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Survival and growth of two heterotrophic hydrothermal vent archaea, *Pyrococcus* strain GB-D and *Thermococcus fumicolans*, under low pH and high sulfide concentrations in combination with high temperature and pressure regimes

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Abstract Growth and survival of hyperthermophilic archaea in their extreme hydrothermal vent and subsurface environments are controlled by chemical and physical key parameters. This study examined the effects of elevated sulfide concentrations, temperature, and acidic pH on growth and survival of two hydrothermal vent archaea (*Pyrococcus* strain GB-D and *Thermococcus fumicolans*) under high temperature and pressure regimes. These two strains are members of the Thermococcales, a family of hyperthermophilic, heterotrophic, sulfur-reducing archaea that occur in high densities at vent sites. As actively growing cells, these two strains tolerated regimes of pH, pressure, and temperature that were in most cases not tolerated under severe substrate limitation. A moderate pH of 5.5–7 extends their survival and growth range over a wider range of sulfide concentrations, temperature and pressure, relative to lower pH conditions. *T. fumicolans* and *Pyrococcus* strain GB-D grew under very high pressures that exceeded in-situ pressures typical of hydrothermal vent depths, and included deep subsurface pressures. However, under the same conditions, but in the absence of carbon substrates and electron acceptors, survival was generally lower, and decreased rapidly when low pH stress was combined with high pressure and high temperature.

Keywords Pressure · Sulfide · Archaea · pH · Survival · Growth · Stress factors · Hyperthermophiles

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Introduction

Anaerobic, hyperthermophilic archaea from hydrothermal vents have been isolated from water samples and from rock and sediment materials on the seafloor that were collected at sites accessible to sampling by deep submergence vehicles (Stetter 1999). Indirect evidence is accumulating that some archaeal species and genera occur in the hydrothermal vent subsurface, and represent candidate organisms for a thermophilic subsurface biosphere of unknown depth and extent (Deming and Baross 1993; Holden et al. 1998; Summit and Baross 1998, 2001; Huber et al. 2002; Takai et al. 2004a, b). The physical and chemical controls and physiological limits for growth and survival of hyperthermophilic archaea

at hydrothermal vents and in the hydrothermal vent subsurface are therefore of considerable interest.

Environmental conditions at hydrothermal vents are not always conducive to steady growth; fluctuations in temperature regime, fluid flux, and carbon substrate supply are creating a spatial and temporal mosaic of microenvironments. For example, carbon substrate availability in hydrothermal metal ores and porous metal sulfides is limited and patchy (Orem et al. 1990; Wirsen et al. 1993). As a consequence, vent archaea in their natural habitat most likely fluctuate through episodes of growth and survival. Growth and survival are two very different physiological states that are characterized by different sensitivities to environmental stress factors and physical and chemical controls. For example, Trent and Yayanos (1985) show that the mesophilic bacterium *V. harveyi* has temperature and pressure ranges that are significantly wider for survival than for growth. Hyperthermophilic archaea show the opposite trend; metabolically active, growing cells were more tolerant to pH, temperature and sulfide concentration extremes than inactive, surviving cells (Lloyd et al. 2005). Since the results of growth and survival experiments cannot be extrapolated and derived from each other, this study tests growth and survival responses of hyperthermophilic archaea to environmental stress factors in parallel.

Of the environmental controls that determine growth and survival of hydrothermal vent archaea in pure culture experiments and enrichments, temperature and pressure have received far more attention (examples in Reysenbach and Deming 1991; Jannasch et al. 1992; Pledger et al. 1994; Holden and Baross 1995; Canganella et al. 1997, 2000; Miller et al. 1988; Marteinsson et al. 1999) than combinations of chemical factors such as pH, sulfide and metal concentrations (Edgcomb et al. 2004; Lloyd et al. 2005) that are highly characteristic for hydrothermal vent environments. Most hydrothermal endmember fluids have low pH values (pH 3–4) (Von Damm 1990), and contain extremely variable concentrations of sulfide and metal (Von Damm 1990; Von Damm 1995). Sulfide concentrations are typically in the millimolar range, with maxima at 12 mmol/kg (Von Damm 1990) and 17 mmol/kg (Ding et al. 2001). Seawater in-mixing attenuates these chemical extremes to varying degrees (McCollom and Shock 1997).

Extremes of pH and sulfide concentrations are most likely major factors determining growth and survival of vent archaea. Mesophilic bacteria show enhanced sensitivity to low pH under pressure (Matsamura et al. 1974). Four archaeal species (*Methanocaldococcus jannaschii*, *Archaeoglobus profundus*, *T. fumicolans* and *Pyrococcus* strain GB-D) show a narrowing pH range for growth and survival under increasing temperatures at atmospheric pressure (0.101 MPa) (Lloyd et al. 2005). Corresponding pH sensitivity data for archaea under high pressure regimes are lacking. Metabolically active anaerobic hyperthermophilic archaea isolated from hydrothermal vents tolerate sulfide in very high concentrations (up to 80–90 mM) that can exceed the wide range of sulfide concentrations in hydrothermal vent endmember fluids (Jannasch et al. 1988, 1992; Lloyd et al. 2005). At atmospheric pressure, metabolically active archaea in full media tolerate sulfide generally better than non-growing hyperthermophiles under substrate limitation; however, the interaction of sulfide and high pressure remains to be determined (Lloyd et al. 2005). To extend these observations, this study examines sulfide and pH sensitivity of hyperthermophilic archaea (both substrate-limited and metabolically active cells) under high pressure, at different temperatures.

