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Pressure and Temperature Effects on Growth and Methane Production of the Extreme Thermophile *Methanococcus jannaschii*

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The marine archaebacterium *Methanococcus jannaschii* was studied at high temperatures and hyperbaric pressures of helium to investigate the effect of pressure on the behavior of a deep-sea thermophile. Methanogenesis and growth (as measured by protein production) at both 86 and 90°C were accelerated by pressure up to 750 atm (1 atm = 101.29 kPa), but growth was not observed above 90°C at either 7.8 or 250 atm. However, growth and methanogenesis were uncoupled above 90°C, and the high-temperature limit for methanogenesis was increased by pressure. Substantial methane formation was evident at 98°C and 250 atm, whereas no methane formation was observed at 94°C and 7.8 atm. In contrast, when argon was substituted for helium as the pressurizing gas at 250 atm, no methane was produced at 86°C. Methanogenesis was also suppressed at 86°C and 250 atm when the culture was pressurized with a 4:1 mix of H_2 and CO_2, although limited methanogenesis did occur when the culture was pressurized with H_2.

Thermophilic bacteria are present in many terrestrial and shallow marine environments where the boiling point of water is about 100°C (2). However, the recent discovery of deep-sea vents that emit superheated hydrothermal fluid at temperatures up to 350°C raises the possibility of organisms living at temperatures well above 100°C. Microbial populations have indeed been found in the vicinity of deep-sea vents (11), and the growth of extremely thermophilic black smoker bacteria in a solid medium at 120°C and a hydrostatic pressure of 265 atm (1 atm = 101.29 kPa) has been reported (6). High pressure was required to obtain substantial growth at 120°C, suggesting that the bacteria were barophilic at high temperatures. This interpretation leads to the general question of how pressure affects the temperature range for growth of thermophiles from high-pressure environments.

Pressure is known to increase the upper temperature for growth of many bacteria isolated from the cold deep sea (ca. 4°C), but this response has not been seen with mesophilic or thermophilic bacteria from atmospheric habitats (17). In recent studies of the thermophilic methanogen *Methanococcus thermolithotrophicus* (originally isolated from geothermally heated sea sediments at a depth of about 0.5 m [8]), hydrostatic pressure up to 500 atm enhanced the growth rate at the optimum temperature but did not extend the temperature range of viability (1). Likewise, hydrostatic pressures of 200 to 300 atm had little or no effect on the temperature range of growth of two extremely thermophilic *Desulfurococcus* species isolated from deep-sea hydrothermal vents (12), and a pressure of about 250 atm decreased the growth rate of each bacterium. In the present work, we examine the effects of pressure on another vent bacterium, *Methanococcus jannaschii* (13). This archaebacterium is the only extremely thermophilic and obligately chemolithotrophic methanogen yet isolated from a deep-sea hot vent (21°N East Pacific Rise at a depth of 2,610 m). It is worth noting, however, that extremely thermophilic methanogens have been isolated from other environments. For example, three isolates of the family *Methanothermaceae* that grow up to 97°C were recently obtained from solfatara fields in Iceland (14).

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MATERIALS AND METHODS

M. jannaschii was grown in a high-temperature, high-pressure bioreactor similar to a prototypical device described elsewhere (15) with the following modifications. The synthetic sapphire pressure vessel was replaced with a larger (internal volume, 167 cm^3) stainless steel 316 vessel (High Pressure Equipment Co.) suitable for pressures up to 1,000 atm, and the screw-actuated piston pump was replaced with a more powerful pneumatic pump (model AFS-150; Haskel Inc.). The latter change facilitated the rapid addition of fresh medium to the pressure vessel. The growth medium was the same as described previously (15) except that $NaHCO_3$ was replaced by 50 mM PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)] and Na_2S was replaced with a combination of 4 mM $Na_2S_2O_3$ and 5 mM $HSCH_2CH_2OH$. Sodium thiosulfate and 2-mercaptoethanol yielded lower growth rates than sodium sulfide but, unlike sodium sulfide, did not result in precipitates that clogged the gas-recirculation pump. The pH was adjusted to 6.8 at 20°C by adding NaOH. At this pH, the ΔpK_a/°C of PIPES is −0.009 (7) and the ΔpH/1,000 atm of standard medium containing PIPES and CO_2 is −0.07 (10).

