) remain coherent with high bootstrap values. Isolates MZ6, -10, and -12 fall within a group of poorly differentiated *Thermococcus* spp.; the 16S rRNA does not have enough resolution to distinguish clusters within this group (Fig. 3, bootstrap values less than 50% not shown).

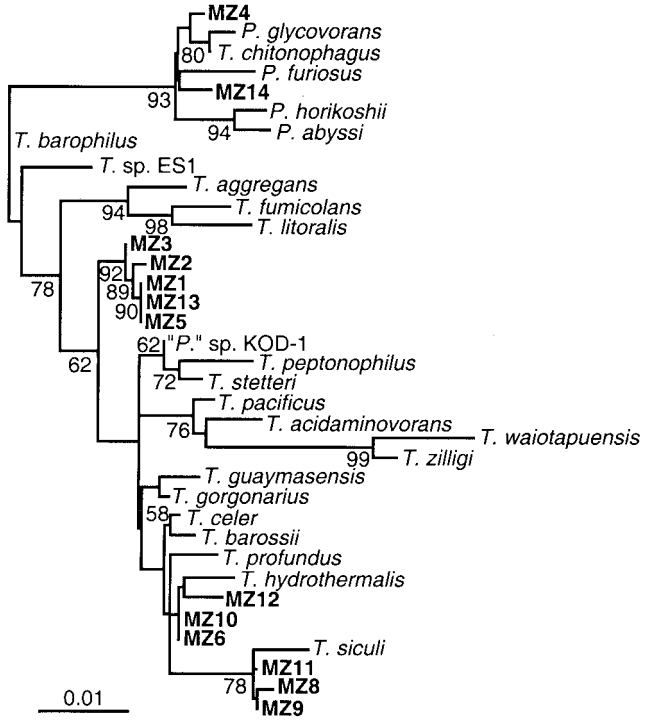

Fig. 3. Minimum-evolution distance tree made with the full length of the 16S rRNA gene showing position of Endeavour isolates relative to characterized species in the genera *Thermococcus* and *Pyrococcus*. Bootstrap values are shown for all groups with over 50% bootstrap support (162 replicates). Scale is one change per hundred nucleotides.

Physiological Diversity. The isolates from sulfide environments (those from sulfides, worms, and hot fluids) had significantly higher temperature maxima (95°C) than those from the low-temperature diffuse fluids (90°C; Wilcoxon two-sample test, $P < 0.01$), although their temperature minima were similar (50°C). The sulfide-associated organisms also were able to grow at much lower salt concentrations (0.4× sea salts) than the group from the diffuse fluids (0.8× sea salts; Wilcoxon two-sample test, $P < 0.1$).

The 13 isolates were remarkably similar in nutritional characteristics. No isolate grew on any of the simple carbon sources presented (glycine, alanine, citrate, acetate, maltose, starch, or ethanol). Some isolates grew on mixtures of 20 amino acids. All isolates grew well on 0.1% total organic carbon (0.05% each yeast extract and peptone), whereas a few isolates could grow given 0.02% total organic carbon. No isolate showed growth with only 0.01% total organic carbon. No significant differences for any of these tests were found between the sulfide or diffuse fluid isolates (Wilcoxon two-sample test).

Only one isolate required elemental sulfur for growth, but other isolates showed stimulation of growth when elemental sulfur was present in the medium. Stimulation by sulfur did not correlate with diffuse fluid or sulfide origin.

Most isolates released an extracellular protease into the growth medium that was active at 80°C. These proteases were not active at room temperature, and only a few (MZ1, -4, and -5) showed appreciable activity at 55°C. Protease production was significantly different between sulfide and diffuse fluid isolates (ANOVA, $P = 0.02$) but did not correlate with growth on amino acids.

Isolates from sulfide structures showed slightly but significantly (Wilcoxon two-sample test, $P < 0.1$) more robust growth in the presence of $ZnSO_4$. Growth differences in $CuSO_4$ were not significant ($P > 0.1$).

Discussion

Hyperthermophilic indicator organisms cultured from low-temperature vents indicate that there is a microbial habitat within subseafloor zones of mixing between hot hydrothermal fluid and seawater. Although similar fluid endmembers mix within the walls of metal sulfide structures, hyperthermophiles from the subseafloor are phylogenetically distinct from their sulfide chimney cousins. This distinction implies that the two habitats are different enough to select for these two groups of organisms. Hypotheses regarding the habitat differences can be generated through comparison of the physiology of the two groups of hyperthermophiles: some potential differences between these habitats include stability of fluid flow, metal ion concentrations, and sources of complex organic matter.

