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Effects of Dissolved Sulfide, pH, and Temperature on Growth and Survival of Marine Hyperthermophilic Archaea

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The ability of metabolically diverse hyperthermophilic archaea to withstand high temperatures, low pHs, high sulfide concentrations, and the absence of carbon and energy sources was investigated. Close relatives of our study organisms, *Methanocaldococcus jannaschii*, *Archaeoglobus profundus*, *Thermococcus fumicolans*, and *Pyrococcus* sp. strain GB-D, are commonly found in hydrothermal vent chimney walls and hot sediments and possibly deeper in the subsurface, where highly dynamic hydrothermal flow patterns and steep chemical and temperature gradients provide an ever-changing mosaic of microhabitats. These organisms (with the possible exception of *Pyrococcus* strain GB-D) tolerated greater extremes of low pH, high sulfide concentration, and high temperature when actively growing and metabolizing than when starved of carbon sources and electron donors/acceptors. Therefore these organisms must be actively metabolizing in the hydrothermal vent chimneys, sediments, and subsurface in order to withstand at least 24 h of exposure to extremes of pH, sulfide, and temperature that occur in these environments.

Laboratory-based physiological studies of hyperthermophilic archaea often take place under specific cultivation and growth conditions, with empirically optimized electron acceptors and donors, pH, temperature, and carbon sources (28). However, conditions in and around hydrothermal vents and hot springs are often dynamic, with steep chemical and temperature gradients and highly variable fluid flow (14). Hyperthermophilic archaea living in vent chimneys, basement basalt, or overlying seafloor sediments are exposed in various degrees to the combined effects of high temperature, low pH, high concentrations of sulfide, and fluctuating levels of carbon and energy sources. Understanding the tolerance limits of known vent microorganisms under in situ chemical and physical stresses allows a more detailed definition of the environmental niches they are capable of occupying. This may also help inform investigations of the putative deep subsurface biosphere. The existence of such a microbial ecosystem is indicated by the diverse communities flushed out in various hydrothermal fluids (9, 31, 32).

We examined growth and nongrowth survival of four species of anaerobic hyperthermophilic archaea with combinations of high sulfide concentrations, low pHs, and high temperatures. *Methanocaldococcus jannaschii*, a methanogen, *Archaeoglobus profundus*, a sulfate reducer, and *Thermococcus fumicolans* and *Pyrococcus* sp. strain GB-D, both sulfur reducers, represent some of the dominant anaerobic physiological types in hydrothermal vent environments (7, 33).

M. jannaschii, an obligate H_2-CO_2 autotroph, was first isolated from hydrothermal vent sediments at 21°N East Pacific Rise (13). Close relatives of this type strain have also been obtained from hydrothermal vents in Guaymas Basin (38) and the Mid-Atlantic Ridge (12). In addition, small subunit 16S rRNA sequences closely related to that of *M. jannaschii* have been found in hydrothermal vent fluids and a sulfide chimney from the Juan de Fuca Ridge (10) and the Kairei hydrothermal vent field (32). *M. jannaschii* has an optimal doubling time of 26 min at 85°C, with a maximum growth temperature of 88°C (13). *A. profundus*, an acetate-utilizing mixotroph, has been isolated from hydrothermal vent chimney material and sediments at Guaymas Basin (2) and from deep North Sea oil reservoirs (29). Cultures and 16S rRNA sequences from the genus *Archaeoglobus* have been retrieved from hydrothermal vent environments at the Mid-Atlantic Ridge (26), the Juan de Fuca Ridge (27), the Guaymas Basin (35), and the Kairei hydrothermal vent field (32). *A. profundus* has an optimal doubling time of 4 h at 82°C and pH 6.5, with a maximum growth temperature of 90°C (2). *T. fumicolans* and *Pyrococcus* strain GB-D were originally isolated from chimney fragments in the North Fiji Basin (6) and Guaymas Basin (11), respectively. *Pyrococcus* strain GB-D has a maximum doubling time of 36 min at 95°C and has a maximum growth temperature of 103°C (11). *T. fumicolans* has a maximum doubling time of 86 min at 85°C and can grow in temperatures up to 103°C (6). Closely related organisms have also been found via culturing and molecular methods in nearly all of the well-studied marine hydrothermal vents (25). Members of the genus *Thermococcus* are among the most frequently recovered archaea in hydrothermal vent chimneys and subsurface areas (33).

