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Reproduction of *Bacillus stearothermophilus* as a Function of Temperature and Pressure

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The colony-forming ability and the rate of reproduction of *Bacillus stearothermophilus* were determined as a function of temperature and pressure. Colonies were formed between 39 and 70°C at atmospheric pressure and between 54 and 67°C at 45 MPa. Colonies did not form at 55.9 MPa. The rate of reproduction in broth cultures decreased with increasing pressure at all temperatures. The rate of reproduction diminished rapidly with pressure above 10.4 MPa. Therefore, increased hydrostatic pressure was not sufficient to enable *B. stearothermophilus* to function beyond the temperature limiting growth and reproduction at atmospheric pressure, and *B. stearothermophilus* should grow in naturally or artificially warmed regions of the deep sea, where the pressure is less than approximately 50 MPa, although growth rates would be low above 10 MPa.

Most of the deep ocean has a temperature near 2°C. Some exceptions are the Sulu Sea, the Mediterranean Sea, and the Red Sea, which have at their greatest depths temperatures of 9.8°C at 5,580 m, 13.3°C at 4,755 m, and 58°C (hot brines) at 2,150 m, respectively (6). Until the discovery of hydrothermal vents and their fascinating microbiology (1, 10), the occurrence of thermophilic organisms in deep-sea sediments (2) was enigmatic. The problem is still not resolved since, among other things, the effects of variations in both temperature and pressure on the physiology of thermophiles (allochthonous or autochthonous to the deep ocean) are not known.

Reported here is a study of the effects of temperature and pressure on the reproduction of a well-studied thermophilic bacterium, *Bacillus stearothermophilus*, found in seawater enrichments of sediments (2) of the deep sea. Since this bacterium has terrestrial habitats, it is probably allochthonous to the deep ocean. Increased hydrostatic pressure ameliorates some denaturing effects of increasing temperatures (7). There is speculation that such interactive effects of temperature and pressure allow cells to function at temperatures greater than possible at atmospheric pressure (11). A study with a deep-sea barophilic and psychrophilic bacterial strain (isolate CNPT3) supports such speculation, since the maximum temperature permitting growth was shifted to higher values by increasing the pressure (A. A. Yayanos, R. Van Boxtel, A. S. Dietz, and K. M. Jones, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, N59, p. 173). Studies with mesophiles (see, for example, reference 9, p. 364) reveal that increased pressure does not increase the maximum temperature permitting growth. Among our conclusions from the study reported here was that incubation at high pressure was not a sufficient condition to extend the maximum temperature allowing growth of a thermophilic bacterial strain.

MATERIALS AND METHODS

Bacterial strain. *B. stearothermophilus* was purchased from the American Type Culture Collection, Rockville, Md. (ATCC 7953).

Medium and growth at atmospheric pressure. Cultures were grown in nutrient broth (Difco Laboratories, Detroit, Mich.) and in nutrient agar (Difco). Solutions of glucose (50 g/liter) were prepared from reagent-grade glucose (Mallinckrodt) and water (Nanopure grade; Barnstead Co., Boston, Mass.) and then sterilized by filtration (0.22-μm-pore-size filters; Millipore Corp., Bedford, Mass.).

Determination of the concentration of cells. The number of cells in diluted samples of cultures was determined with a model H Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) with an aperture tube having a 30-μm orifice. The diluent was an aqueous solution containing 0.85% NaCl, 0.05% sodium azide, and 0.025% Formalin. The azide-containing solutions were handled with glassware and plasticware and were discarded into drains plumbed with plastic pipe. Correction was made for coincidence counting where necessary.

Temperature-pressure limits of colony-forming ability. A high-pressure temperature gradient apparatus (15; A. A. Yayanos, R. Van Boxtel, and A. S. Dietz, manuscript in preparation) was used to find the ranges of temperature and pressure over which *B. stearothermophilus* formed colonies. Liquid agar at 50°C was inoculated to contain 10^4 cells per ml. The agar was aspirated into glass tubes 76 cm in length and 0.7 cm in

