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Activity and Distribution of Thermophilic Prokaryotes in Hydrothermal Fluid, Sulfidic Structures, and Sheaths of Alvinellids (East Pacific Rise, 13°N)▽†

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Processes of inorganic carbon assimilation, methanogenesis, sulfate reduction, and acetate oxidation to CO_2 occurring in samples from the East Pacific Rise at 13°N were traced, using radioisotopically labeled substrates, at temperatures ranging from 65 to 100°C. Molecular hydrogen stimulated lithotrophic methanogenesis and sulfate reduction but inhibited inorganic carbon assimilation. Active mineralization of acetate was observed in an organic-rich *Alvinella*-associated system at 80°C. Members of the *Thermococcales* were the most numerous hyperthermophilic archaea in these samples, their density achieving 10^8 cells per cm^3, while the numbers of cultured hydrogen-utilizing thermophilic lithotrophs were several orders of magnitude lower.

Deep-sea hot vents constitute a unique habitat in which chemolithoautotrophic microorganisms thrive by using the energy of inorganic substrates (8). The hot zones of deep-sea hydrothermal environments are inhabited by diverse thermophilic or hyperthermophilic archaea and bacteria, growing at temperatures ranging from moderate to the highest at which microbial growth has been demonstrated (11, 18, 19). The hydrothermal chimneys of the East Pacific Rise are colonized by alvinellids, *Polychaeta* worms, which were reported to have no chemosynthetic symbionts but to be accompanied by uncultured proteobacterial epibionts (2, 5) and which live in tubes built from organomineral material of proteinaceous nature (23). The goal of this work was to characterize the thermophilic processes of organic matter production and destruction in the hydrothermal samples from the East Pacific Rise at 13°N and to reveal the influence of molecular hydrogen, a possible electron donor, on the rate of these processes.

Sampling procedure and sample characteristics. Deep-sea hydrothermal samples were collected at the 13°N hydrothermal vent field on the East Pacific Rise (depth, 2,650 m) during the AMISTAD (Advanced Microbiological Studies of Thermophiles: Adaptations and Diversity) cruise in 1999. Our study focused on the vent field lying between 12°48′18″ and 12°50′32″N and between 103°56′39″ and 103°56′80″W (see Fig. S1 in the supplemental material). Dense populations of *Alvinella pompejana*, a thermotolerant polychaetous annelid that makes tubes in direct contact with sulfides (3), colonized active structures at the La Chaînette and Grandbonum sites. Pieces of sulfide structures and parts of *Alvinella* sheaths were collected by the arm of the *Nautile*, a deep-sea submersible vessel, and lifted to the surface in a submersible insulated basket. Characteristics of these samples are given in Table S1 in the supplemental material.

Radioisotopic assays of microbial activity. The samples were prepared for radioisotopic assays immediately following their delivery aboard ship. Fluid/seawater samples were dispensed into 15-ml bottles in 10-ml portions under a flow of nitrogen. Samples of sulfidic structures and tubes of *Alvinella* were divided into parts of approximately equal volume (~1 cm^3), placed in 15-ml bottles, and covered with a fluid/seawater mixture from the same site to a final volume of 10 ml under a flow of nitrogen. Labeled substrate in sterile degassed water solutions ($H^{14}CO_3^{2-}$ and $^{14}CH_3COO^-$, 10 μCi per sample; $^{35}SO_4^{2-}$, 15 μCi per sample) was injected into the bottles (0.2 ml per bottle). In order to study the effect of molecular hydrogen on process rates, in some of the samples, nitrogen in the headspace was replaced by 100% hydrogen. The bottles were hermetically closed, sealed with aluminum caps, and incubated for 24 h at temperatures chosen according to the available data on the sampling site. After incubation, all microbiological processes were stopped by adding 0.5 ml of saturated KOH solution to each experimental bottle. All experiments were made in triplicates. Parallel abiotic controls were carried out by adding 0.5 ml of saturated KOH to one bottle in each series before incubation. After the experiments were completed, the bottles were stored at −4°C. The presence of radioactivity in the experimental and control bottles was determined back in the laboratory according to standard protocols (17). The actual rates of transformation of substrates into certain products in the samples studied were calculated by the following equation: $I = (r - r_c)C\alpha/RT$, where I is the rate of product formation by microorganisms, r is the radioactivity of the product formed, r_c is the radioactivity of the same product formed in the abiotic control, R is the initial radioactivity of the labeled substrate