The subseafloor organisms come out of low-temperature vents issuing from cracks in the basaltic seafloor. The environment tapped by these vents is thought to be one created mainly through subseafloor mixing of hot (≈350°C) hydrothermal fluid and cold seawater (35, 36). This mixing likely takes place in the highly porous layers of upper oceanic crust, built from seafloor eruptions (Fig. 4). Layers of pillow basalts have porosities that range from 10% to 38%, estimated from seafloor gravity measurements (e.g., ref. 37), examination of ophiolites (39), and borehole measurements (38). Porosity of sheet flows is estimated at somewhat lower values (10%; ref. 39). Permeability of these layers has been directly measured in boreholes in young oceanic crust; the formations can be extremely permeable (10^{-13} to 10^{-12}

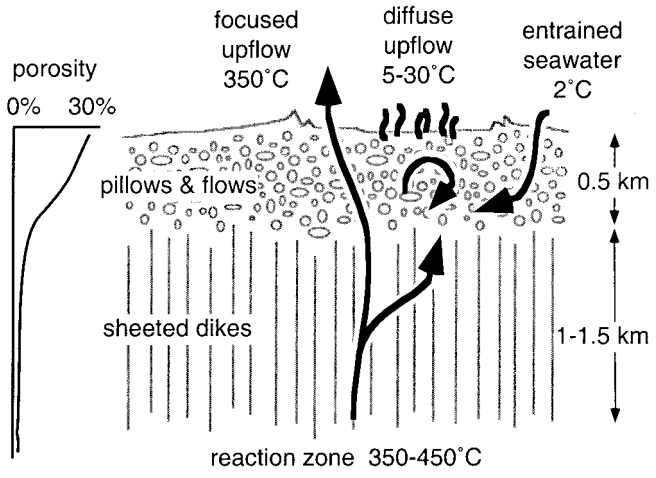

Fig. 4. Schematic cross-section of the oceanic crust at a spreading center showing subseafloor mixing of hydrothermal fluid and seawater in the extrusive layer. Porosity values in the axial pillow lavas/extrusives are taken from gravity measurements of axial pillow flows (37) and extrusive/dike transition and dike porosity taken from Ocean Drilling Program Hole 504B (38).

m^2 or even higher in zones; ref. 40), similar to a clean medium-grained sand. Porosity decreases sharply in the dike sequence (Fig. 4) and therefore mixing may be confined to the extrusive layers.

Modeling of these mixing systems (41, 42) predicts that if the temperature is controlled solely by mixing, as the temperature drops the pH will remain close to 6 until the temperature is near 50°C, where it rises toward the seawater value of 7.8. A transition occurs from anoxic conditions at high temperature to oxic conditions at low temperature; in calculations by McCollom and Shock (42), this transition temperature is also at 50°C but may be affected by varying amounts of reductant in the hydrothermal fluid endmember. These same calculations predict that such mixing environments should be excellent microbial habitats. The mixing of seawater electron acceptors and hydrothermal electron donors creates an energy-rich zone (42). Energy is also available from methanogenesis, a reaction independent of seawater electron acceptors.

The hyperthermophiles cultured from the subseafloor are in accord with the temperature, pH, and redox conditions in this predicted habitat, as they are anaerobic and grow well at a pH near 6. Methanogenesis is also predicted to yield energy in the hyperthermophilic range, and methanogenic hyperthermophiles were cultured from these same subseafloor samples (9). Attempts to culture hyperthermophilic organisms with lower pH optima were unsuccessful (data not shown), but further explicit and systematic testing of predictions of microbial physiology and metabolisms should be conducted.

Fluid mixing in the subseafloor may be similar to the mixing and reaction between hydrothermal fluid and seawater that occurs in the walls of metal sulfide structures (43). The sulfide environment is much easier to sample than the subseafloor environment, and it would be convenient if sulfides could be considered as a model for the subseafloor. To test whether the high-temperature habitats are similar in sulfide structures and within the subseafloor, isolates were collected from each of the two environments and compared physiologically and phylogenetically.

