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Effects of Hyperbaric Pressure on a Deep-Sea Archaebacterium in Stainless Steel and Glass-Lined Vessels

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The effects of hyperbaric helium pressures on the growth and metabolism of the deep-sea isolate ES4 were investigated. In a stainless steel reactor, cell growth was completely inhibited but metabolic gas production was observed. From 85 to 100°C, CO_2 production proceeded two to three times faster at 500 atm (1 atm = 101.29 kPa) than at 8 atm. At 105°C, no CO_2 was produced until the pressure was increased to 500 atm. Hydrogen and H_2S were also produced biotically but were not quantifiable at pressures above 8 atm because of the high concentration of helium. In a glass-lined vessel, growth occurred but the growth rate was not accelerated by pressure. In most cases at temperatures below 100°C, the growth rate was lower at elevated pressures; at 100°C, the growth rates at 8, 250, and 500 atm were nearly identical. Unlike in the stainless steel vessel, CO_2 production was exponential during growth and continued for only a short time after growth. In addition, relatively little H_2 was produced in the glass-lined vessel, and there was no growth or gas production at 105°C at any pressure. The behavior of ES4 as a function of temperature and pressure was thus very sensitive to the experimental conditions.

As pointed out by Zobell and Oppenheimer (34), observations of hydrostatic pressure influencing biological activity date back to the late 1800s. Increased pressure can induce changes in the activity of enzymes (12, 19, 21) as well as alter the production of proteins (13), the regulation of genes (3, 16), and the composition of lipids (10, 32) in bacteria. Some psychrophilic bacteria from the cold deep sea are obligate barophiles and thus require elevated pressure for growth (33). Lately, considerable attention has focused on the role of pressure in high-temperature environments. Renewed interest in pressure effects on life at high temperatures has been triggered in large part by the discovery of submarine hydrothermal vents (9). High pressure has recently been shown to increase the growth rates of some thermophilic microorganisms (4, 11, 20, 24) and to extend the upper temperature limit for metabolism (20). On the other hand, elevated pressure (specifically, the in situ pressure of 250 atm [1 atm = 101.29 kPa]) reduced the growth rates of two extremely thermophilic *Desulfurococcus* strains obtained from deep-vent sites (14), and a pressure of 750 atm inhibited growth of microorganisms from deep-sea (7,000 m) water and sediment samples (25).

In the present study, we investigated the effects of hyperbaric pressure on the growth rate and metabolism of the hyperthermophilic archaebacterium, strain designate ES4. Strain ES4 was isolated from flange fragments collected at a vent site and has been grown at temperatures up to 110°C (23). Reported here is a comparison of growth studies done at pressures up to 500 atm in a stainless steel vessel with and without a glass lining. The results indicate that the behavior of ES4, specifically, its ability to grow and its response to elevated hyperbaric pressure, depends dramatically on its environment.

MATERIALS AND METHODS

A pure culture of ES4 was obtained from Robert M. Kelly (Johns Hopkins University, Baltimore, Md.). The medium consisted of artificial seawater (described elsewhere [7]) with the addition of 30 g of elemental sulfur per liter (rhombic, unsterilized [31]), 1 g of yeast extract (YE) per liter, 5 g of Bacto Tryptone per liter, and 10 ml of 5% (wt/vol) $Na_2S \cdot 9H_2S$ per liter. Before adding the phosphorus, nitrogen, and sulfide sources, we sterilized the medium by autoclaving for 20 min at 125°C. Atomic absorption spectrometry analysis of a single medium sample gave the following metal concentrations: Fe, 7.7 μM; Co, 3.9 μM; Ni, 4.8 μM; Mn, 2.5 μM; and Cr, 1.1 μM. Medium was preheated to 80°C before inoculation.