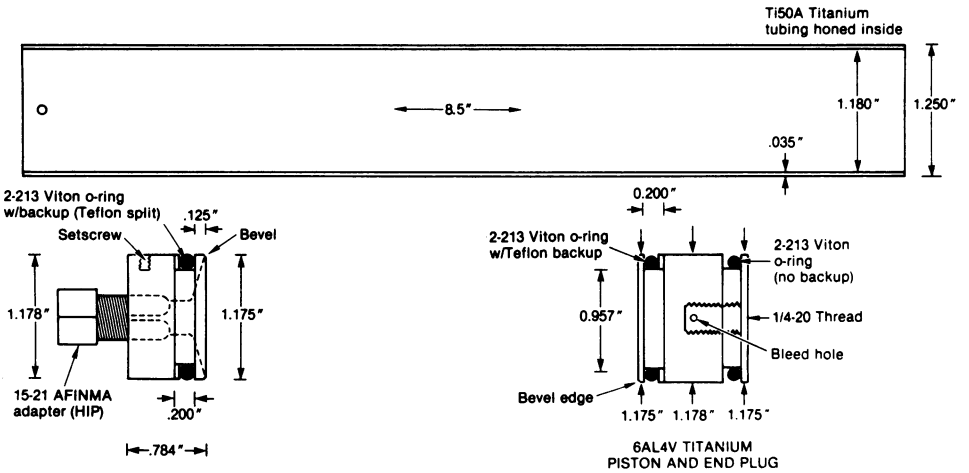

FIG. 1. Details of a titanium (type Ti50) syringe used to confine cultures at high pressures. The first O-ring on the plunger (piston) of the syringe isolated the contents of the syringe from the hydraulic fluid. The second O-ring helped to keep the plunger aligned in the syringe. The threaded hole in the plunger allowed insertion of a threaded rod to assist loading and disassembling of the syringe. HIP, High Pressure Equipment Co.

outer diameter. The ends of the glass tube were sealed with rubber pistons. After the agar gelled, each tube was placed into one of the eight pressure vessels of the gradient apparatus. The eight pressures used were 1, 124, 228, 331, 449, 559, 656, and 759 bars (10 bars = 1 MPa). The pressures were accurate to ±1.4 bars. The temperature gradient along each vessel was from 35.5 to 84.1°C. The temperatures were accurate to ±0.1°C. The vessels were opened after an incubation of 2 days, and the temperatures bounding the distribution of colonies along the length of each tube were determined. Thus, at any given pressure the presence of

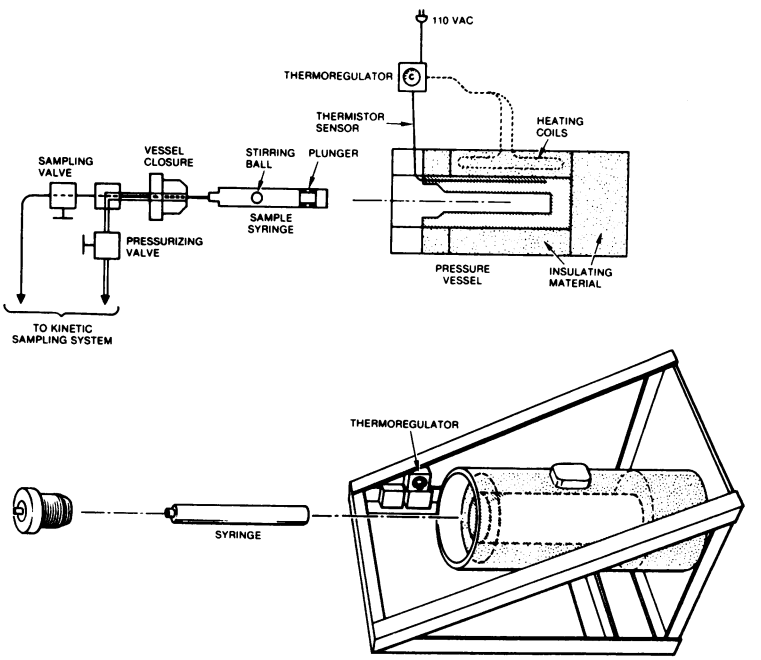

FIG. 2. Schematic representation showing the components of a system used to incubate bacterial cultures at high pressures and high temperatures. The system could be used to 350°C (with the use of appropriate O-rings and hydraulic fluid) and to 1,034 bars. Samples were transferred under a small pressure gradient (less than 1 bar) to another pressure vessel (not shown), isolated, cooled, decompressed, and removed for assay.

colonies along a portion of the temperature gradient served to define the upper and lower temperatures for growth at that pressure.