Subseafloor *Thermococcus* isolates are phylogenetically distinct from sulfide-associated *Thermococcus* isolates at the Endeavour Segment (Fig. 2). Differential sampling and enrichment was avoided in this study as far as possible so that the two groups of organisms, from sulfides and from the subseafloor, would be as comparable as possible. However, there were some unavoidable differences between the two groups. The subseafloor organisms cultured were only those that were carried out of the subseafloor habitat by fluids and thus perhaps only a subset of the population. All organisms from the subseafloor were exposed to oxygenated seawater during sampling, whereas the various sulfide-associated samples were better protected. All initial enrichment cultures were slightly different because of the inoculum type, although subsequent transfers and pure cultures were grown under identical conditions. Interestingly, all of these conditions would tend to favor the sulfide samples as more representative of the various microhabitats contained within the sampled macrohabitat: more diverse sample types, more protected (or at least more variable) sampling and sample handling, and more variable enrichment conditions. This is exactly the reverse of what is seen; the variable sulfide samples produced five nearly identical strains of *Thermococcus* (Fig. 2), whereas the potentially biased and stressed subseafloor samples produced at least three genetically distinct groups of *Thermococcus*. We believe these two distinct groups of organisms, while subject to the problems of small sample size that are always an issue in deep-sea studies, are representative of their respective macrohabitats.

A genetic distinction between two groups of organisms can indicate a difference in habitat conditions that allows selection of a group of specifically adapted organisms, or it may instead be caused by restricted exchange of organisms between habitats. There is no evidence for restricted exchange between the subseafloor and sulfide habitats. Hyperthermophiles most likely move between specific seafloor locations through seawater; organisms from both habitat types are ejected into ocean bottom water (*Thermococcus* species are notorious for their tolerance for long-term exposure to cold, oxygenated conditions; ref. 11). Seawater reenters sulfide and subseafloor habitats through seawater entrainment and therefore organisms from sulfides should be drawn into the subseafloor and *vice versa*. The lack of genetic distinction between organisms found in the Main Endeavour and High Rise fields, 2 km apart (Fig. 1), indicates that exchange of organisms may be occurring through the water column over this spatial scale. Macrofauna also show similarities between these vent fields and exchange of these organisms is believed to take place across the same distance (44). If there are no physical barriers to exchange between the sulfide habitat and subseafloor habitat, the habitats must instead be different enough to select for and maintain separate groups of dominant hyperthermophilic heterotrophs, which indicates that there may be differences throughout the microbial communities within these two environments.

If selective forces are keeping the subseafloor and sulfide-associated organisms separate, the niches that the two groups of organisms inhabit must differ. The physiology of an organism necessarily encompasses its niche parameters, and therefore the differences in the subseafloor and sulfide hyperthermophilic heterotrophic niches is encoded in the physiology of the 13 isolates in this study. These 13 strains were initially isolated under identical conditions to ensure that the organisms would be comparable to each other, inhabiting niches as similar as may exist between these habitats. If systematic physiological distinctions between the two groups of organisms can be discovered, these differences may be adaptations that reflect differences in their respective niches and thus between their respective habitats.

The two groups were extremely similar nutritionally; neither group appears adapted to eat anything less complex than proteins or a suite of amino acids. However, sulfide-associated organisms produced more active proteases, which indicates that they may be better adapted to foraging at a distance and perhaps to life in high organic or restricted flow environments (45). Isolates from sulfides were slightly but significantly better

adapted to grow under high zinc ion concentrations than were isolates from the subseafloor, showing a potential adaptation to their habitat. However, all organisms showed some tolerance of the maximum zinc and copper ion concentrations that might be experienced naturally (27). The organisms from sulfide structures were adapted to live over a slightly wider temperature range and a much wider salinity range than their subseafloor counterparts, implying that they are better adapted to changing fluid flow conditions.

These physiological differences potentially reflect habitat differences in organic matter source, metal ion concentration, and fluid flow: they represent hypotheses waiting to be tested. Many other physical and chemical parameters could also be examined, but it is difficult to determine *a priori* how two habitats that are poorly understood should differ. Use of biological techniques (physiological and genetic, individual and community level) to complement the physical studies of subseafloor processes such as geochemistry and fluid flow will allow faster characterization of this newly recognized habitat.

Conclusions

Within the ocean crust at the ridge crest, there is a novel habitat created by the mixing of hydrothermal fluids and seawater. Its only known inhabitants are hyperthermophilic microorganisms, but there are predicted to be many other metabolic and thermal classes of microbes also living in these systems. While metal sulfide structures contain some similar organisms, comparison between hyperthermophiles isolated from the two environments shows that the subseafloor in vent systems is a biotope distinct from massive sulfide environments, with unique characteristics and a separate group of indigenous hyperthermophilic species. As this fertile subseafloor environment is arguably the site of the origin of life on Earth (46) and an early Earth safe haven, there is a powerful rationale for closely examining the mid-ocean ridge subseafloor as an analog for habitats on other planets and moons, such as Mars and Europa, that currently have or had sometime during their history liquid water and volcanic activity.

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