High-pressure reactor and sampling system. ES4 was grown at hyperbaric pressures in a high-pressure-temperature reactor described elsewhere (20) with the following modifications. Three pneumatically actuated valves (HIPCO type; High Pressure Equipment Co., Erie, Pa.) were installed in the gas sampling line to facilitate automated sampling of the gas phase. The compressed air supplied to these valves was controlled by electronically actuated solenoid valves (Red Hat; Automatic Switch Co., Florham Park, N.J.). These valves were controlled by an HP 3393A integrater through an HP 19405A sample/event control module (Hewlett-Packard, Palo Alto, Calif.). The integrater was programmed to take samples at any desired interval with less than a 0.75% drop in total system pressure at 500 atm. The pressure drop was about 2.5% with each sample at 8 atm. The sample loop had an internal volume of 0.20 cm^3 for pressures up to 10 atm; for higher pressures, a sample loop volume of 0.03 cm^3 was used. The larger sample volume at lower pressures was necessary to purge all lines. At each sampling time, two samples were taken: the first to purge the sample lines, and the second for analysis. The lines were evacuated with a vacuum pump between all samples. For pressures above 10 atm, a 20-cm^3 reservoir was included between the gas chromatograph and the reactor to allow decompression of the gas sample before it entered the chromatograph.

A back-pressure regulator maintained the gas sample pressure at 120 kPa while the temperature was constant at 100°C. Helium was used to pressurize the system, and the

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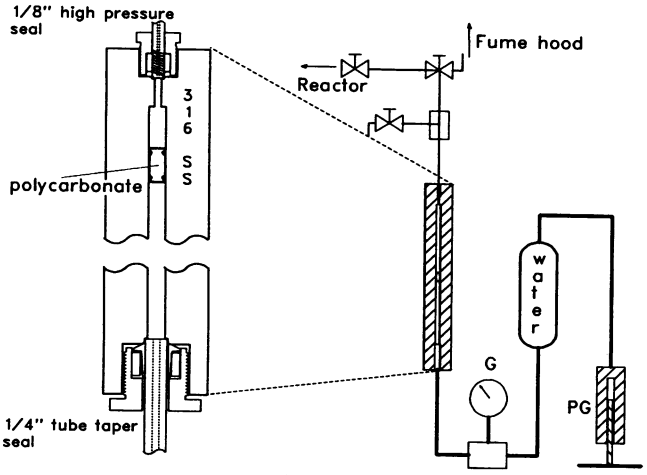

FIG. 1. Schematic diagram of liquid-sampling system with expanded view of high-pressure syringe. PG, pressure generator; G, pressure gauge; SS, stainless steel.

concentrations of CO_2 and H_2S were measured with an HP 3390 gas chromatograph. The peak area for CO_2 was proportional to its mole fraction in the gas phase and was used to determine the total amount of CO_2 produced. With automated sampling, it was possible to obtain precise, reproducible sample volumes and achieve around-the-clock gas sampling.

The final modification was the addition of a liquid sampling device that enables slow decompression of liquid samples before their removal from the system. A schematic of this apparatus is shown in Fig. 1. A high-pressure syringe was constructed of 316 stainless steel. The internal diameter is 0.64 cm and the length is 8.0 cm, providing an internal volume of 2.5 ml. A piston constructed from polycarbonate, equipped with two Viton O rings, separates the fermentation broth from the hydraulic fluid. The syringe has a maximum working pressure in excess of 1,000 atm. A minimum pressure drop of 100 lb/in^2 across the piston is required for movement. Also constructed was a second high-pressure syringe that includes polycarbonate windows for direct viewing of the sample during decompression. The maximum working pressure of this system is only 250 atm, however, and the internal volume is only 1 ml.

The high-pressure syringe can be prepressurized to a pressure equal to that in the reactor. The valve is then opened and about 1 ml of liquid is withdrawn by slowly backing out the pressure generator. This procedure allows sample removal with a near-zero pressure drop and very low shear. The valve to the pressure vessel is then closed, and the pressure of the syringe is slowly reduced. A 32-ml reservoir of water between the syringe and the pressure generator provides ballast to reduce the rate of decompression per turn of the pressure generator. As with gas sampling, two samples were removed at each sampling time to purge liquid lines. In these experiments, water was used as the pressurizing fluid for the sampling system; however, by choosing a fluid with a higher compressibility, or by increasing the ballast volume, the decompression rate per turn could be further reduced. Vertical alignment of the syringe allows venting of gas generated upon decompression without loss of sample. At 250 atm and 95°C, a 10-min decompression time was sufficient to increase the apparent cell density by 10-fold compared with samples removed directly.