The gas atmosphere contained H_2 and CO_2 in about a 4:1 mix. The partial pressure of the H_2:CO_2 substrate was roughly 7.8 atm at each temperature and final pressure. Therefore, on the basis of solubilities calculated from the Krichevsky-Kasarnovsky equation (which describes the dependence of Henry's constant on pressure [16]), the initial concentration of each gaseous substrate in the liquid medium was nearly the same under all conditions. Pressures above 7.8 atm were obtained by adding helium (except where noted), and helium was added to maintain constant pressure during methanogenesis and sampling. Gas analysis was performed on-line by gas chromatography (15). To promote equilibrium between the vapor and liquid phases, the gas phase was continuously recirculated through the liquid medium by a magnetically driven pump. Recirculation of substrate gas had a positive effect on growth; in a 7-h control experiment at 86°C and 7.8 atm with the pump on, exponential methane formation began 4 h after inoculation; however, in a separate experiment of the same duration with the pump off, no methane was observed.

Methanogenesis and protein production in single cultures

TABLE 1. Specific growth rates for methanogenesis of *M. jannaschii* as a function of temperature and pressure

Temp (°C)	Specific growth rate (h^{-1}) at pressure (atm)[a]			
	7.8	250	500	750
86	0.5 ± 0.1	0.96 ± 0.04	1.8 ± 0.2	2.36 ± 0.01
90	0.26 ± 0.03	0.5 ± 0.1		0.83

[a] Values are averages from two parallel cultures, except for the rate at 90°C and 750 atm, which was measured once. The lag phase in this particular case lasted nearly 7 days; in contrast, duplicate experiments at 90°C and 500 atm were carried out for only 5 days, during which time no methane was observed.

were measured at different temperatures along an isobar. Growth studies were started by transferring approximately 60 ml of a 1% (vol/vol) bacterial suspension into the pressure vessel preheated to 86°C. Gaseous substrate was then added, followed by helium, if necessary. To replenish the medium during a run, about three-fourths of the liquid was removed from the reactor vessel and subsequently replaced by fresh medium at room temperature. The gaseous substrate was replaced in similar fashion when methane production began to level off. Calculations of transient temperature profiles indicated that the temperature of the reactor contents increased from 25 to 90°C in less than 1 min when the steel vessel was preheated to 90°C and that temperature increases beyond 90°C (e.g., when the oven temperature was raised from 90 to 93°C) took no longer than an hour. Therefore, after each temperature increase at least an hour elapsed before the next methane concentration was measured. This methane level then served as the starting point for determining the methane production rate at the new temperature.

At various times, 0.3-ml liquid samples were removed from the reactor via an external sample line. Protein concentrations in these samples were measured by the Bio-Rad microassay technique (15). The optical density at 600 nm of liquid samples removed from the reactor during high-pressure runs was always below 0.1 because of cell lysis caused by the sudden decrease in pressure upon sampling. However, samples collected during a run at about 3 atm and 86°C showed an increase in optical density that paralleled protein production.

RESULTS AND DISCUSSION

The optimal growth temperature of *M. jannaschii* at 3 atm is about 85°C (13). Specific growth rates based on methane production at 86 and 90°C are presented in Table 1 for different pressures. Under these conditions, protein production (the criterion used for growth) and methanogenesis proceeded in parallel, which is typical behavior for methanogens. At both temperatures, the bacterium grew at pressures up to 750 atm, with the fastest growth occurring at the highest pressure. Slower growth rates and much longer lag phases were obtained at 90°C. However, under no circumstances was growth observed at higher temperatures, as evidenced by the absence of protein production in cultures above 90°C.

Methanogenesis proceeded at temperatures beyond 90°C, indicating that cell growth and methane production were uncoupled at higher temperatures. As shown in Fig. 1, methane formation rates measured above 90°C were higher at 250 atm, the in situ pressure of *M. jannaschii*. Moreover, the maximum temperature for methanogenesis was extended by increased pressure. Interestingly, the highest production rate was measured at 93°C and this rate was consistently higher than the rate at 92°C.