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TABLE 1. Rates of microbiological processes as determined by radioisotopic methods

Gas in headspace, sample	Sample type[a]	Incubation temp (°C)	Mean rate (ng C cm^{-3} day^{-1})[b] ± SD of:			Mean rate of sulfate reduction (μg S cm^{-3} day^{-1})[b] ± SD
			Inorganic carbon assimilation	Lithotrophic methanogenesis	Acetate oxidation[c]	
N_2						
AM-05	F	65	0	0	1.4 ± 0.3	0
AM-06	F	80	0	0	0.87 ± 0.2	0
AM-12	F	80	0	0	0	0
AM-03	F+S	65	2.8 ± 0.4	4.5 ± 0.8	2.4 ± 0.5	0
AM-03	F+S	80	10.5 ± 2.4	0.97 ± 0.2	4.9 ± 0.8	0
AM-05	F+S	80	0.18 ± 0.03	0	—	0
AM-15	F+S	80	1.4 ± 0.3	0	2.8 ± 0.5	0
AM-15	F+S	90	1.9 ± 0.2	0.16 ± 0.03	1.5 ± 0.3	0
AM-15	F+S	100	2.0 ± 0.2	1.4 ± 0.2	1.2 ± 0.2	0
AM-16	F+A	80	0.30 ± 0.05	0.09 ± 0.02	35.0 ± 6	0
AM-17	F+A	80	5.4 ± 1.0	0.11 ± 0.02	25.0 ± 5	0
H_2						
AM-03	F+S	65	—	13.1 ± 2	—	250 ± 45
AM-03	F+S	80	1.0 ± 0.2	22.1 ± 4	—	0
AM-05	F+S	80	0.12 ± 0.02	22.6 ± 4.5	—	135 ± 30
AM-06	F	80	0	0	—	0
AM-16	F+A	80	1.7 ± 0.3	3.1 ± 0.6	—	200 ± 30
AM-17	F+A	80	0.24 ± 0.05	0.8 ± 0.2	—	0

[a] F, fluid; S, sulfidic structure; A, sheaths of *Alvinella*.
[b] 0, background level; —, not determined.
[c] Potential rate of acetate transformation.

added to the sample, C is the natural concentration of this substrate in the sample, α is the correction factor for isotope fractionation (1.06 for ^{14}C and 1.045 for ^{35}S), and T is the incubation time.

Rates of microbial processes in the deep-sea samples. Of the samples studied, the only one with no activity was AM-12 (Table 1), which due to a low sulfate concentration (see Table S1 in the supplemental material), was the only undiluted fluid sample. Active inorganic carbon assimilation was observed in sulfidic structures and in microbial communities associated with *Alvinella* tubes, and it increased with increased incubation temperature both in the moderately thermophilic sample AM-03 and in the high-temperature sample AM-15, where it achieved its maximum at 100°C (Table 1). An intensive process of inorganic carbon assimilation occurring at 80°C was previously detected in hydrothermal samples from the Mid-Atlantic Ridge, but no stimulation effect of thiosulfate or hydrogen sulfide was observed (25). Given that molecular hydrogen of volcanic origin is the energy substrate for numerous thermophilic and hyperthermophilic lithoautotrophs inhabiting deep-sea hydrothermal sites (11, 19), we expected it to have a stimulating effect on the inorganic carbon assimilation process. Surprisingly, in three of the five samples, the rate of this process in the presence of hydrogen decreased (10- to 20-fold in samples AM-03 and AM-17 and 33% in sample AM-05) compared to its rate in the same samples incubated under N_2 (Table 1). This can most likely be explained by the stimulation of hydrogen sulfide production by lithotrophic sulfate and sulfur reducers, which inhibits the activity of aerobic hyperthermophilic lithoautotrophs present in these samples. However, one of the *Alvinella* samples (AM-16) assimilated five times as much inorganic carbon under a hydrogen atmosphere as when incubated with nitrogen (1.7 versus 0.3 ng of C per cm^3 per day).