Determination of the kinetics of reproduction at high temperatures and pressures. A culture in nutrient broth was placed into an autoclaved titanium syringe (Fig. 1 and 2) containing a glass ball. The syringe was secured to high-pressure tubing (0.159-cm outer diameter; 0.076-cm inner diameter) with an adapter connector (type AF1 NMA; High Pressure Equipment Co., Erie, Pa.), as shown in Fig. 1 to 3. The tubing was rated for a maximum working pressure of 1,035 bars. The details of how the small high-pressure tubing was passed through the closure of the pressure vessel, through the T fitting, and coaxially through 6.35-mm high-pressure tubing are shown in Fig. 3. This manner of connecting the sample syringe allowed two things: (i) the culture was securely connected to the sample valve with minimal chance for contamination by hydraulic fluid, and (ii) both pressurizing and sampling were accomplished through the single port at the top of the pressure vessel. The pressure vessel was purchased from Autoclave Engineers, Inc. (Erie, Pa.), was made of 4340 alloy steel, and had an internal volume of 500 ml, an inner diameter of 5.08 cm, an outer diameter of 10.16 cm, and a working pressure of 2,069 bars (206.9 MPa). The hydraulic fluid for the temperature range studied here was 50% (vol/vol) ethylene glycol in water. The vessel was surrounded by an aluminum sleeve adapting it to the heating mantle (Parr Instrument Co., Moline, Ill.). The temperature was regulated to ±0.5°C. The small (0.159-cm outer diameter) tubing connecting the culture at high temperature and pressure to the kinetic sampling system (Fig. 2 and 3) was flexible. This allowed the vessel under pressure (Fig. 2) to be placed horizontally and rocked so that the motion of the ball in the syringe kept the culture stirred during incubation. The system to which the sampling and pressurizing valves were connected allowed removal and decompression of samples without decompression of the culture in the syringe and without exposure of the sample to any damaging shear forces. This kinetic sampling system is a modified version of one already described (14). Two or more sequential samples were always taken, and all but the last of them were discarded. This procedure purged the tubing and valve connecting the culture being incubated (Fig. 2) to the syringe at 2°C in the pressure vessel of the kinetic sampling system (14).

RESULTS

The domain of temperature-pressure values over which *B. stearothermophilus* could form colonies is shown in Fig. 4. The data were obtained from the distribution of colonies in gels incubated in the high-pressure temperature gradient apparatus. At each pressure, two data