Determination of cell numbers. To quantitate growth, we removed 1-ml liquid samples at a rate of about one per hour during the exponential growth phase. Lag phases varied from a minimum of 1 h to a maximum of 14 h. Growth rates were not measured in the relatively few cases in which the lag phase exceeded 6 h. The liquid samples were fixed in 10% formalin for a minimum of 1 h and stained with 0.5 μg of DAPI (4',6-diamidino-2-phenylindole) per ml for a minimum of 10 min. Samples ranging in volume from 100 μl to 1 ml were then filtered onto 0.22-μm-pore-size isoporous black polycarbonate filters. The filters were mounted on slides, and cell densities were determined by epifluorescence microscopy (Nikon Optiphote). A minimum of 10 fields per slide were counted.

Procedures for stainless steel vessel. Each run was started by transferring 2.0 ml of a cell suspension containing ca. 5×10^8 cells per ml into 100 ml of medium preheated to 80°C. The liquid inlet system was purged with 40 ml of medium, and 60 ml was transferred to the preheated pressure vessel (reactor) preheated to either 85 or 95°C. Within 5 min, the medium was heated to within 1% of the desired temperature. Elemental sulfur (30 g liter of medium; 1.8 g total) was retained in a stainless steel cup that could be lowered into the vessel prior to sealing and evacuation.

For temperatures of 95 and 100°C, gas production rates were measured along an isobar. The reactor was inoculated at 95°C and pressurized with helium. Gas concentrations were then measured for a minimum of 12 h. Following removal of nearly all liquid (ca. 3 ml remained in the cup), 60 ml of freshly inoculated medium was added, and the temperature was increased to 100°C. The pressure dropped 30 to 50% upon removal of the liquid and increased again as new medium was added. Helium was used to restore the pressure to its initial value. Two hours were required for the reactor to reach thermal equilibrium after the 5°C temperature increase; thus, gas measurements taken during the first 2 h at 100°C were disregarded. To check for gas production at 105°C, we measured gas production first at 98 and 102°C (data not shown). During each run, the cell density did not change by more than 10% as determined by epifluorescence microscopy. The reactor was opened, cleaned with water and then 70% ethanol, and loaded with fresh sulfur after a maximum of four runs.

Procedures for glass-lined vessel. To limit exposure of the culture to stainless steel and to facilitate addition and removal of solid sulfur, a removable glass liner was added to the reactor. Liquid was added and removed through a 6-mm glass capillary tube extending about 3 cm above the bottom of the vessel. This clearance prevented the solid sulfur from entering and clogging the liquid-sampling line. The inoculation procedure was as described above except that 100 ml of medium were added to the vessel to provide a greater liquid-sampling capacity. The amount of sulfur was increased accordingly (3.0 g total). After a maximum of four runs, the glass liner was removed and washed with soap, rinsed with water and then 70% ethanol, and dried in an oven before being loaded with fresh sulfur.

RESULTS

Stainless steel vessel. In the stainless steel vessel, metabolic CO_2 production was measured at temperatures of 85, 95, and 100°C at total pressures of 8, 250, and 500 atm (Table 1). At all temperatures between 85 and 100°C, the rate of CO_2 production at 500 atm was significantly higher than the rates of 8 and 250 atm. Figure 2 compares the CO_2 produc-