Since the environmental variables in this study (temperature, pH, sulfide, hydrostatic pressure) were tested in multiple combinations and also separately for metabolically active (in full media) versus surviving (non-growing cells under substrate limitation), resulting in a complex data matrix, we limited the number of test strains to two heterotrophic hyperthermophilic archaea, *Pyrococcus* strain GB-D (Jannasch et al. 1992) and *Thermococcus fumicolans* (Godfroy et al. 1996). Numerous species and strains of these two genera have been isolated from hydrothermal environments world-wide with such frequency that *Pyrococcus* and *Thermococcus* spp. are now the most commonly cultured indicator organisms for hydrothermal activity (Zillig and Reysenbach 2001; Kelley et al. 2002). Sequences of *Thermococcus* and *Pyrococcus* are recovered frequently in molecular environmental surveys at hydrothermal vents (Summit and Baross 2001; Takai et al. 2001; Takai et al. 2004a, b). Dilution series data indicate high population densities of these archaea at hydrothermal vents (Harmsen et al. 1997; Takai et al. 2004a). *Pyrococcus* strain GB-D and *T. fumicolans* were originally isolated from the walls of black smoker chimneys, at the Guaymas Basin (Jannasch et al. 1992) and the North Fiji vents (Godfroy et al. 1996). They are in several respects well adapted to the environmental stresses of the hydrothermal vent environment and the vent subsurface, and thus represent suitable model organisms. The upper growth temperature limit of *Pyrococcus* strain GB-D increases towards higher pressure (95–100°C at 0.1 MPa, 102°C at 0.5 MPa, 104°C at 10.1 and at 20.3 MPa). Exposure to 120°C is survived for at least 5 min. *Pyrococcus* strain GB-D maintains short doubling

times, approx. 36–45 min at 85°C, 90°C and 95°C over a wide pressure range from 0.1 to 20.3 MPa (Jannasch et al. 1992). To date, extreme sulfide tolerance has been indicated by a single-point growth measurement at 44 mM sulfide (Jannasch et al. 1992). *T. fumicolans* has a wide temperature (73–103°C) and pH range (pH 4.5–9.5) for growth. Unusual for the genus *Thermococcus*, *T. fumicolans* can grow on pyruvate alone, a possible adaptation to nutrient limitations (Godfroy et al. 1996).

The effects of low pH and high sulfide concentrations in conjunction with hydrostatic pressure and temperatures on growth and survival, extend our knowledge of archaeal adaptations and physiological tolerances to the complex matrix of physical and chemical stress factors that characterize their hydrothermal vent habitat.

Materials and methods

Archaeal strains and media

Thermococcus fumicolans was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). *Pyrococcus* strain GB-D was isolated and maintained in our laboratory (Jannasch et al. 1992). Growth and survival media for *Pyrococcus* and *Thermococcus* were prepared as described previously (Lloyd et al. 2005). Survival media were prepared in the same way as growth media with the exception that electron donors and acceptors and organic growth substrates were omitted (Lloyd et al. 2005).

Growth experiments with high sulfide concentrations required special media preparations in order to ensure accurate sulfide concentrations. High sulfide media were prepared by mixing standard media (0.4 mM sulfide) with a high sulfide stock medium. High sulfide stock was prepared by bubbling media in a sealed bottle with H_2S gas for a minimum of 10 min while stirring. The headspace was then gassed with H_2S for a minimum of 5 min to allow equilibration of dissolved sulfide. The final pH of the high sulfide stock was adjusted by adding 8 N NaOH. The sulfide concentration in high-sulfide medium was determined spectrophotometrically (Cline 1969). For zero sulfide growth experiments, sulfide was stripped off by bubbling standard 0.4 mM sulfide medium with nitrogen. All sulfide growth media were buffered to a starting pH 7 with PIPES; the pH was checked after each growth experiment using PIPES-buffered media at 90–100°C. The final pH at these temperatures was in the range of 6.3–6.7, which is consistent with theoretical predictions ($\Delta pH/\Delta T = -0.0085$; Dawson et al. 1989). For experiments at lower pH, acetate buffer was used. The ratio of acetic acid to sodium acetate was adjusted as needed for the desired pH.

Selection criteria for experimental parameters of pressure, pH, sulfide and temperature

We selected high-pressure regimes (25.3–105.3 MPa) where preliminary experiments indicated that (a) survival within the experimental time frame of our survival experiments (19–24 h) was visibly affected, and (b) where growth within the experimental time frame (12–22 h) was limited. The lower pressures used here, 25.3 and 35.5 MPa, correspond to the in situ pressure of most hydrothermal vents, between 2,500 and 3,500 m depth (Kelley et al. 2002); *Pyrococcus* strain GB-D and *Thermococcus fumicolans* were isolated from 2,000 m depth. Significantly higher pressures (86.1–105.3 MPa) were used to test the ability of both strains to tolerate extreme hydrostatic pressures in the deep subsurface. The experimental pH values for growth and survival (4.5, 5.0, 5.5, 7.5; see Tables 1, 2) represent pH values within the range generated by seawater in-mixing into acidic (pH 3–4) endmember fluid over a wide range of mixing ratios (McCollom and Shock 1997). The temperature regimes (90°C and 100°C) covered the range from near-optimal growth temperatures (90°C) to near the upper limit (100°C) for both strains. Sulfide concentrations for growth and survival experiments (0–80 and 0–95 mM, respectively; see Tables 3, 4) included and exceeded the range of sulfide concentrations measured in endmember fluids of most hydrothermal vents (Von Damm 1990; Von Damm 1995; Ding et al. 2001). For growth experiments under elevated sulfide concentrations, only pH 7 could be used because sulfide concentrations would not remain stable at acidic pH (Table 4).

Growth experiments under pressure

For growth experiments, freshly grown cells in exponential growth phase were diluted to ca. 1×10^6 cells/ml into Hungate tubes (Bellco Glass, Vineland, NJ) containing growth media at specific pH and sulfide concentration. Preliminary experiments demonstrated that growth curves for both organisms generated from cultures inoculated with cells pre-grown at atmospheric pressure or at 25.3 MPa were consistent. The pH of all media was adjusted preceding and checked following incubations using an InLab 412 sulfide-resistant pH electrode (Mettler-Toledo, Schwerzenbach,