Since *M. jannaschii* and *A. profundus* grow well at 82°C and *T. fumicolans* and *Pyrococcus* strain GB-D grow well at 90°C, we used these as control growth temperatures in our experiments. Cultures were incubated at 88°C (for *M. jannaschii* and *A. profundus*) and 100°C (for *T. fumicolans* and *Pyrococcus*

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strain GB-D) to determine the effect of heat shock on their tolerance to various pH values and/or sulfide concentrations. Growth and survival for *M. jannaschii* and *A. profundus* were tested at a pH range of 4.5 to 6.5 and a sulfide range of 0 mM to 80 mM, similar to the observed ranges for hydrothermal vent fluid discharges (37). Sulfide refers to H_2S, HS^-, and S^{2-}, although at low pH values, H_2S is expected to be the dominant form, with a pK_a (H_2S-HS^-) of 7.1 (30). Growth and survival of *T. fumicolans* and *Pyrococcus* strain GB-D were tested at a pH range of 4.5 to 7.5. Growth and survival experiments were conducted over periods of 12 to 24 h, since control experiments showed that all strains grew by 2 orders of magnitude within this time period under optimized culture conditions.

Media. *M. jannaschii*, *A. profundus*, and *T. fumicolans* were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Braunschweig, Germany). *Pyrococcus* strain GB-D was isolated and maintained in our laboratory (11). For *M. jannaschii* and *A. profundus*, growth medium consisted of DSMZ medium 282 (http://www.dsmz.de/media/med282.htm) modified with 25 g/liter NaCl, 4.0 g/liter $MgCl_2 \cdot 6H_2O$, and 3.3 mM (final concentration) citrate buffer, with both 1.0 g/liter sodium acetate and 0.5 g/liter yeast extract (Difco) added for *A. profundus*. Anoxic growth media were pressurized to 3 atm with 4:1 (vol/vol) H_2-CO_2. For *T. fumicolans* and *Pyrococcus* strain GB-D, growth media consisted of half-strength marine broth 2216 (Difco) diluted into Turk's Island artificial seawater (11) and supplemented with ~1% (wt/vol) elemental sulfur. For media at pH 7.5, 6.93 g/liter PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer was used; all lower pH values were buffered with 0.82 g/liter CH_3COONa. Anoxic survival media consisted of DSMZ medium 282 seawater, for *M. jannaschii* and *A. profundus*, or Turk's Island artificial seawater, for *T. fumicolans* and *Pyrococcus* strain GB-D, containing no carbon or energy sources, under an N_2 atmosphere. All growth and survival media were prepared under N_2, with 1 mg/ml of the redox indicator resazurin, and were reduced with 96 mg/liter $Na_2S \cdot 9H_2O$. This minimal concentration of sulfide (0.4 mM) was required to keep the media reduced.

Growth experiments. For growth experiments, freshly grown cells were diluted to between 10^5 and 10^6 cells/ml into Hungate tubes containing growth media. For sulfide experiments, the growth media were adjusted to a sulfide concentration of 0.4, 20, 40, 60, or 80 mM at a pH of 6.0 for *M. jannaschii* and 6.5 for *A. profundus*. Sulfide concentrations were determined spectrophotometrically (3). For growth experiments at a range of pH values, the pH of the growth media was adjusted to 4.5, 5.0, or 6.0 for *M. jannaschii* and *A. profundus*, as well as pH 6.5 for *A. profundus* and pH 4.5, 5.0, 5.5, or 7.5 for *T. fumicolans* and *Pyrococcus* strain GB-D. The pH was checked before and after growth at pH 5.0, 6.0, and 6.5 (for *M. jannaschii* and *A. profundus*) and at pH 4.5 and 5.0 (for *T. fumicolans* and *Pyrococcus* strain GB-D) by using a sulfide-resistant pH electrode (model MP 220; Mettler-Toledo GmbH, Schwerzenbach, Switzerland), to make sure the values changed by no more than 0.3. After strains were incubated in 82°C-, 88°C-, 90°C-, or 100°C-water baths for 24 h (sulfide experiments) or 12 to 22 h (pH experiments), growth was assessed by direct counts of cells stained with acridine orange (8).