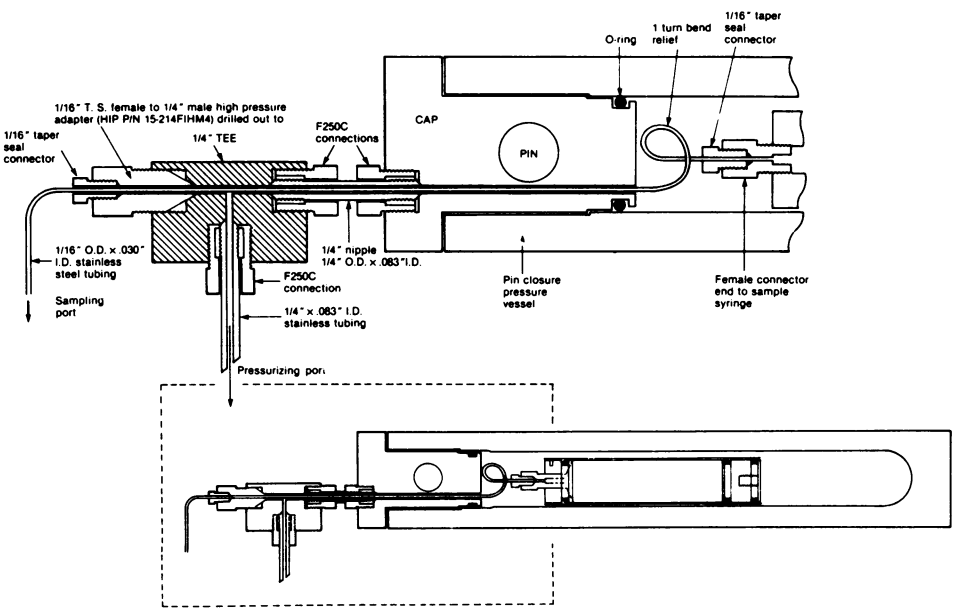

FIG. 3. Dual valving through a single pressure port of a pin-closure piston-seal pressure vessel. A single piece of small-bore high-pressure tubing was used to connect a culture in a syringe inside one pressure vessel to a syringe inside another pressure vessel. The tube of the sampling port was connected to the high-pressure sampling system (based on that described by Yayanos [14]). This tube formed a continuous path to the culture in the syringe. This was done by first drilling out the High Pressure Equipment Co. (HIP) adapter, passing the tubing through the adapter, TEE, F250C connections, and pressure vessel cap, and then connecting it to the female connector end of the sample syringe. The high-pressure connection at the female end of the HIP adapter was made in the customary way. This method allowed hydraulic fluid to enter through the other port of the TEE, around the outside of the tube, and into the pressure vessel. I.D., Inner diameter; O.D., outer diameter.

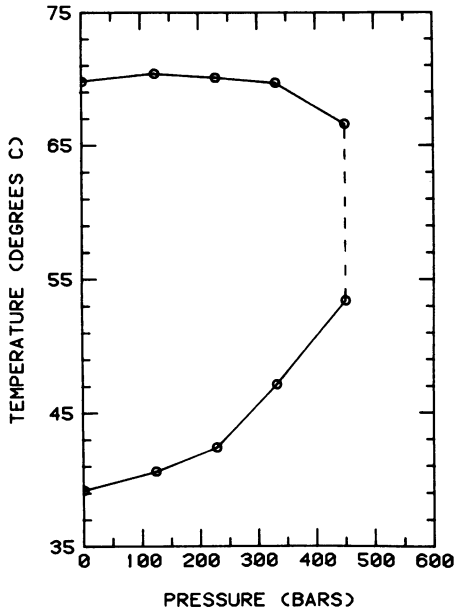

FIG. 4. Summary of data from the high-pressure temperature gradient apparatus. Each pair of points along an isobar came from a single pressure vessel. One point defines the upper temperature at which colonies formed, and the other point defines the lower temperature. The region of temperatures and pressures over which *B. stearothermophilus* grew is defined by connecting the points as shown. The dashed line is at 450 bars, the highest pressure at which colonies were observed. No colonies formed at 559 bars.

points, one representing the minimum and the other the maximum temperature at which colonies could develop, were generated for the graph shown in Fig. 4. Colonies grew at five of the eight pressures used. The data connected as shown form a boundary around those values of temperature and pressure at which *B. stearothermophilus* could form colonies. The dashed line connecting the two points at 450 bars (45 MPa) represents the maximum pressure at which growth of colonies was observed. Colonies were absent in the incubation at 559 bars (55.9 MPa). The maximum pressure allowing colony formation during a 2-day incubation of this organism was therefore between 450 bars (45 MPa) and 559 bars (55.9 MPa). The maximum temperature at which cells reproduced (formed colonies) was nearly invariant with pressure, but the minimum temperature significantly increased with increasing pressure (Fig. 4). We concluded from results with other strains studied with this method that extension of the time of incubation would not have substantially modified our results with *B. stearothermophilus*.

Replicated determinations of the data in Fig. 4 agreed within ±0.5°C and ±2 bars. Identical results were obtained in nutrient agar with and without glucose.

The effect of temperature on the rate of reproduction in nutrient medium without glucose is shown in Fig. 5 for the experiments at atmospheric pressure and in Fig. 6 for those at 104 bars (10.4 MPa). At these two pressures, the effect of temperature on the generation time was slight between 55 and 69°C. The effect of pressure was determined in more detail at 65°C and was found to be substantial only above 139 bars. The effect of pressure between 1 bar and 104 bars was not large enough to warrant, for the