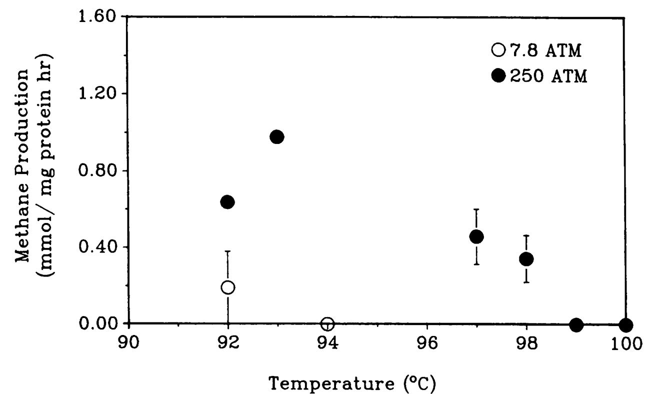

FIG. 1. Methane production by *M. jannaschii* as a function of temperature and pressure. In each experiment, the partial pressure of the substrate gas was 7.8 atm. Production rates were determined from straight-line fits of the methane production data collected over a minimum of 2 h. All values are averages of at least two parallel cultures; the production rate at 93°C is the average of three runs. Mean values with standard deviations ≤0.05 mmol mg^{-1} h^{-1} are shown without error bars. Protein concentrations at each temperature in each run were determined from at least two samples collected at least 3 h apart. The mean deviation of each average protein concentration was less than 12%.

To obtain methanogenesis at 97 and 98°C, the temperature of the culture had to be increased gradually; a single-step increase in temperature from the starting point of 86°C resulted in no methane production above 90°C. A typical temperature progression for methanogenesis at 250 atm is shown in Fig. 2. After methane production was observed at 92°C, the temperature was lowered to 90°C and fresh medium and substrate were added. Raising the temperature to 93°C and then to 97°C resulted in methane formation, but no methane was observed for 100°C following the addition of fresh medium and another period at 90°C (during which protein was again produced). Moreover, at this point the culture was no longer viable, as evidenced by a lack of methanogenesis when the temperature was lowered from 100°C to 90°C. This result is consistent with the decrease in protein observed in 100°C, which may have been due to cell lysis and the precipitation of protein from solution (an increase in the turbidity of liquid samples removed from the reactor at 100°C was also noted). Protein precipitation was visually evident in separate experiments when sonicated cells were heated to 100°C for 10 h at 2 atm.

In all of the experiments described so far, helium was used as the pressurizing gas. Helium was chosen because of its very low anaesthetic potency relative to other noble gases (3, 4). When Ar was substituted for He as the pressurizing medium at 86°C and 250 atm, methane production was not observed. Studies at superatmospheric pressures with other organisms (3, 4) have shown that Ar is a much more effective inhibitor of cellular growth than He, possibly because of its greater solubility in lipids. It is significant, if not surprising, that the same result was obtained with *M. jannaschii*, whose major membrane lipid is a macrocyclic diether not yet found in any other species of archaebacteria (5).

As with Ar, no growth or methane formation was evident at 86°C and 250 atm when the culture was pressurized with a 4:1 mix of H_2 and CO_2. To distinguish between the potentially inhibitory effects of high substrate levels and acidification of the medium due to dissolved CO_2, experiments were also performed at 86°C and 250 atm by using 1.6 atm of