Survival experiments. For survival experiments, freshly grown cells (density, 0.5×10^8 to 1×10^8 cells/ml) were diluted to 10^6 cells/ml into Hungate tubes containing survival media. In this way, the inoculum volume and carbon carryover into the survival medium were limited to 0.1 ml, or 1% of the test volume. In some cases, cultures with lower cell densities allowed only for concentrations of 10^5 cells/ml in the Hungate tubes with survival media; inoculum volume was no greater than 0.2 ml, or 2%, to limit carbon carryover.

In our survival experiments, cell counts could not distinguish dead cells from living cells, since the morphology remained largely intact. Therefore, survival was assessed after 24 h by using a six-step decimal dilution series method. After exposure to stress conditions, cell suspensions were diluted into six-step decimal dilution series into complete growth medium and incubated at optimal temperature for up to 5 days. Tubes were checked daily for regrowth, on the basis of visible turbidity and microscopic examination. Data are presented as the highest decimal dilution step out of six steps in each dilution series that produced regrowth after each survival experiment. Selected single dilution series were checked for repeatability in triplicate most-probable-number experiments (data not shown).

For sulfide survival experiments, the media were adjusted to sulfide concentrations of 0.4, 10, 20, 30, 40, and 75 mM, at a pH of 6.0 for *M. jannaschii* and a pH of 6.5 for *A. profundus*. The media for pH survival experiments covered a pH range similar to that of the growth experiments (pH 4.5 to 6.5 for *M. jannaschii* and *A. profundus*; pH 4.5 to 7.5 for *T. fumicolans* and *Pyrococcus* strain GB-D). pH values were checked for consistency (within 0.3) before and after each experiment.

pH experiments. The in situ pH in the matrix of vent chimneys and in hydrothermally flushed sediments is a function of mixing of hydrothermal vent end-member fluid (pH, ca. 3 to 4) with near-neutral seawater (pH 7.8) (21). *M. jannaschii*, *T. fumicolans*, and *Pyrococcus* strain GB-D were all capable of growth at a pH as low as 4.5 at both temperatures. The lower pH limits of growth were not reached in these experiments; thus, it is possible that these organisms can tolerate even lower pHs. *A. profundus*, on the other hand, grew only at pH 5.5, at both 82°C and 88°C. The ability of *M. jannaschii*, *T. fumicolans*, and *Pyrococcus* strain GB-D to grow at lower pH values than *A. profundus* may indicate a difference in these organisms' environmental adaptations. Based on mixing model calculations for seawater and hydrothermal vent end-member fluid in vent chimney walls, seawater in-mixing raises the pH from typical end-member fluid values (~4) to 5.5 to 6.0 over a wide range of mixing ratios (21). The sensitivity of *A. profundus* to low pH indicates that it is adapted to habitats with seawater in-mixing, characterized by moderate pH and elevated sulfate concentrations. *M. jannaschii*, *T. fumicolans*, and *Pyrococcus* strain GB-D, however, are not dependent on sulfate, and their growth at pH 5.0 and below may indicate that they are better equipped to deal with low-sulfate, low-pH environments where seawater in-mixing is limited.

In most cases, temperature tolerances were not greatly affected by low pH. Growth and survival were similar at the two temperatures tested for *M. jannaschii*, *A. profundus*, and *Pyrococcus* strain GB-D. The same was true for *T. fumicolans* in growth experiments (Table 1). The only exception to this trend is *T. fumicolans* in survival experiments, which lost its high

TABLE 1. Effect of pH on the growth and survival of *M. jannaschii*, *A. profundus*, *T. fumicolans*, and *Pyrococcus* strain GB-D at optimal (82°C or 90°C) and superoptimal (88°C or 100°C) temperatures with and without nutrients

pH for indicated species	Growth[a] with growth nutrients at:		Survival[b] without growth nutrients at:	
	82°C	88°C	82°C	88°C
M. jannaschii				
6.5	ND[f]	ND	6	6
6.0	++	++	3.5[c]	3[c]
5.0	++	++	3[c]	0
4.5	++	++	0	0
A. profundus				
6.5	++	++	6	6
6.0	++	++	6[c]	6[c]
5.0	−	−	0[d]	0[d]
4.5	−	−	ND	ND
	90°C	100°C	90°C	100°C
T. fumicolans				
7.5	++	++	6	0
5.5	++	++	6	0
5.0	++	++	6	0
4.5	+	+	0	0
Pyrococcus sp.				
7.5	++	++	6	6
5.5	++	++	6[e]	6
5.0	++	+	6	6
4.5	++	+	6[e]	6

[a] Growth is indicated by an increase in cell number of greater than or equal to 1 order of magnitude (++), less than 1 order of magnitude (+), or less than one doubling (−).
[b] Survival is indicated by the number of dilution steps in the sixfold dilution series that exhibited regrowth after 24-h exposure to nongrowth conditions.
[c] These numbers are averages from two experiments.
[d] At pH 4.7, good survival was found (5 at 82°C and 6 at 88°C), indicating highly variable results in the pH range of 4.7 to 5.
[e] Number verified by most-probable-number calculation.
[f] ND, no data collected.