Table 1 pH tolerance for survival of *Thermococcus fumicolans* and *Pyrococcus* strain GB-D under different temperature and pressure regimes

pH	1 atm/90°C [a]	90°C/86.1 (MPa)				90°C/98.3 (MPa)				1 atm/100°C [a]	100°C/98.3 (MPa)				100°C/105.3 (MPa)			
	Survival time (h)																	
	24	4.5	9.5	16	24	4.5	9.5	16	24	24	4.5	9.5	16	24	4.5	9.5	16	24
T. fumicolans																		
7.5	6	6	6	6	5	6	3	3	0	0	0	0	0	0	0	0	0	0
5.5	6	6	6	6	5	3	0	0	0	0	0	0	0	0	0	0	0	0
5.0	6	6	6	5	6	3	0	0	0	0	0	0	0	0	0	0	0	0
4.5	0	6	5	6	4	3	0	0	0	0	0	0	0	0	0	0	0	0
Pyrococcus GB-D																		
7.5	6	6	6	6	3	–	–	–	–	6	6	5	0	0	–	–	–	–
5.5	6[b]	6[b]	4[b]	3[b]	2[b]	–	–	–	–	6	5	0	0	0	–	–	–	–
5.0	6	6	3	2	1	5	5	2	2	6	3	0	0	0	5	0	0	0
4.5	6[b]	5[b]	4[b]	2[b]	0[b]	3	0	0	0	6	0	0	0	0	5	0	0	0

pH tolerance for survival of *Thermococcus fumicolans* and *Pyrococcus* strain GB-D under different temperature and pressure regimes. Survival was evaluated by regrowth of pH-stressed cells in dilution series (steps 1–6). Start concentration is near $1 \times 10 \times 10^6$ cells/ml. After timed exposure to stress conditions in non-growth medium (survival time), cell suspensions were diluted into six-step decimal dilution series in growth medium, and incubated at optimal temperature and 1 atm pressure for at least 5 days. Tubes were checked daily for re-growth, on the basis of visible turbidity and microscopic examination. The symbol "–" indicate tests not performed for that organism

[a] Results previously reported in Lloyd et al. (2005)

[b] Experiments checked by MPN quantification (Supplementary Data Table S3)

Switzerland). Hungate tubes were completely filled with ca. 17 ml media and sealed with butyl rubber septum stoppers (Bellco) that transmitted external hydrostatic pressure to the media-filled interior of each tube. For control experiments at atmospheric pressure, tubes had ca. 7 ml nitrogen headspace. A maximum of nine Hungate tubes were placed into stainless steel pressure incubation vessels 54 cm long and 3.4 cm internal diameter (High Pressure Equipment Co. (HiP); Erie, Pa). Hydrostatic pressure was applied with a hand-operated pressure generator (HiP). Pre-set pressures were maintained using adjustable pressure relief valves (Swagelok, Willoughby, OH). Temperature was controlled using water baths (Precision, Winchester, VA) that accommodated up to 20 l of water and four high-pressure incubators. The pressure vessels heated up to water bath temperature within 1 h. All physical and chemical variables were, repeatedly, checked during experiments. Pressures were measured at the beginning of each experiment, and pressure gauges and relief valves were incorporated to maintain experimental pressures.

Incubation times for growth and survival experiments at pressure

Incubation times were based on laboratory observations of time frames required for sustained cell growth. In previous experiments, *Pyrococcus* strain GB-D exhibited doubling times of ca. 30–60 min, at pH 7.2, at temperatures up to 100°C and at pressures of up to 20.3 MPa (Jannasch et al. 1992). *T. fumicolans* had a specific growth rate of ca. 0.2/h, or a doubling time of 5 h at low pH values (4.5, 5.0, and 5.5), and a specific growth rate of 0.5/h (doubling time of 2 h) at pH 7.5 (Godfroy et al. 1996). In multiple pre-experiments with three replicate growth curves each, we found doubling times averaging 78 min for *Pyrococcus* strain GB-D and 84 min for *T. fumicolans* at pH 7, 90°C and atmospheric pressure. Pre-experiments at pH 5, 90°C and atmospheric pressure demonstrated doubling times of 2–3 h for both strains. Incubation periods above 24 h had to be avoided since cell counts of both strains decreased due to cell lysis after reaching peak density within this time frame. Laboratory incubations ranged from 12 to 22 h. In order to obtain comparable data, survival experiments were conducted within the same timeframe. Survival and growth (along with pH and sulfide concentration as appropriate) were measured four times at evenly spaced intervals during experiments. For each measurement, a separate pressure vessel (containing one replicate tube for each unique variable combination within an experimental series) was depressurized and opened, in order to avoid multiple depressurizations for the remaining samples in the time series. Cell counts for growth experiments

Table 2 Change in cell concentration of *Thermococcus fumicolans* and *Pyrococcus* strain GB-D at different pH, pressure and temperature over 12–21 h

Temperature	Pressure (MPa)	pH	*T. fumicolans* Δ in cells/ml	*P.* strain GB-D Δ in cells/ml
90°C	0.1[a]	7.5	++[c]	++[c]
	0.1[a]	7.5	++	++
	0.1[a]	5.5	++	++
	0.1[a]	5.0	++	++
	0.1[a]	5.0	++	++
	0.1[a]	4.5	+	++
	25.3	7.5	++	++
	35.5	7.5	++	++
	86.1	7.5	ND	++
	86.1	7.5	++	–
	86.1	5.0	++	++
	86.1[b]	4.5	+	+
	98.3[b]	7.5	+	+
	98.3[b]	5.5	+	+
	98.3[b]	5.0	–	–
	98.3[b]	4.5	ND	–
	98.3[b]	4.5	–	–
100°C	0.1[a]	7.5	++	++
	0.1[a]	7.5	++	++
	0.1[a]	5.5	+	++
	0.1[a]	5.0	++	++
	0.1[a]	5.0	++	++
	0.1[a]	4.5	+	–
	0.1[a]	4.5	++[b]	+
	25.3	7.5	Total lysis	++
	35.5	7.5	Total lysis	++
	98.3	7.5	++	++
	98.3	7.5	+	++
	98.3	5.0	++	ND
	98.3	5.0	++	++
	98.3[b]	4.5	–	–
	105.3[b]	7.5	–	–
	105.3[b]	7.5	–	Total lysis
	105.3	5.5	–	ND
	105.3[b]	5.5	–	–
	105.3[b]	5.0	–	–
	105.3[b]	4.5	–	–

Effect of low pH on growth of *Thermococcus fumicolans* and *Pyrococcus* strain GB-D under different temperature and pressure regimes. Freshly grown cells were diluted to between 10×10^5 and 10×10^6 cells/ml into Hungate tubes containing growth media. Incubation continued for a minimum of 12 h, and for 21 h in cases where significant growth was not observed after 12 h. Cell numbers increased by more or equal to one order of magnitude (++), less than one order of magnitude (+), or remained below one doubling (–). When available, results for replicate experiments are reported. Incubations continued for a minimum of 12 h, and for 21 h in cases where significant growth was not observed after 12 h. See full data Table S1 in Supplementary Materials for more detail

[a] Experiments previously reported in Lloyd et al. (2005) and provided here for comparison. In some cases results are reported for replicate experiments

[b] AO counts of final cell concentrations difficult to interpret due to partial cell lysis (total cell lysis noted, and confirmed by >3 experiments each)

[c] Result of this experiment confirmed by >10 replicate experiments

were determined using AODC staining under epifluorescence (Hobbie et al. 1977), and direct counts under phase contrast in a Petroff–Hauser chamber. In a separate study, temperature, pH, and sulfide growth experiments were also conducted at atmospheric pressure (Lloyd et al. 2005).