Lithotrophic methanogenesis was prominent in the samples of sulfidic structures and, at a much lower rate, in the thermophilic microbial communities associated with *Alvinella* tubes, showing different temperature profiles in different samples (Table 1). It was detected at temperatures as high as 100°C and, thus, could be attributed to the activity of *Methanopyrus kandleri* detected in this habitat by molecular methods (14). However, the rate of lithotrophic methanogenesis, even when stimulated by the addition of molecular hydrogen, was too low to support the estimated rate of carbon assimilation. Its rate increased significantly in the presence of hydrogen (Table 1). This finding suggests that the rate of lithotrophic methanogenesis in deep-sea hydrothermal ecosystems exposed to high hydrostatic pressure might be much higher than that measured at atmospheric pressure due to the presence of significant amounts of dissolved hydrogen.

Acetate oxidation—the process that closes the anaerobic carbon cycle and is therefore associated with complete organic matter mineralization—was followed by the production of CO_2 from the methyl group of acetate. Given that the concentration of acetate in water-fluid samples was lower than the sensitivity of the analytical methods employed, only the potential rate of acetate oxidation to CO_2 (acetate mineralization) was estimated from the concentration of labeled acetate introduced into the samples (0.2 μmol per sample). The formation of CO_2 from acetate was found to be a potentially prominent process in the high-temperature hydrothermal environment. It was observed in two of the three fluid samples and in all samples of sulfidic structures; it was particularly intensive in organic-rich *Alvinella* samples (Table 1). Its rate increased with tempera-

ture in sample AM-03 but decreased in sample AM-15, where it was still detectable at 100°C (Table 1).

The rate of sulfate reduction, measured as the rate of acid-labile $^{35}S^{2-}$ formation from $^{35}SO_4^{2-}$, showed no difference from that in abiotic controls when samples were incubated in an atmosphere of 100% N_2. However, in the presence of molecular hydrogen (100%), this process was detected in three samples (Table 1). The potential rate of lithotrophic sulfate reduction in the presence of molecular hydrogen was extremely high, reaching 125 to 250 μg S cm^{-3} day^{-1}, while in abiotic control bottles filled with hydrogen, it amounted to just 1 to 2% of the experimental values. The addition of acetate (2 g $liter^{-1}$) had no effect on the rate of biological sulfate reduction: the rate remained indistinguishable from that in the abiotic controls (data not shown). It could be assumed, therefore, that vigorous lithotrophic sulfate reduction is effective in deep-sea hydrothermal environments under a high concentration of dissolved hydrogen. Under our experimental conditions, its rate was of the same order of magnitude as reported for sulfate reduction in the Guaymas Basin (6, 7, 24).

Dilution-to-extinction enumeration and identification of thermophilic prokaryotes. Immediately after delivery aboard ship, the samples of internal or external parts of sulfide structures and sheaths of alvinellids (site PP57, sample AM-09) were disintegrated and dispensed in 1-cm^3 portions in 15-ml N_2-filled Hungate tubes to be mixed with 5 or 10 ml of substrate-free reduced marine medium of the following composition (g $liter^{-1}$): NH_4Cl, 0.33; KCl, 0.33; KH_2PO_4, 0.33; $CaCl_2$ $2H_2O$, 0.33; $MgCl_2$ $6H_2O$, 0.33; NaCl, 25; Na_2S $9H_2O$, 0.5; $NaHCO_3$, 0.5; resazurin, 0.002; trace elements (1), 1 ml $liter^{-1}$; and vitamins (26), 1 ml $liter^{-1}$. The slurries obtained were used for serial 10-fold dilutions prepared in the same medium with different substrates and/or electron acceptors as follows: with headspace filled with an H_2/CO_2 mixture (80:20; 5-ml portions of the medium), with no additions, with sodium sulfate (2 g $liter^{-1}$), or with elemental sulfur (10 g $liter^{-1}$). For cultivation of organotrophs, the same medium supplemented with 2 g $liter^{-1}$ peptone and 0.1 g $liter^{-1}$ yeast extract was dispensed in 10-ml portions, and the headspace was filled with a mixture of N_2/CO_2 (80:20). The incubation temperatures were 65 or 80°C, and for two series of dilutions with organic substrates, 90 and 99°C (see Table S2 in the supplemental material). Microbial growth was monitored by light microscopy and by qualitative determination of the end products methane and sulfide by gas-liquid chromatography (10) and a modification of Trüper and Schlegel's colorimetric method (22), respectively. In samples of sulfidic structures, the numbers of moderately thermophilic and hyperthermophilic lithotrophs varied from 10^2 to 10^3 and the numbers of organotrophs from 10^3 to 10^4 cells per cm^3. Most numerous (10^5 cells per cm^3) were sulfur-reducing organotrophs growing at 99°C (see Table S2 in the supplemental material). *Alvinella* sheaths were found to be populated by an abundant microbial community, dominated by hyperthermophilic anaerobic organotrophs numbering 10^8 cells per cm^3, while the numbers of cultivated hydrogen-utilizing lithotrophs were two to six orders of magnitude lower (see Table S2 in the supplemental material).