TABLE 1. Gas production by ES4 in stainless steel vessel

Temp (°C)	8 atm			CO_2 at 250 atm (mmol h^{-1} $liter^{-1}$)	CO_2 at 500 atm (mmol h^{-1} $liter^{-1}$)
	H_2 (area h^{-1} $liter^{-1}$)	H_2S (area h^{-1} $liter^{-1}$)	CO_2[a] (mmol h^{-1} $liter^{-1}$)		
85	5.39	420	0.59	0.50	1.13
95	32.1 ± 1.6	418 ± 202	0.72 ± 0.26	1.10 ± 0.06	1.46 ± 0.21
100	11.3 ± 3.4	314 ± 75	0.48 ± 0.07	0.53 ± 0.05	1.35
105	ND[b]	ND	ND	ND	0.45

[a] Under all conditions CO_2 production continued at a constant rate for at least 10 h. The production rates at 95°C are averages from at least two parallel cultures, as are the rates at 100°C at 8 and 250 atm. At 105°C, CO_2 was not produced above the background rate at either 8 or 250 atm.
[b] ND, not detected.

tion trajectories at 95°C for pressures of 8, 250, and 500 atm. Epifluorescence microscopy showed that cell numbers did not change over the period that gas production was measured. At 105°C, no gas was produced until the pressure was increased to 500 atm.

H_2 and H_2S were also produced at all pressures (see Table 1 for the 8 atm results), but the large amounts of helium at 250 and 500 atm precluded accurate measurement of these gases at these higher pressures. The method of containment of the sulfur greatly influenced relative production of H_2 and H_2S. At 95°C and 8 atm, H_2 was produced exclusively, with no production of H_2S, when the elemental sulfur was completely enclosed by a microporous (0.45-μm pores) membrane (data not shown). The final concentration of CO_2 was also about 10-fold lower in this case.

Glass-lined vessel. In the glass-lined vessel, growth of ES4 was measured at temperatures ranging from 85 to 100°C at pressures of 8, 250, and 500 atm (Fig. 3). In most cases at temperatures of 98°C and below, pressures above 8 atm resulted in slower growth. At 100°C, however, the specific growth rate was essentially identical for all three pressures. Growth was not observed at any pressure at 105°C in our system.

Metabolic CO_2 production was also measured in the glass-lined vessel (Table 2). Carbon dioxide production was exponential during growth, and in contrast to the results in the unlined vessel, CO_2 was produced for only a very short time after cessation of growth. Under most conditions, the specific rate of CO_2 production did not equal the specific growth rate. No metabolic CO_2 production was observed at 105°C at any pressure, nor was there quantifiable H_2 production at any temperature, i.e., H_2 levels were below 0.5 mol% in all cases. Hydrogen sulfide was produced under all conditions, but the production rate was highly variable (e.g., the production rate ranged from 170 to 2,200 area h^{-1} 1 $liter^{-1}$ at 8 atm, where area refers to integrated peak area of the chromatogram).

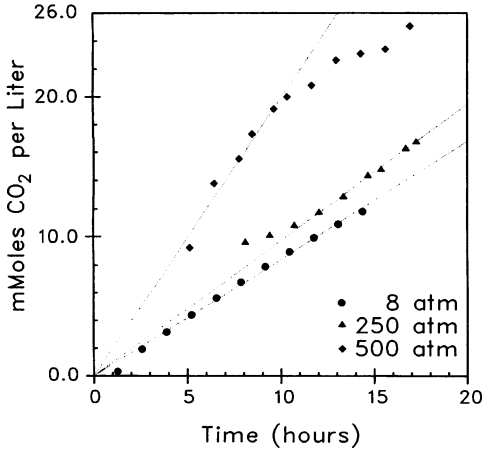

FIG. 2. CO_2 production by ES4 in stainless steel vessel at 95°C and hyperbaric pressures of 8, 250, and 500 atm. CO_2 concentrations are expressed as millimoles of gas produced per liter of medium. The time scales have been adjusted to account for the variable lag phases. Background CO_2 production at 95°C and 8 atm was 0.11 mmol h^{-1} $liter^{-1}$. At higher pressures, background CO_2 production was not detectable during the first 20 h. In each experiment, the cell concentration remained constant at (1 ± 0.1) × 10^7 cells per ml.