Survival experiments at pressure under selected temperatures, sulfide concentrations and pH values

Anaerobic media for survival experiments (without electron donors and acceptors and organic growth substrates) were prepared and dispensed into Hungate

Table 3 Sulfide tolerance for survival of *Thermococcus fumicolans* and *Pyrococcus*strain GB-D under different temperature, pressure and pH regimes

Sulfide concentration (mM)	pH 5/90°C/86.1 (MPa)				pH 5/100°C/98.3 (MPa)				pH 7/90°C/86.1 (MPa)				pH 7/100°C/98.3 (MPa)			
	Survival time (h)															
	3	6	10	18	3	6	10	18	3	6	10	18	3	6	10	18
T. fumicolans																
0	6	6	6	6	0	0	0	0	6	6	6	6	0	0	0	0
5	6	6	6	6	0	0	0	0	6	6	6	6	5	6	5	6
10	6	5	4	1	0	0	0	0	6	6	6	6	/	–	–	–
25	1	0	0	0	0	0	0	0	–	–	–	–	–	–	–	–
30	–	–	–	–	0	0	0	0	6	6	6	6	–	–	–	–
40	–	–	–	–	0	0	0	0	4	5	5	4	–	–	–	–
50	–	–	–	–	–	–	–	–	5	3	3	3	6	6	6	6
60	–	–	–	–	–	–	–	–	–	–	–	–	5	6	6	6
70	–	–	–	–	–	–	–	–	0	0	0	0	6	6	6	5
80	–	–	–	–	–	–	–	–	0	0	0	0	–	–	–	–
95	–	–	–	–	–	–	–	–	0	0	0	0	4	0	0	0
Pyrococcus sp.																
0	6	6	5	6	5	1	0	0	6	6	6	6	6	5	4	3
5	6	6	6	6	–	–	–	–	6	6	6	6	6	5	4	3
15	–	–	–	–	0	0	0	0	6	6	6	6	6	5	4	2
20	3	1	1	0	–	–	–	–	–	–	–	–	4	2	0	2
25	–	–	–	–	–	–	–	–	–	–	–	–	5	3	0	0
35	2	1	0	0	0	0	0	0	6	6	6	6	–	–	–	–
40	–	–	–	–	–	–	–	–	6	5	4	5	4[a]	2[a]	0[a]	0[a]
60	–	–	–	–	–	–	–	–	6[a]	5[a]	4[a]	5[a]	2	1	0	0
85	–	–	–	–	–	–	–	–	5	4	3	3	1	0	0	0

Sulfide tolerance of *Thermococcus fumicolans* and *Pyrococcus* strain GB-D under different temperature, pressure and pH regimes. Start concentration is near $1 \times 10 \times 10^6$ cells/ml. After exposure to stress conditions in non-growth media, cell suspensions were diluted into six-step decimal dilution series in growth medium, and incubated at optimal temperature and 1 atm pressure for at least 5 days. Tubes were checked daily for re-growth, 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 re-growth after each survival experiment. The symbol "–" indicate tests not performed for that organism

[a] Experiments checked by MPN quantification (Supplementary Data Table S3)

tubes in the same way as for growth experiments described above. Inoculum volume was limited to 1% of survival media (final concentration 1×10^6 cells/ml) to prevent excess carbon carryover. Survival was assessed at pH 4.5, 5.0, 5.5 and 7.5 using freshly grown exponential phase cells of all test strains at 86.1 and 98.3 MPa at 90°C, and 98.3 and 105.3 MPa at 100°C. Tubes were incubated under pressure for 24 h. At 4.5, 9.5, 16 and 24 h, a separate pressure vessel (containing replicate experimental tubes) was depressurized and opened, in order to avoid multiple depressurizations for the remaining samples in the time series. For our survival experiments, AODC staining could not be used to distinguish dead cells from living cells, a limitation of the AO technique. Therefore, survival was assessed at each time point using a 6-step decimal dilution series method. After exposure to stress conditions, cell suspensions were diluted into six-step decimal dilution series in growth medium (half strength Marine Broth 2216 supplemented with elemental sulfur; pH 7.5) as described previously (Lloyd et al. 2005), and incubated at optimal temperature and 0.1 kPa pressure for at least 5 days. Tubes were checked daily for re-growth, on the basis of visible turbidity and microscopic examination. Survival is tabulated as the highest decimal dilution step (out of six steps in each dilution series) that produced re-growth (Tables 1, 3). The dilution steps indicate statistically unsupported order-of-magnitude estimates for surviving cell concentrations: Step 1: 1–10 cells/ml; step 2: 10^1–10^2 cells/ml; step 3: 10^2–10^3 cells/ml; step 4: 10^3–10^4 cells/ml; step 5: 10^4–10^5 cells/ml; step 6: 10^5–10^6 ells/ml.