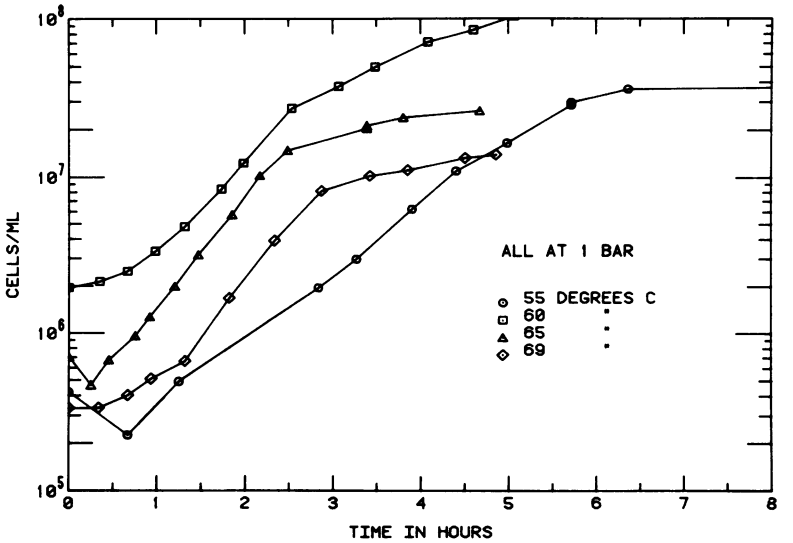

FIG. 5. Kinetics of reproduction as a function of temperature at atmospheric pressure.

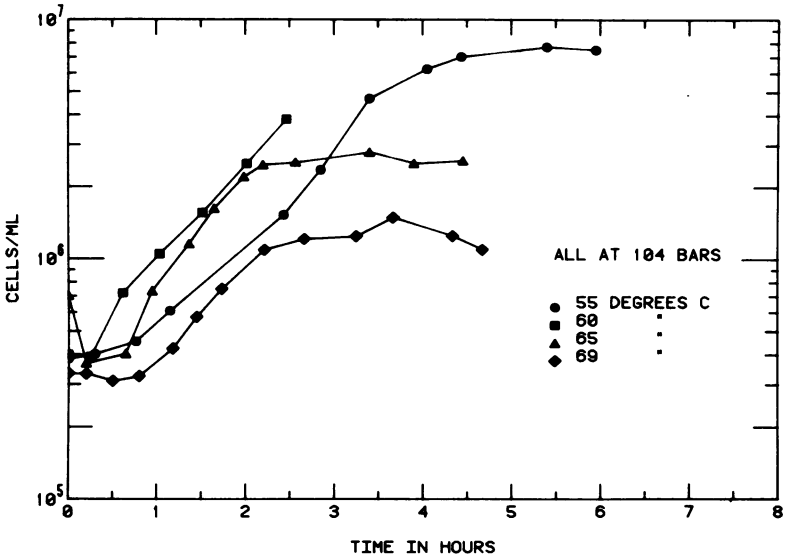

FIG. 6. Kinetics of reproduction as a function of temperature at 10.4 MPa.

purpose of this paper, additional studies at 1 atm in the high-pressure apparatus. Table 1 summarizes all of the determined generation times.

The dependence of final cell concentration on the temperature and pressure was not determined in detail (Table 1). The final cell concentration at 104 bars (10.4 MPa) decreased with temperature, as seen from the data in Fig. 6 for 55, 65, and 69°C. The final cell concentration at 65°C decreased with increasing pressure.

All but one of the experiments at atmospheric pressure were done in screw-cap tubes. For the reason stated above, only a single experiment was done at 65°C in the pressure vessel at atmospheric pressure. Table 1 shows that this experiment resulted in a generation time slightly longer than and a final cell concentration less than that found with a culture in a screw-cap tube.

TABLE 1. Generation times for *B. stearothermophilus*

Temp (°C)	Pressure (bars)	Generation time (h)	*n*	R^2 error	Final titer (cells per ml)
55	1	0.6	3	1.000	3.7×10^7
55	104	0.81	5	0.986	7.5×10^6
60	1	0.48	4	0.999	1.0×10^8
60	1	0.46	4	0.998	
60	1	0.44	5	0.983	
60	104	0.77	5	0.999	
65	1	0.55[a]	5	1.000	8.0×10^6
65	1	0.43	8	0.998	2.8×10^7
65	104	0.64	4	0.998	2.6×10^6
65	139	0.80	4	0.984	1.8×10^6
65	208	6.0	10	0.539	
69	1	0.40	3	0.999	1.5×10^7
69	104	0.67	3	0.997	1.2×10^6

[a] Experiment done in high-pressure apparatus at 1 bar.