DISCUSSION

A significant change in behavior was observed when ES4 was incubated in a stainless steel vessel compared with the same vessel subsequently lined with glass. Although there were no apparent effects of the steel vessel in previous high-pressure experiments with the deep-sea methanogen *Methanococcus jannaschii* (20), stainless steel inhibits the growth of some thermophilic aerobes (28), and corrosion of steel vessels by H_2, sulfide, and seawater is common even at moderate temperatures (31). Potentially inhibitory metal ions that can leach from corroding steel include Fe, Co, Ni,

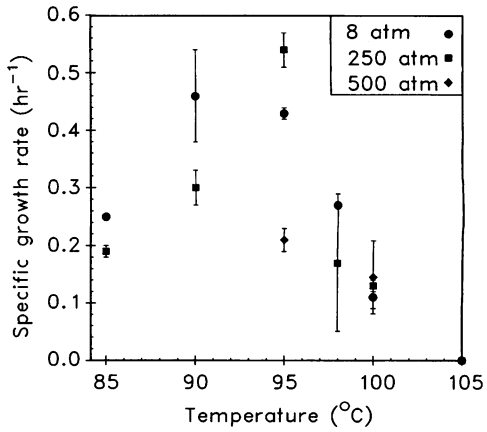

FIG. 3. Specific growth rate of ES4 as a function of temperature at three pressures in the glass-lined vessel. The rates at 85 and 98°C and 8 atm are based on single measurements. All other rates are the averages of at least two separate cultures, with the error bars representing standard deviations. Experiments at 500 atm were done only at 95, 100, and 105°C.

TABLE 2. Specific production rates of CO_2 by ES4 in the glass-lined vessel

Temp (°C)	Specific production rate (h^{-1}) at pressure of[a]:		
	8 atm	250 atm	500 atm
85	0.28	0.23	NP[b]
95	0.29 ± 0.18	0.48 ± 0.17	0.26
100	0.50	0.25 ± 0.11	0.12 ± 0.04
105	ND[c]	ND	ND

[a] Production rates without standard deviations are based on single measurements. All other values are the average of at least two separate runs.
[b] NP, not performed.
[c] ND, not detected.

Mn, and Cr (28). The initial concentrations of these metals in our medium were less than 10 μM. However, calculations based on the composition of 316 stainless steel (15) indicate that a uniform corrosion layer of 0.1 μm over the available surface of the steel vessel could result in Cr and Ni concentrations greater than 300 μM and an Fe concentration well over 1 mM.

Chromium compounds have been shown to inhibit DNA synthesis in activated sludge, resulting in increased generation times and decreased cell division (22). Impaired DNA synthesis is thus one possible mechanism for growth inhibition in the presence of metabolic gas production. Cobalt can also inhibit bacterial growth, as evidenced by growth studies of *Escherichia coli* (1) and the cyanobacteria *Anabaena doliolum* and *Anacystis nidulans* (27).

In a study of hydrostatic pressure effects on the metal tolerance of three deep-sea bacteria, Arcuri and Ehrlich (2) found that in some cases, increasing pressure reduced the toxicity of metal ions and reduced inhibitory effects noted at lower pressures. For one isolate cultured in 10 mg of Co^{2+} per liter, a higher cell yield was obtained at 340 atm than at 1 atm. This result was opposite to the lower cell yields measured at the higher pressure for Co^{2+} concentrations ranging from 0 to 1 mg/liter. There was also evidence that abrupt changes in cell behavior occurred at critical pressures, analogous to the change in CO_2 production exhibited by ES4 as the pressure increased from 250 to 500 atm. It was suggested that pressure induces a structural change in the site of action of the metal ions (2), a hypothesis consistent with our observation. It is worth noting, however, that microorganisms can respond differently to hydrostatic and hyperbaric pressure (6, 17, 29, 30); thus, comparing effects of hyperbaric and strictly hydrostatic pressure may not be appropriate in many cases.