For sulfide experiments, freshly grown cells were exposed to sulfide (H_2S, $HS^-$and S^{2-}) at concentrations from 0 up to 95 mM, at pH 5 and pH 7, and at 90 and 100°C. Previous experiments had demonstrated that the two strains survived in the presence of very high (60 mM) sulfide at atmospheric pressure and 90°C (Lloyd et al. 2005). Additional survival experiments in

Table 4 Change in cell abundance of *Thermococcus fumicolans* and *Pyrococcus* strain GB-D at pH 7.0, under different pressure, temperature and sulfide concentrations over 18–24 h

Temperature	Pressure (MPa)	Sulfide (mM)	T. fumicolans Δ in cell abundance	*P.* strain GB-D Δ in cell abundance
90°C	0.1	0.4[b]	++	++
	0.1	10	+	++
	0.1	20	+	+
	0.1	30	+	+
	0.1	40[a]	+	–
	0.1	60[ba]	–	–
	0.1	80[a]	total lysis	–
	86.1	0.4[b]	++	++
	86.1	10	++	+
	86.1	10	+	+
	86.1	20	++	++
	86.1	20	+	+
	86.1	30	+	+
	86.1	30	++	+
	86.1	40	+	+
	86.1	40	++	++
	86.1	60	+	++
	86.1	60	+	++
	86.1	80	–	ND
	86.1	80[a]	+	+
100°C	0.1	0.4[b]	++	++
	0.1	10	+	+
	0.1	20	+	+
	0.1	30	+	++
	0.1	40	+	–
	0.1	60[a]	–	–
	0.1	80	–	–
	98.3	0.4	–	ND
	98.3	0.4	+	++
	98.3	10	+	+
	98.3	10	+	+
	98.3	20	+	+
	98.3	20	+	+
	98.3	30	+	++
	98.3	30	+	+
	98.3	40	++	++
	98.3	40	++	+
	98.3	60[a]	+	++
	98.3	60[a]	+	+
	98.3	80[a]	+	ND
	98.3	80	Total lysis	ND

Effect of high sulfide concentrations on growth of *Thermococcus fumicolans* and *Pyrococcus* strain GB-D at pH 7.0, under different temperature and pressure regimes. Freshly grown cells were diluted to between 10×10^5 and 10×10^6 cells/ml into Hungate tubes containing growth media. Incubation times were between 18 and 24 h. Cell numbers increased by more or equal to one order of magnitude (++), less than one order of magnitude (+), or remained below one doubling (–). When available, results for replicate experiments are reported. See full data Table S2 in Supplementary Materials for more detail

ND no data

[a] AO counts of final cell concentrations difficult to interpret due to partial cell lysis (total cell lysis noted, and confirmed by >3 experiments each)

[b] Result of this experiment confirmed by >10 replicate experiments

this study were conducted at high pressure (86.1 and 98.3 kPa). For pH 5, gassing with H_2S lowered the pH of this acetate-buffered medium to the target pH. Inoculation, timed incubations at high pressure, and determination of survival by re-growth in decimal dilution series were performed as described above. Stable sulfide concentrations during the survival experiments were confirmed at the start and at the end of the incubation period.

Under high temperature, the pH of neutrality (equal concentrations of protons and hydroxy ions) changes from 7.0 at 25°C to 6.3 at 100°C (Whitfield 1975).

Therefore, acidity effects are measured within a pH range of 5.5–4; the near-neutral pH ranges (6 or 6.5) are omitted. For reference purposes, pH 7 is retained in this study as a baseline parameter to ensure comparability to previously published studies; most published work on pH tolerance of hyperthermophilic archaea is based on growth media buffered to pH 7 or 7.2 at room temperature (Jannasch et al. 1992; Canganella et al. 1997).

Most probable number (MPN) counts and reproducibility of experiments

In order to evaluate the results of dilution series with a quantitative method, six survival experiments with *Pyrococcus* strain GB-D were checked with triplicate MPN counts (American Public Health Association, 1969): pH 4.5, 90°C, 0.1 and 86.1 MPa; pH 5.5, 90°C, 0.1 and 86.1 MPa (Table 1; four experiments); and 60 mM sulfide, pH 7, 90°C and 86.1 MPa, and 40 mM sulfide, pH 7, 100°C and 86.1 MPa (Table 3, two experiments).

To test the reproducibility of experiments, each of the abovementioned six survival experiments was performed three times in parallel, by dividing a test culture into three survival experiments that were run in parallel, at the same time under identical conditions. Survival results of the three parallel runs were in each case quantified by MPN separately.

Results

pH tolerance

In the absence of growth nutrients, *Pyrococcus* strain GB-D and *T. fumicolans* survived exposure to low pH values only within narrowing limits of temperature and pressure. In contrast to broad pH tolerance at atmospheric pressure and 90°C (Lloyd et al. 2005), at 86.1 MPa and 90°C *Pyrococcus* strain GB-D lost viability at all pH values, in particular at lower pH values (5.5 and lower) (Table 1). These trends are also substantiated by MPN counts (see Table S3, Supplemental data). Higher temperature and pressure (98.3 MPa, 100°C) led to complete die-off within the incubation period (Table 1). In brief, under increasingly severe conditions (higher pressure coupled with 100°C and decreasing pH), *Pyrococcus* strain GB-D survival decreased rapidly within the 24 h experimental timeframe.

A similar pattern was observed for survival of *T. fumicolans*. In the absence of growth nutrients at higher pressures (86.1 and 98.3 MPa, 90°C), *T. fumicolans* cells under pH 7.5 survived better than cells at pH 4.5, 5.0 and 5.5 (Table 1). *T. fumicolans* appeared to be more sensitive to temperature stress than *Pyrococcus* strain GB-D; *T. fumicolans* did not survive exposure to 100°C under any of the conditions and time frames tested (Table 1).

In the presence of carbon and energy sources in growth media, both strains frequently tolerated harsher conditions that led to quick cell death in survival media. Under increased pressure in growth media (86.1 MPa, 90°C), *T. fumicolans* and *Pyrococcus* strain GB-D grew well (Table 2). Good growth under these conditions contrasted to significantly declining survival over a 24 h time span in the absence of growth nutrients at all pHs, particularly for *Pyrococcus* strain GB-D (Table 1). More severe conditions (98.3 MPa/90°C and 98.3 MPa/100°C) are close to the limits for growth of *T. fumicolans* and *Pyrococcus* strain GB-D. At 98.3 MPa, growth was limited to pH 5.5 and 7.5, in both cases less than one order magnitude increase in cell abundance over 20 h. Under the most severe conditions (105.3 MPa/100°C) neither organism grew at all (Table 2), consistent with rapid die-off at 100°C and/or 105.3 MPa pressure (Table 1).

The sensitivity of both strains to very high pressures (above 81.0 MPa) required a control experiment to check their sensitivity at pressures that are characteristic for their hydrothermal vent habitat (25.3 and 35.5 MPa). At 25.3 and 35.5 MPa and 90°C both organisms grew by more than an order of magnitude increase in cell abundance. Only *Pyrococcus* strain GB-D was able to grow at 100°C at 25.3 and 35.5 MPa (Table 2).