TABLE 2. Effect of sulfide on the growth and survival of *M. jannaschii* and *A. profundus* at optimal (82°C) and superoptimal (88°C) temperatures with and without nutrients

Sulfide concn (mM) for indicated species	Growth with growth nutrients[a]		Survival without growth nutrients[b]	
	82°C	88°C	82°C	88°C
M. jannaschii				
0.4	++	++	4.5[d]	3[d]
10	ND[e]	ND	5[d]	3[d]
20	++	++	2	0
30	ND	ND	1	0
40	++	++	1	0
60	++	−	ND	ND
75/80[c]	+	−	0	0
A. profundus				
0.4	++	++	4.5[d]	2.5[d]
10	ND	ND	2	2
20	++	++	1	0
30	ND	ND	0	0
40	++	−	0	0
60	+	−	ND	ND
75/80[c]	−	−	ND	ND

[a] Growth is indicated by an increase in cell number of greater than or equal to 1 order of magnitude (++), less than 1 order of magnitude (+), or less than one doubling (−).
[b] Survival is indicated by the number of dilution steps in replicates of the sixfold dilution series that exhibited regrowth after 24-h exposure to nongrowth conditions.
[c] Concentrations were 80 mM for the growth experiments and 75 mM for the survival experiments.
[d] These numbers are the average of two experiments.
[e] ND, no data collected.

temperature tolerance at every pH tested. With the exception of the *T. fumicolans* survival results, the stresses of low pH do not seem to greatly impair temperature tolerance.

When deprived of the energy and carbon sources necessary for growth (H_2 and CO_2 for *M. jannaschii*; H_2, CO_2, yeast extract, and acetate for *A. profundus*; and sulfur and yeast extract for *T. fumicolans* and *Pyrococcus* strain GB-D), *M. jannaschii* and *T. fumicolans* did not survive at pH 4.5 (Table 1), in contrast to having good growth under the same conditions in growth medium. *M. jannaschii* did not survive well at a pH of ≤6.0. In the absence of growth nutrients, *T. fumicolans* lost all temperature tolerance and could not survive at any of the pH values tested when at 100°C. *A. profundus* had variable survival below pH 5.5, in agreement with its poor growth at low pH. *Pyrococcus* strain GB-D was more resistant than the others to low pH, starvation, and high temperature. It survived and grew in the full range of temperatures and pH values tested. Apparently, *Pyrococcus* strain GB-D is more tolerant of harsh fluctuations in hydrothermal vent conditions than are the other test organisms.

Sulfide experiments. Sulfide in high concentrations is a characteristic feature of hydrothermal vents, where it contributes to alleviating the toxicity of metals by metal-sulfide complex formation (4). However, uncomplexed sulfide has been shown to be highly toxic to methanogenic archaea (19, 23, 24). At the control temperature (82°C), both *M. jannaschii* and *A. profundus* were able to grow over 24 h at very high sulfide levels, up to 80 mM and 60 mM, respectively (Table 2). These sulfide tolerances far exceed the 4- to 8-mmol/kg sulfide concentrations measured for vent end-member fluid from the sites where these archaea were originally isolated, at 21°N East Pacific Rise, and at Guaymas Basin, respectively (37). This high sulfide tolerance may favor wide dispersal and distribution of *M. jannaschii* and *A. profundus*, since sulfide concentrations as high as 110 mmol/kg have been measured at other hydrothermal vents (reviewed in reference 14).