DNA from the last positive dilutions was isolated as described previously (16), and a two-step PCR with several sets of primers, universal and specific for the domains of *Bacteria* and *Archaea* (9) and the phylum *Crenarchaeota* (16), was carried out. Denaturing gradient gel electrophoresis (DGGE) analysis of PCR products was performed as described previously (16). *Crenarchaeota*-positive PCR products were used for hybridization with a genus-level probe, Ign447 (5′-CGCTTATTACCC CCGCCTGTTTACA-3′), that is specific for *Ignicoccus.* DNAs were transferred to a positively charged nylon membrane by the Southern method, and further steps were performed according to the protocols described elsewhere (15). Representatives of the genus *Thermoanaerobacter* were identified by using the PCR products obtained with *Bacteria*-specific primers for the Southern blot hybridization with a genus-specific oligonucleotide probe (20).

The PCR-DGGE 16S rRNA analysis with archaeal primers revealed the presence of *Methanocaldococcus* spp. and *Methanococcus* spp. in enrichments developing on the H_2/CO_2 mixture (see Table S2 in the supplemental material). *Thermococcus* spp. were present in lithotrophic cultures, as well as in an anaerobic peptone-degrading culture obtained from the 10^{-8} dilution of the sample. Representatives of the genus *Ignicoccus*, although not exposed by the DGGE analysis, were detected by a genus-specific fluorescent probe in H_2-utilizing methanogenic and sulfate-reducing enrichments.

The PCR-DGGE analysis with *Bacteria*-specific primers showed the presence in the samples of different groups of *Bacteria* (see Table S2 in the supplemental material): *Deferribacter* and *Coprothermobacter* (H_2/S^0-containing medium incubated at 65°C) and *Thermoanaerobacter* (organotrophic culture growing at 65°C; detected with a genus-specific probe). Both the moderately thermophilic and hyperthermophilic enrichments contained representatives of mesophilic taxa—*Pseudomonas* spp., *Bacteroidetes*, or organisms closely related to *Methylovorus mays* that originate, most probably, from surrounding cold zones.

Polychaeta worms of the genus *Alvinella* have been shown to be exposed *in situ* to temperatures as high as 80°C (3, 4). Our data on the numbers of thermophilic prokaryotes in *Alvinella* tubes indirectly support this contention, as members of all physiological groups were an order of magnitude more numerous at 80°C than at 65°C. These data are in agreement with findings reported by Moussard et al. (12), who showed that *Alvinella pompejana* tubes were inhabited by diverse hyperthermophilic archaea. The population of *Thermococcales* was found to be extremely dense in this habitat, achieving 10^8 cells per cm^3. This is one to two orders of magnitude higher than reported for other deep-sea hydrothermal environments (13, 21). *Thermococcales* degrade complex proteinaceous substrates, forming acetate as the main fermentation product. Our radioisotopic experiments showed that acetate is readily oxidized to CO_2 by hyperthermophilic microorganisms that are part of the same microbial community.

Nucleotide sequence accession numbers. The 16S rRNA gene partial sequences of bacterial and archaeal DGGE bands retrieved in this study have been deposited in GenBank under the accession numbers GU084822 to GU084828.

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