Sulfide tolerance

Increasing sulfide concentrations decreased survival for both test strains. For example, at pH 5, *Pyrococcus* strain GB-D did not tolerate sulfide concentrations above 5 mM over the 18 h experiment (Table 3). Survival experiments at 100°C, 98.3 MPa and pH 5 in the presence of increasing sulfide concentrations (Table 3) showed no significant difference compared to non-sulfide experiments (Table 1); in both cases, rapid die-off was observed for both strains.

At pH 7, *Pyrococcus* strain GB-D and *T. fumicolans* showed significantly increased sulfide tolerance compared to pH 5. At pH 7, both organisms showed good sulfide tolerance at 86.1 MPa and 90°C (30 mM sulfide for *T. fumicolans* and 35 mM sulfide for *Pyrococcus*

strain GB-D) (Table 3). Increasing temperature and pressure (100°C and 98.3 MPa) reduced the ability of *Pyrococcus* strain GB-D to survive high sulfide concentrations; the cells quickly lost viability at all sulfide concentrations, and died off completely at 25 mM or higher (Table 3). Interestingly, sulfide appeared to alleviate the high sensitivity of *T. fumicolans* to high temperature and pressure (100°C and 98.3 MPa) (Table 3). *T. fumicolans* did not survive in media without sulfide additions at 100°C, 98.3 kPa, and pH 7.5 (Table 1), but showed good survival at up to 70 mM sulfide under these conditions (Table 3).

Sulfide tests in growth media were performed at pH 7 only (Table 4). Sulfide growth experiments at pH 5 could not be performed due to the formation of precipitates in the growth medium and difficulties in maintaining this pH under high sulfide concentrations. Under growth conditions, both strains grew in the presence of very high sulfide concentrations. *T. fumicolans* tolerated up to 60 mM sulfide at 86.1 MPa/90°C (80 mM sulfide results were conflicting). *Pyrococcus* strain GB-D tolerated up to 80 mM sulfide at 86.1 MPa. At 100°C and high pressure (98.3 MPa), both strains showed similar sulfide tolerances (Table 4).

In comparing sulfide tolerances under growth and survival conditions at pH 7, growing cells often tolerated higher sulfide levels than cells under survival conditions. In the absence of growth nutrients at 90°C and 86.1 MPa, *Pyrococcus* strain GB-D rapidly lost viability at sulfide concentrations of 20 and 35 mM (Table 3), but in growth medium this strain grew at maximally 80 mM sulfide (Table 4). At 100°C and 98.3 MPa, *Pyrococcus* strain GB-D grew in the presence of a wide range of sulfide levels, up to 60 mM (Table 4); under survival conditions, these sulfide concentrations would either kill cells completely within 24 h (25 mM and higher) or allow only a small fraction to survive (20 mM and less) (Table 3). A similar pattern was observed for *T. fumicolans*; at 90°C and 86.1 MPa, cells grew in the presence of up to 60 mM sulfide (Table 4), but survived fully only at max. 30 mM sulfide (Table 3). Under the harshest regimes tested (100°C and 98.3 MPa), growth tolerances of the two test organisms under high sulfide concentrations were quite comparable. Consistent growth was observed at up to 60 mM sulfide (Table 4). In survival media, *T. fumicolans* tolerated up to 60 mM sulfide without apparent loss in viability during the experiment. Under these same conditions *Pyrococcus* strain GB-D showed loss in viability over the duration of the experiment at all sulfide levels, with marked losses in viability above 15 mM sulfide (Table 3).

Most probable number counts and reproducibility

For six experiments (Table S3, Supplemental Data), reproducibility of survival was checked in three parallel pH/temperature/pressure/sulfide incubations, beginning with the same start culture and proceeding in parallel, using the same incubation vessels under the same conditions. These three parallel incubations allowed a measure of reproducibility within each experiment. Cell survival for every incubation was individually quantified using MPN; the results are listed in Table S3. In most cases (21 of 24 experiments), the MPN counts for the three parallel incubations within each experiment are either identical or overlap extensively in their confidence intervals. In three cases, one MPN result is incompatible with the other two. These divergences are limited to experiments near the survival limit, when one MPN gives a result of zero surviving cells, and the other two MPN series indicate low-level survival of 1–10 or 10–100 cells/ml. The diverging MPN counts are marked with a ≠ symbol in Table S3. These data show that internal variability within each experiment is noticeable only at very low cell numbers, when fluctuating survival or die-off of small cell numbers impacts the outcome of the experiment.

Direct comparisons of serial dilutions and MPN counts show that in most cases, the serial dilution results remain within the 95% confidence interval of the MPN counts (Table S3). In 20 out of 24 direct comparisons of MPN and serial dilution data, the result of these serial dilution experiments was within the range of the MPN 95% confidence intervals (for at least two of three parallel MPN series). However, discrepancies of one order of magnitude were found in four direct comparisons (marked with asterisks in Table S3) and show that individual measurements in serial dilutions can include errors of one order of magnitude. To avoid misinterpreting serial dilution results for survival, survival trends obtained by serial dilution should be internally consistent over the time period measured, and should be confirmed by multiple measurements within a time series.

Discussion

Growth and survival of hyperthermophilic archaea in the hydrothermal vent environment and the vent subsurface appear to be strongly modulated by the coexisting stress factors that characterize their in situ environment. In addition to temperature and pressure, pH and sulfide concentrations were significant factors.

Carbon substrate and electron acceptor limitation

The test strains *T. fumicolans* and *Pyrococcus* str. GB-D responded differently to key stress factors under growth (with growth nutrients) than under survival (without growth nutrients) conditions. With adequate carbon and energy sources, these archaea grow under environmental regimes that they could not even tolerate for short time periods in survival mode under severe substrate limitation. For example, *Pyrococcus* strain GB-D can grow at 100°C, 98.3 kPa, pH 5 and 7.5 (Table 2), but could not survive as a non-growing population without growth substrates under these same conditions for more than 4.5–9.5 h (Table 1). Experiments with *T. fumicolans*, *Pyrococcus* strain GB-D, the methanogen *Methanocaldococcus jannaschii*, and the sulfate reducer *Archaeoglobus profundus* under 0.1 kPa pressure demonstrated the same trend, decreasing environmental tolerances to a wide range of stress factors under substrate limitation (Lloyd et al. 2005).