High-temperature stress (88°C) limited the range of sulfide concentrations in which *M. jannaschii* and *A. profundus* could grow over 24 h (Table 1). At 88°C, the maximum sulfide concentration at which *M. jannaschii* was capable of growth was reduced to 40 mM. Similarly, *A. profundus* responded to 88°C with a decrease in the growth limit to 20 mM sulfide. Although high temperatures decreased their sulfide tolerance limits, both organisms were capable of growth at sulfide concentrations much higher than those present at their vents of origin. High sulfide tolerances have also been noted for the hyperthermophiles *Pyrococcus* strain GB-D (44 mM) and *Desulfurococcus* sp. strain SY (90 mM) (11).

Without energy and carbon sources, *M. jannaschii* and *A. profundus* were less tolerant of sulfide over 24 h (Table 2). At 82°C, both strains lost viability at moderate sulfide concentra-

tions. At 88°C, survival of both strains was more sensitive to high sulfide concentration exposure than at 82°C; 24-h exposure resulted in essentially complete mortality at sulfide concentrations above 10 mM for both species. Nongrowing cells of *M. jannaschii* and *A. profundus* were much less able to withstand high sulfide concentrations than cells that were well supplied with electron donors and carbon substrates. These effects were magnified when the temperature was increased to 88°C.

The growth of *M. jannaschii* and *T. fumicolans* under conditions of high temperature and low pH contrasts with lack of survival of the organisms under the same temperature and pH conditions but without nutrients. Similarly, growth of *M. jannaschii* and *A. profundus* under conditions of high temperature and high sulfide concentrations contrasts with the inability of the organisms to survive under the same conditions but without nutrients. As a caveat, the limits of pH tolerance for *Pyrococcus* strain GB-D were not reached in this experiment.

The ability of actively growing cells to withstand greater stresses than carbon- and energy-deprived cells indicates that essential stress adaptation mechanisms require a basic carbon and energy supply. The starved cells in our study could have been unable to adequately maintain essential enzymatic activities and structural components. This, in turn, weakens the cellular defenses against a wide range of physiological stress factors, such as pH, high sulfide concentrations, and high-temperature shocks.

When under heat stress, many hyperthermophilic archaea synthesize heat shock proteins, or chaperonins, which act as molecular chaperones stabilizing cellular components (17, 36). For example, *Archaeoglobus fulgidus*, a close relative to *A. profundus*, expresses two chaperonin subunits, cpnα and cpnβ, at temperatures near 89°C (5). *M. jannaschii* has been found to express the chaperonin HSP16.5, which is activated at 85°C (15, 16). However, these adaptation mechanisms to high temperatures, as well as other physiological defenses against different stress factors (e.g., pH and sulfide), are likely to break down without a basic nutrient supply.

Conclusions. The physiological responses of *A. profundus*, *M. jannaschii*, *T. fumicolans*, and *Pyrococcus* strain GB-D were studied under combinations of extremes of pH, temperature, and sulfide concentrations, with and without carbon substrates and electron donors/acceptors. Previous studies have shown that some stress factors at hydrothermal vents alleviate each other. For example, high metal and sulfide concentrations can be tolerated by *M. jannaschii*, *T. fumicolans*, and *Pyrococcus* strain GB-D due to the formation of metal-sulfide complexes (4). Elevated seafloor and subseafloor hydrostatic pressures may also increase the tolerances of some archaea to hydrothermal vent conditions (1, 20, 22). In addition, biofilm formation and attachment to mineral surfaces may allow hydrothermal vent archaea to expand the range of tolerable temperature, pH, and sulfide stresses (18, 27).

Pyrococcus strain GB-D is more tolerant to acidic and high-temperature conditions than are the other organisms and therefore may be able to access areas in the hydrothermal vent subsurface or chimneys that are exposed to vent fluids less diluted by seawater. This finding agrees with clone libraries that retrieved members of the *Thermococcales* on the internal chimney walls nearest to the hydrothermal vent fluid conduit (27, 34). We found that the negative effects of high temperature and high sulfide concentrations tend to compound each other for *M. jannaschii* and *A. profundus*. The greatest decrease in tolerance to adverse conditions in our study occurred when the archaea were deprived of electron acceptors, electron donors, and carbon substrates. Without energy and carbon sources, nongrowing cells of *M. jannaschii*, *A. profundus*, and *T. fumicolans* could not survive the stress levels that they tolerated easily as growing cells. Thus, the responses of these archaea to stress factors depend primarily on active metabolism and the ability to synthesize new biomolecules; extended survival in a nongrowing, metabolically inactive, suspended state appears improbable in environmental extremes found in hydrothermal vent environments.

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