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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 at both 86°C and 90°C were accelerated by pressure up to 750 atm, but growth was not observed above 90°C at either 7.8 atm 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.

We have also constructed a novel bioreactor suitable for precise studies of enzymic reactions at elevated temperatures and pressures. Initial studies in this bioreactor at 86°C indicate that the methyl viologen-reducing activity of hydrogenase in crude extracts of *M. jannaschii* is more than tripled by an increase in pressure from about 7.5 atm to 260 atm.

Finally, we have purified a single hydrogenase from *M. jannaschii* 40-fold to apparent homogeneity.

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FINAL REPORT

Contract: N00014-87-K-0347

Title: Pressure-Temperature Effects on Thermophilic Archaebacteria

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I. Project Goals

The primary objective of this project was to investigate the interaction of temperature and pressure in the heat tolerance of *Methanococcus jannaschii*, an extremely thermophilic marine archaebacterium isolated from a deep-sea hydrothermal vent. Toward this end, it was necessary to expand the operating range and analytical capabilities of a high temperature-pressure bioreactor previously constructed in our laboratory.

II. Summary of Accomplishments

A. Pressure-Temperature Effects on *Methanococcus jannaschii*

Initial studies focused on the growth and methane production of *Methanococcus jannaschii*, an extremely thermophilic methanogen isolated from a deep-sea "white smoker" chimney (21°N East Pacific Rise at a depth of 2,610 m). Using a specialized bioreactor designed for high temperatures and pressures, we have shown that *M. jannaschii* grows nearly 5 times faster at 750 atm than at 7.8 atm (Table 1 and Figure 1), and that the maximum temperature for methane production is extended from 92°C at 7.8 atm to 98°C at 250 atm, the pressure at the vent site (Figure 2). No growth was observed above 90°C at either 7.8 atm or 250 atm, however, indicating that methane production and cell growth are uncoupled at temperatures above 90°C.

Temperature (°C)	Pressure (atm) 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	Not measured	0.83

Table 1. Specific growth rates for methanogenesis (hr^{-1}) of *M. jannaschii* as a function of temperature and pressure. 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.

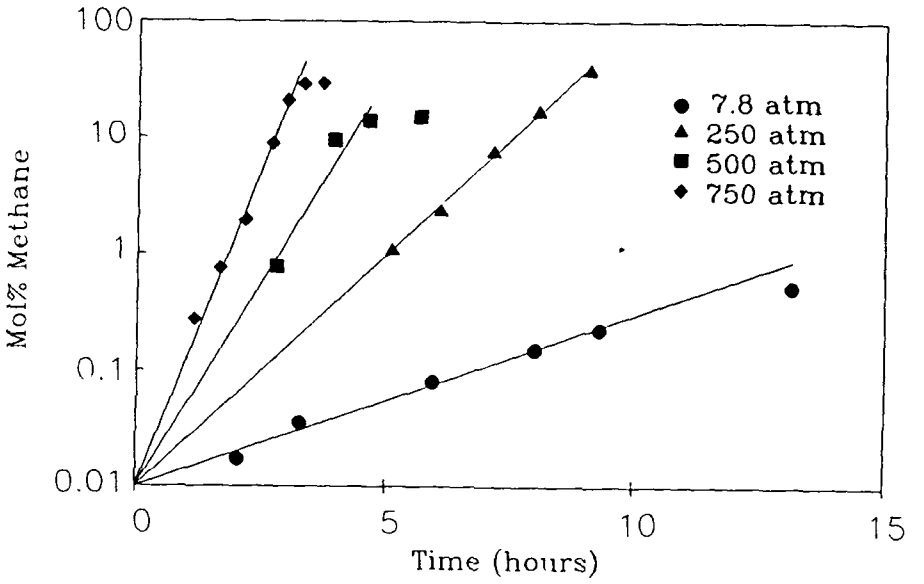

Figure 1. Effect of pressure on methane production by *M. jannaschii* at 86°C.