DISCUSSION

High pressure did not extend the upper temperature at which cells of *B. stearothermophilus* reproduced. The rate of reproduction was also inhibited in incubations at high pressure. There is evidence suggesting that the effects of elevated temperature on macromolecules can be antagonized by high pressure. For example, the rate of heat inactivation of transforming DNA is slowed (7) by increasing the pressure from 1 to 2,736 bars. In addition, the melting of DNA occurs at higher temperatures the higher the pressure (7). These effects of increased pressure on nucleic acid stability are small. There are similar examples with proteins whose stability is extended to higher temperatures by elevated pressure (9). The thermal inactivation of enzymes from a thermophile often occurs at temperatures only a few degrees above the maximum temperature at which cell growth occurs (4). Again this suggests that increased pressure might extend the upper limit at which a thermophile could function. There is also evidence that deep-sea bacteria can grow at temperatures somewhat higher than otherwise possible by increasing the hydrostatic pressure on the cultures (Yayanos et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, N59, p. 173). In contrast to this evidence, the data reported here force the rejection of the hypothesis (11) that pressure

effects per se can extend the upper temperature at which cells can reproduce.

We did not observe any evidence of spore formation. If *B. stearothermophilus* is like other spore-forming microorganisms that have been studied at high pressures (5, 12), then the germination of its spores rather than sporulation of its vegetative cells should be favored at high pressures.

The results of this study partly address the question of what might happen in a spherical shell of warmed sediment surrounding buried radioactive waste. Such waste, in one scheme under consideration (13), would heat nearby sediments for tens of years to temperatures ranging from over 200°C at the surface of the waste container to ambient values at about 2 m from the container. At the sites where the properties of sediments are thought to be appropriate, the hydrostatic pressure is in excess of 500 bars. The data in Fig. 4 to 7 show that hydrostatic pressure would probably inhibit organisms like *B. stearothermophilus* from growing in the artificially warmed environment around radioactive waste. In the proximity of the waste, where the radiation field would be high, there would probably be synergistic effects of heat and radiation (8), making it even less likely that such bacteria could function. Further work needs to be done on the effects of temperature, pressure, and radiation on deep-sea thermophiles whose presence in the cold deep ocean was once paradoxical (2) but now seems expected (1). It is interesting to note that *B. stearothermophilus* was one of the thermophilic strains isolated from deep-sea sediment cores (2) with seawater-based media.

The effects of pressure on the reproduction of *B. stearothermophilus* were what would have been surmised from the effects of pressure on the mesophile *Escherichia coli*. That is, the rate of reproduction was slowed with increasing pressure and the ability to reproduce was stopped at about 500 bars. This result is totally different from that with deep-sea bacteria, whose rate of reproduction is a maximum at a high pressure and whose ability to reproduce extends, depending on depth of origin, well beyond 1,000 bars (16, 17). All of these results point to the importance of two types of studies on the role of high pressures in ecology. One is to understand the limits of temperature (3) and pressure (16–18) at which a species can evolve. A second is to determine how evolution of a species in one region of the temperature-pressure plane restricts its excursion to environments of other temperatures and pressures.

Physiological properties of some of the microbes present in a sample of the deep ocean may, moreover, reveal interesting pathways for the dispersal of microorganisms and the existence of unusual environments for their sources. In this light, the presence of thermophiles in deep-sea sediments (2) could have been used to predict the presence of submarine geothermal activity. Using this kind of rationale, the result (18) showing microbial growth at 30°C and 1,000 bars and the fact that bacteria adapted to cold waters at that depth are psychrophilic (16, 17) could be used as a predictor of geothermal activity at the great depths of 10,000 m in the Philippine Trench.

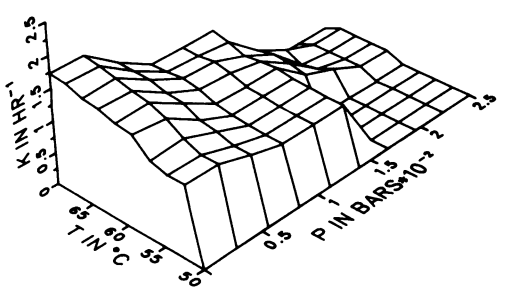

FIG. 7. Specific growth rate constant (K) for *B. stearothermophilus* [defined as ln(2)/generation time] as a function of temperature (T) and pressure (P). The data from Fig. 4 and Table 1 were fit with a spline-fit algorithm (DISSPLA, version 9.0 software package; Integrated Systems Software Corp., San Diego, Calif.).

ACKNOWLEDGMENTS

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