These results indicate a constant requirement for substrate and energy sources by hyperthermophilic vent archaea; there is no evidence for resting stages and extended survival under non-growth or starvation conditions. As suggested by experiments with *Escherichia coli* and the moderate piezophile *Photobacterium profundum* (Bartlett 2002), an increase in pressure tolerance for actively growing cells may be accounted for by alterations of membrane lipid and protein composition in order to maintain membrane fluidity under pressure. Growing cells can also invoke pressure-induced protections of the DNA structure and function, as well as high-pressure regulation of gene expression (Bartlett 2002). Without the carbon and energy sources necessary for growth, cells may not be able to perform these adjustments. Specific response mechanisms to temperature and pressure stress aim at providing sufficient substrates. For example, under increasing temperature and pressure stress, *Thermococcus* spp. synthesize and over-express proteases and carbohydrate-degrading enzymes that meet increasing substrate demand (Canganella et al. 1997; Canganella et al. 2000). Analogous considerations also apply to sulfide stress; in this study, cells in substrate-rich growth media showed higher sulfide tolerance, compared to non-growing cells without substrate.

Since the test strains survived hydrothermal vent conditions only with adequate substrate and energy supply, hydrothermal vent environments apparently provide sufficient organic carbon to support the high densities of heterotrophic Thermococcales that have been found repeatedly (Jannasch et al. 1992; Harmsen et al. 1997; Summit and Baross 1998; Holden et al. 1998; Summit and Baross 2001; Takai et al. 2004a, b). Organic compounds from different sources are available in the hydrothermal vent environment, and can supply heterotrophic vent archaea with growth substrates. Complex organic substrates are produced in the microbiologically active water column surrounding hydrothermal vent zones, which is characterized by an unusually high DOC content (Comita et al. 1984). Organic substrates from this source may enter the shallow subsurface by diffusion, or could be pulled into the deep hydrothermal subsurface by seawater entrainment (Von Damm 1995). As a result of entrainment of organics in seawater and of chemoautotrophic microbial growth in-situ, vent chimney polymetal sulfides have a patchy, but sometimes surprisingly high content of organic carbon, in the range of 0.5–3% (w/w) (Wirsen et al. 1993). Also, geothermal reactions within the hot subsurface could provide ammonia and methane as reduction products of CO_2 and N_2, and low-molecular-weight, incompletely reduced species (organic acids) that exist in metastable equilibrium with their source compounds (Shock 1990).

While most described species of the Thermococcales are obligate heterotrophs, a new *Thermococcus* strain isolated from the East Pacific Rise (13°N) was capable of lithotrophic growth on CO, producing H_2 and CO_2; yeast extract in small amounts was an essential supplement for growth (Sokolova et al. 2004). Thus, hydrothermally produced CO has to be taken into account as a potential carbon source for at least some members of the Thermococcales. The ability to utilize a variety of growth substrates may equip a population of hyperthermophiles with the energy necessary to withstand subsurface stresses.

pH tolerance

For all published *Thermococcus* and *Pyrococcus* species, the published optimal growth pH is in the range of pH 6–8, for media buffered at atmospheric pressure and room temperature. The range of pH for growth is typically between 4.5 and 9, with pH extremes between 3.5 and 10 for a few specialized species (Canganella et al. 1997; Zillig and Reysenbach 2001). This study has shown that, with increasingly severe temperature and pressure stress, the pH range for growth and survival of these hyperthermophiles narrows towards near-neutral pH (Table 1). A moderately acidic or near-neutral pH (at least pH 5.5) is required for survival and growth range of *Pyrococcus* strain GB-D and *T. fumicolans* over a wide range of temperature, pressure, and sulfide

concentrations (Tables 1, 2, 3). A moderate pH requires mixing of acidic vent fluids (pH around 4.0) with entrained seawater in the vent chimney matrix (McCollom and Shock 1997), or in the subsurface underneath hydrothermal vents (Von Damm 1995). This mixing of vent fluid and seawater is also necessary to lower the fluid temperature into an acceptable range for microorganisms. Incremental mixing models of seawater and hydrothermal endmember fluid, using the 21°N East Pacific Rise fluids as a model component, show that in the relevant temperature range near 100°C, the pH is between 5.5 and 6 (McCollom and Shock 1997).

At low pH organisms must expend energy in order to maintain their near-neutral internal pH using various proton transporters or by maintaining a proton-impermeable membrane. Under starvation conditions there may be insufficient energy for maintenance of near-neutral internal pH (Albers et al. 2001) if combined stress factors exceed some threshold unique for each organism (Table 1). Marquis (1982) observed that when growing under pressure, the ATPases of a strain of the bacterium *Streptococcus faecalis* became inefficient at transporting protons out of the cell, resulting in increased sensitivity to acidic conditions and reduced growth. Similar physiological limits most likely apply to the hyperthermophilic archaea *P.* strain GB-D and *T. fumicolans*.

Sulfide tolerance

This study has shown that sulfide is tolerated in very high concentrations by both model organisms under vent-typical temperatures and pressure. Both test strains survived exposure to sulfide concentrations typical for hydrothermal vent endmember fluids (5–10 mM) at 90°C/86.1 MPa (Tables 1, 3). Generally, sulfide occurs in the order of magnitude of 1–10 mM in hydrothermal vent endmember fluids (Von Damm 1995; Von Damm 1990); maximal concentrations of 17 mM have been observed during continuous monitoring of hydrothermal vents (Ding et al. 2001). Sulfide up to 60 mM is tolerated during growth under high pressure (86.1 and 98.3 MPa); 80 mM appears to be the limit where growth stops or becomes unreliable (Table 4). However, growth experiments were performed at a starting pH of 7, since pH 5 was unstable during growth experiments. The high sulfide tolerances observed here are likely moderated for growth at lower pH. All survival tests indicated that both strains are more sensitive to sulfide at pH 5 than at pH 7. This effect may be due to the enhanced sulfide exposure at pH 5; with decreasing pH, the predominant form of sulfide shifts from HS^- to H_2S, and the uncharged H_2S form has a higher membrane permeability relative to HS^-.