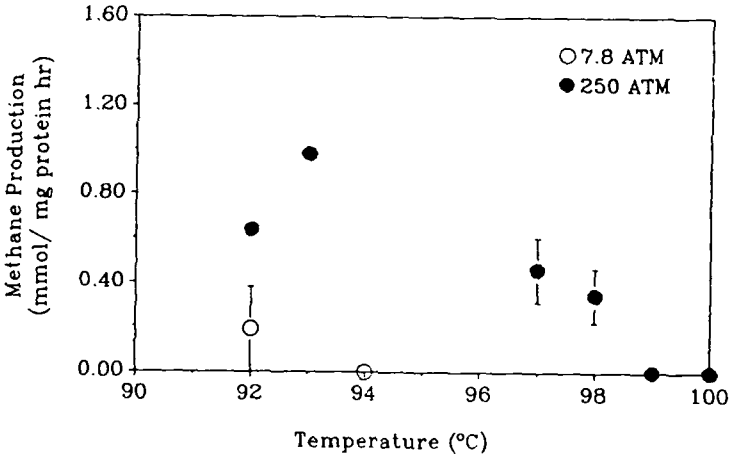

Figure 2. Methane production by *M. jannaschii* as a function of temperature and pressure. In each experiment the partial pressure of the substrate gas was 7.8 atm. Production rates were determined from straight-line fits of the methane production data collected over a minimum of 2 hr. All values are 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 mmols mg^{-1} hr^{-1} are shown without error bars. Protein concentrations at each temperature in each run were determined from at least 2 samples collected at least 3 hr apart. The mean deviation of each average protein concentration was less than 12%.

B. Hydrogenase Activity at Elevated Pressure.

A second bioreactor originally designed for enzyme assays at high temperatures and pressures has been used to study hydrogenases. In preliminary studies of pressure effects on hydrogenase activity, methylviologen-reducing hydrogenase activities of cell lysates were measured at 86°C and at ca. 8.5 and 250 atm. Representative results are presented in Table 2. In each case, the partial pressure of the hydrogen substrate was about 8.5 atm, and the remaining pressure in the high-pressure assays was obtained by adding helium. Thus, the initial concentration of each gaseous substrate in the liquid medium was nearly the same under all conditions and the observed pressure effect was not related to substrate solubility.

Pressure (atm)	**6.5 - 8.5**	**250 - 264**
Reaction rate at 86°C $\left(\frac{\mu mol\ MV\ reduced}{mg\ protein\ min}\right)$	52.7 ± 7.2	171 ± 23

Table 2. Methyl viologen (MV) reduction by hydrogenase(s) in crude extracts of *Methanococcus jannaschii*. Reaction rates are expressed per mg of total protein as determined by the Bio-Rad microassay technique. The low-pressure rate is the average obtained from 5 samples; the high-pressure rate is the average obtained from 3 samples. The methyl viologen concentration in each assay was 1.1 ± 0.1 mM, and the partial pressure of hydrogen was 7.5 ± 1.0 atm. Pressures above 8.5 atm were obtained by adding helium.

For each assay, cell-free extract of *M. jannaschii* was first reduced in TRIS buffer, then added to the preheated vessel and allowed to equilibrate with approximately 8.5 atm of hydrogen for 15 minutes. Methylviologen was then rapidly injected with pressurizing helium to a final concentration of 1.2 mM, and its reduction by hydrogenase monitored at 590 nm with a fiber-optics probe. Data collection was initiated within 5 seconds of substrate addition. As shown in Table 2, the average methylviologen-reducing hydrogenase activity was increased over 3-fold by increased pressure.

C. Hydrogenase Purification

Hydrogenases have been purified from many organisms under aerobic conditions and then deoxygenated with restoration of activity, and we have recently demonstrated that such an approach is suitable for hydrogenases from *M. jannaschii* as well. We have recently purified a single hydrogenase from *M. jannaschii* 40-fold to apparent homogeneity (Figure 3).