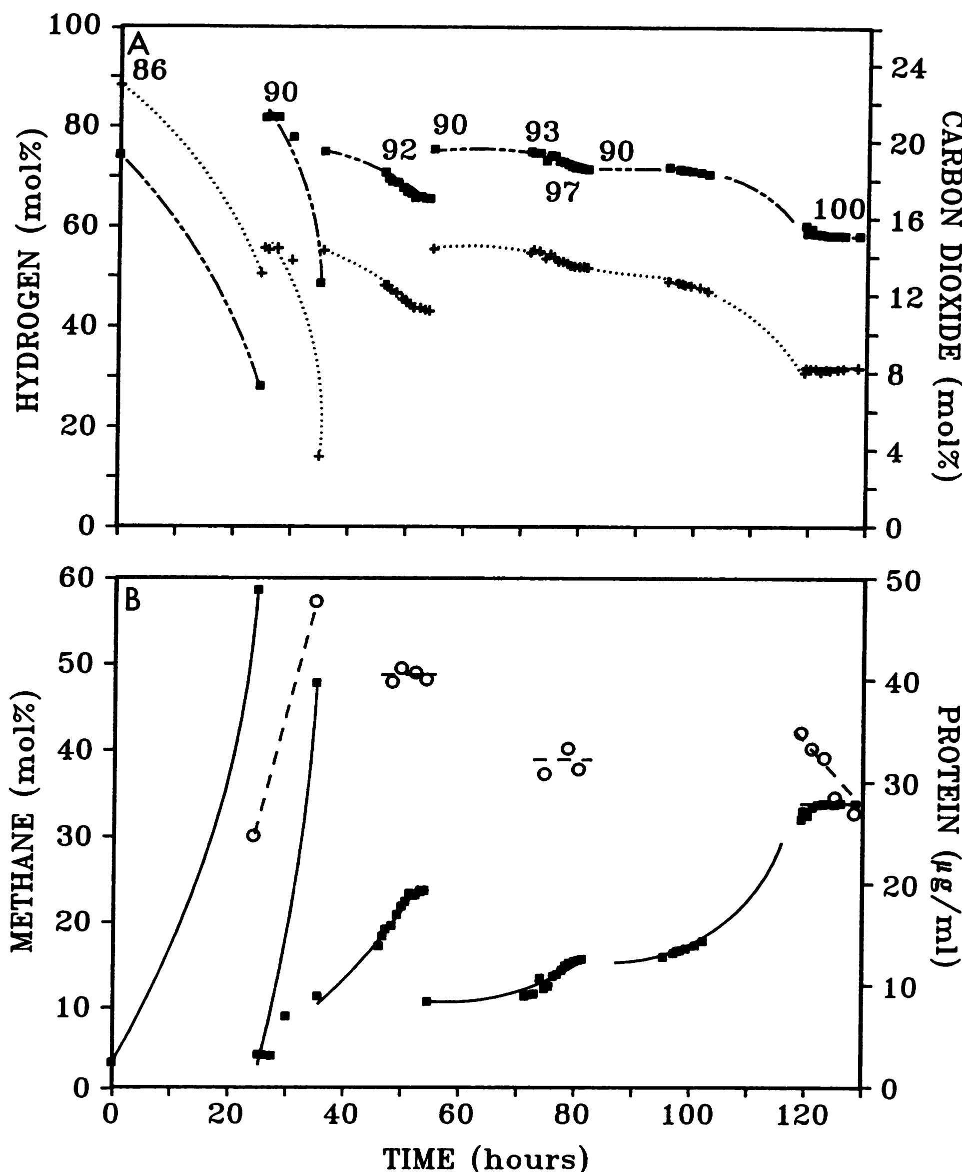

FIG. 2. (A) Consumption of hydrogen (■) and carbon dioxide (+) by *M. jannaschii* at 250 atm and temperatures ranging from 86 to 100°C. Concentrations are expressed on a helium-free basis. Temperatures are shown directly on the figure at the approximate times the temperature was set to the indicated value. (B) Methane (■) and total protein production (O) under the same conditions as above. Decreases in methane and protein concentrations (except at 100°C) were due to dilution of the culture with gaseous substrate and fresh medium, respectively.

CO_2 with H_2 as the pressurizing gas. In this case, the specific growth rate for methanogenesis was 0.06 h^{-1}, indicating significant growth inhibition by H_2.

In summary, high hyperbaric pressure of He up to 750 atm increased the growth rate of *M. jannaschii* at both 86 and 90°C but did not extend the upper temperature limit for growth. That pressure did not increase the maximum growth temperature is consistent with studies of *M. thermolithotrophicus* in nickel tubes under hydrostatic pressure (1). However, increased pressure did extend the maximum temperature for methanogenesis by *M. jannaschii* from less than 94°C at 7.8 atm to 98°C at 250 atm. Therefore, pressure appears to have had a stabilizing effect on an enzyme or enzymes crucial to methane production (for a review of enzymes under extreme conditions, see reference 9). These results illustrate that metabolic events other than growth are important to consider in studies of pressure effects on microorganisms.

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