Passivation with nitric acid has been shown to reduce the growth inhibition by stainless steel of some aerobic thermophiles (28). However, with ES4 the inhibitory effect was not reversed by pretreating the steel vessel with HNO_3. The strongly reducing conditions of the medium quickly degraded the metal oxide, as evidenced by pitting of the reactor surface. After the vessel was lined with glass, surface degradation was eliminated.

Carbon dioxide production was faster in the stainless steel vessel under all conditions, and in some cases, the final yield of CO_2 (millimoles of CO_2 produced per liter of medium) was three times higher in the steel vessel than in the glass-lined vessel. In addition, the concentration of H_2 was at least 10-fold greater. (In the glass-lined vessel, some H_2 was detected, but the amount was too small to be accurately measured.) This gas production resulted from metabolism in the absence of increasing cell mass. The ability to sustain metabolism in the absence of increasing biomass can be very important industrially, since the cellular machinery acts as a catalyst with no substrate utilized for reproduction.

Perhaps more remarkable was the increased CO_2 production with increased pressure when ES4 was incubated in the steel vessel. At temperatures from 85 to 100°C, metabolic CO_2 production proceeded two to three times faster when the pressure was increased from 8 to 500 atm. This effect is similar to the enhanced CO_2 production at 750 atm and 3°C observed by Schwarz et al. (25) for deep-sea microorganisms from water and sediment samples. When the same experiments were done in glass, growth occurred and less gas was produced. Moreover, increasing the pressure to 500 atm did not enhance CO_2 production at any temperature.

The availability of sulfur also played a critical role in the metabolism of ES4. Evidence suggests that in the absence of direct bacterial contact, solid sulfur must first be solubilized before it can be metabolized (see, for example, reference 5). In the particular case of the hyperthermophile *Pyrococcus furiosus*, polysulfides formed from elemental sulfur serve as substrates for the production of H_2S (5). In the absence of sulfur, *P. furiosus* produces H_2 and CO_2 but not H_2S (18). In contrast, ES4 required elemental sulfur for the production of CO_2 and either H_2 or H_2S. In the stainless steel reactor, all three gases were produced when sulfur was readily accessible; however, when direct contact with sulfur was prevented, H_2 was produced exclusively of H_2S. Sulfur was always freely exposed in the glass-lined vessel, but hydrogen production was minimal.

The behavior of ES4 at all pressures in the glass-lined vessel was similar to the reported behavior in glass tubes at ambient pressure (23). Maximum growth rates and the optimum growth temperatures were almost identical (0.6 h^{-1} versus 0.7 h^{-1} and 95°C, respectively); however, we observed a much steeper decline in growth rate as the temperature increased above 95°C and saw no indication of growth or metabolism above 105°C. Metabolic H_2 production by ES4 has not been previously reported under any conditions.

When assessing the role of pressure on morphology, it is important to consider the possible effects of decompression. For example, Chastain and Yayanos (8) observed ultrastructural changes in the obligate barophile MT-41 due to isothermal decompression from 1,021 atm and subsequent incubation at atmospheric pressure. In our experiments with ES4, cell lysis was predominant during rapid decompression from 250 atm or more. However, when samples were slowly decompressed, allowing adequate time for dissolution of gases, intact cells were observed. Lysis was probably due in part to both high shear rates during sampling and foaming as dissolved gases were liberated, but these effects have not been adequately studied.

The deep sea and, more specifically, hydrothermal vents possess many unique environmental characteristics. Extreme temperatures, elevated pressure, and the low influx of organic material are all potentially important factors in the evolution of deep-sea organisms (26). When studying deep-sea bacteria in the laboratory, it is often desirable to reproduce many of the conditions found in the natural environment. New variables introduced while establishing extreme conditions must be carefully considered, however. Medium composition, construction materials, method of pressurization, and so forth may all affect growth as well as a bacterium's response to environmental parameters such as temperature and pressure.

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