Native polyacrylamide electrophoresis gels of crude extract revealed three major hydrogenase bands, indicating that *M. jannaschii* contains at least two hydrogenases (the bottom band of lane 1 could be a distinct hydrogenase or a component of one of the top two hydrogenases). Indeed, several other methanogens have been shown to contain two or three distinct hydrogenases. One type of hydrogenase reduces both the methanogen redox coenzyme F_{420} and the dye methylviologen, whereas the other type reduces only methylviologen. We have

Figure 3. Hydrogenase species of *M. jannaschii* in 4% polyacrylamide gels after electrophoresis. (1) Activity bands of aerobic crude extract following treatment with tetrazolium activity stain. (2) Activity band of hydrogenase isolated from crude extract by elution from a DEAE-Sepharose column, ammonium sulfate precipitation, and preparative electrophoresis. (3) Hydrogenase-containing sample prepared as in (2) and stained with Coomassie Blue.

not yet determined which type of hydrogenase we have purified; however, based on the electrophoresis results of Figure 3, the different hydrogenases of *M. jannaschii* are resolvable and we propose to isolate at least the top two to homogeneity. In addition, hydrogenases will be purified from *M. thermolithotrophicus* to compare the effects of pressure on hydrogenases from methanogens of deep-sea and shallow marine environments.

III. Summary and Plans

These experiments have demonstrated the favorable effects of pressure on the deep-sea thermophile, *M. jannaschii*, and its hydrogenase enzyme(s). Future work will examine pressure-temperature relationships in the growth and productivity of thermophilic archaebacteria isolated from two deep-sea hydrothermal vents (i.e., vent sites along the Juan de Fuca Ridge and at 21^{o} N East Pacific Rise) and from geothermal fluids. Of particular interest are experiments that simulate the natural environments and chemical compositions of hydrothermal

vent fields and geothermal wells. We will also examine pressure and temperature effects on the structure and function of hydrogenases and proteases isolated from extreme thermophiles. For these studies two high pressure-temperature bioreactors have already been constructed. These reactors will be employed for precise analyses of organisms and enzymes under extreme conditions, and can also be used to optimize laboratory-scale bioprocesses involving thermophilic species.

IV. Publications

E.L. Almond, A.J. Clark, and D.S. Clark, "Complementation of a thr-1 Mutation of Escherichia coli by DNA from the Extremely Thermophilic Archaebacterium Methanococcus jannaschii", Appl. Microbiol. Biotechnol., **30**, 148 (1989).

J.F. Miller, N.N. Shah, C.M. Nelson, J.M. Ludlow, and D.S. Clark, "Pressure-Temperature Effects on the Growth and Methane Production of the Extreme Thermophile Methanococcus jannaschii," Appl. Environ. Microbiol., **54**, 3039 (1988).

J.F. Miller, C.M. Nelson, J.M. Ludlow, N.N. Shah, and D.S. Clark, "High Pressure-Temperature Bioreactor: Assays of Thermostable Hydrogenase with Fiber Optics," Biotechnol. Bioeng., in press.

V. Presentations

J.F. Miller, N.N. Shah, J.M. Ludlow, and D.S. Clark, "Pressure Effects on the Function of Thermostable Hydrogenase and the Extreme Thermophile Methanococcus jannaschii," AIChE Annual Meeting, New York City, New York, November, 1987.

J.F. Miller, J.M. Ludlow, and D.S. Clark, "A Novel Bioreactor for Studying the Structure and Function of Enzymes at Extreme Temperatures and Pressures", poster presented at Enzyme Engineering IX, Santa Barbara, California, October 1987.

J.F. Miller, N.N. Shah, and D.S. Clark, "Pressure-Temperature Relationships in the Growth of Methanococcus jannaschii, An Extremely Thermophilic Methanogen," AIChE Summer Meeting, Minneapolis, Minnesota, August 1987.

VI. Personnel Supported

Dr. Jay Miller, Post-doc	March 1987 - February 1988
Mr. Nilesh Shah, Graduate Student	February 1988 - February 1989