In some cases sulfide appears to be an essential reagent for cell survival under high pressure and high temperature regimes. An interesting example is the ability of *T. fumicolans* to survive at pH 7, 100°C, and 98.3 MPa in the presence of up to 50 mM sulfide without any detectable decrease in cell viability (Table 3). This temperature and pressure regime cannot be tolerated without sulfide additions, as seen by zero survival in the pH 7 baseline experiments with no sulfide added (Table 3) and in standard survival media at pH 7.5 (Table 1). At present, the mechanism for sulfide-dependent, increased pressure and temperature tolerance at 98.3 MPa/100°C remains unidentified. We suggest a speculative, but feasible explanation for this observation here. At high temperatures, highly toxic Fenton-type chemical reactions could be occurring either intracellularly or extracellularly, causing toxicity. Fenton reactions involve the catalytic production of the reactive oxygen radical $OH^{\cdot}$ from H_2O_2 and have been demonstrated to be highly detrimental to cellular organic material (Ambroz et al. 2001). Increases in temperature can greatly increase Fenton chemistry reaction kinetics (Lee et al. 2003). The presence of a small amount of sulfide could alleviate these toxic effects by reducing the free extra- or intra-cellular concentration of the two alternate Fenton reactants copper and iron (Edgcomb et al. 2004), and by reducing the redox potential of the medium significantly, making H_2O_2 scarcer. This quenching of Fenton chemistry by sulfide would be less effective at pH < 5 where sulfide acid–base chemistry dictates much higher concentrations of H_2S relative to bisulfide (HS^-) (Millero et al. 1988). Differences in intracellular enzymes (either as expression levels or absence of enzymes from the genome) that counteract oxidative stress, such as superoxide dismutases (Silva et al. 1999; Ursby et al. 1999), could explain the variations in survival of *Thermococcus* and *Pyrococcus* GB-D.

Pressure tolerance

The results of this study and earlier work with diverse microorganisms indicate that some bacteria and archaea are surprisingly pressure-tolerant if other stress parameters remain in the non-critical range. The upper pressure limits for growth of hyperthermophiles found in this study (maximum pressure 98.3 MPa) are high above the in situ pressure at hydrothermal vents, but similar to maximal growth pressures for other hyperthermophiles. For example, the heterotrophic

sulfur-reducing strains ES1 and ES4 grow at up to 89.1 and 66.9 MPa, respectively (Pledger et al. 1994). The hyperthermophilic methanogen common to hydrothermal vents, *Methanocaldococcus jannaschii*, grew faster when incubation pressure was increased to 75.0 MPa (Miller et al. 1988). However, with higher pressure, the temperature range permitting growth narrows significantly, and higher and lower temperature ranges that are permissible under atmospheric or under vent in situ pressure are no longer compatible with growth (Pledger et al. 1994). Such observations apply to hyperthermophiles (strains ES1 and ES4; Pledger et al. 1994), but also to mesophilic bacteria. Some barotolerant bacteria can grow under extremely high pressure, above the in-situ range of hydrothermal vents (ca. 40.5 MPa), but growth close to the pressure limit is accompanied by a narrowing of the growth temperature spectrum (Marquis 1982; Trent and Yayanos 1985).

Implications for deep subsurface microbiology

High pressure tolerance above the in situ pressure of hydrothermal vents would allow metabolically active hyperthermophiles to inhabit not only the vent environment at the deep-sea bottom and on mid-ocean ridges, but also the hydrothermal vent subsurface and the ocean crust, towards the depth range of the heat source below mid-ocean ridges. In the case of the fast-spreading East Pacific Rise, the magmatic heat source is located at a depth of ca 1.6–2.4 km below the sea bottom at ca. 2500 m depth; in the case of the slow-spreading Mid-Atlantic Ridge, the top of the geothermal heat source is located at ca. 3–3.5 km below the Rift Valley floor at 3,500 m water depth (Alt 1995). Not pressure per se, but temperature and permeability constraints are most likely limiting the depth range of the vent subsurface biosphere; low-permeability dikes overlying the heat source possibly limit the microbially accessible habitat to the upper few hundred meters of permeable volcanic basalts in the vent subsurface (Alt 1995).

For a precise location of vent subsurface microbiota, indirect observations, for example of nucleic acids in extremely hot endmember vent fluids (Deming and Baross 1993) and archaeal DNA in warm vent waters produced by subsurface mixing (Holden et al. 1998; Summit and Baross 2001; Huber et al. 2002) have to be substantiated by deep subsurface drilling and sampling to show that hyperthermophilic archaea are indeed present and active in the hydrothermal vent subsurface. Ribosomal RNA gene analysis alone is not sufficient, since it does not rule out fossil DNA and inactive remnant populations (Inagaki et al. 2001, Kormas et al. 2003). The few existing field surveys indicate that prokaryotic populations decrease with depth below hydrothermal vents. Currently, observed depth ranges of hydrothermal vent archaea in the subsurface are largely based on direct microbiological sampling of the vent subsurface biosphere at sedimented sites. For example, direct cell counts (AODC) in hydrothermally heated sediments on a sedimented mid-ocean ridge (Middle Valley, Juan de Fuca Ridge) indicated a dramatic decrease of microbial cell numbers towards hot sediment layers over a vertical range of tens of meters, complicated by considerable site-to-site variation in temperature gradients, sediment composition, and flow patterns (Cragg et al. 2000). In general, cell numbers decreased towards quantification limits in sediment layers with in-situ temperatures near and above 100°C (Cragg et al. 2000). The hydrothermal rock subsurface underneath the TAG hydrothermal field on the Mid-Atlantic Ridge harbored microbial cells at max. 50 m depth; cells were detected with DAPI in a few subsurface samples only, suggesting a very spotty distribution pattern (Reysenbach et al. 1998). These datasets support the existence of at least a shallow (<100 mbsf) subsurface hydrothermal habitat for extremophilic archaea. Thus, the growth requirements of heterotrophic, hyperthermophilic archaea could most likely to be met in the shallow subsurface at hydrothermal vents, where sufficient organic carbon input of water column origin, and moderate pH and temperature as a consequence of seawater mixing, are most likely to create favorable and diversified niches for hyperthermophiles.

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