APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1982, p. 1481–1489
0099-2240/82/061481-09$02.00/0
Vol. 43, No. 6

Thermal Inactivation of a Deep-Sea Barophilic Bacterium, Isolate CNPT-3

A. ARISTIDES YAYANOS* AND ALLAN S. DIETZ

Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92093

Received 4 September 1981/Accepted 3 February 1982

The barophilic deep-sea bacterium, isolate CNPT-3, was inactivated by exposures to temperatures between 10 and 32°C at atmospheric pressure. Inactivation in samples from warmed cell suspensions was measured as the loss of colony-forming ability (CFA) at 10°C and 587 bars. At atmospheric pressure, there was a slow loss of CFA even at 10°C. The loss of CFA was rapid above 20°C and only slightly affected by high pressures. The first-order rate constants for thermal inactivation fit the Arrhenius equation with an activation energy of 43 kcal (ca. 179.9 kJ)/mol. Light microscopy and scanning transmission electron microscopy revealed morphological changes due to warming of the cells. The changes ensued the loss of CFA. The results supported the hypothesis from an earlier work that indigenous (autochthonous) deep-sea bacteria from cold deep seas are both barophilic and psychrophilic. If ultimately sustained, these characteristics may be useful in designing experiments to assess the relative importance of the autochthonous and allochthonous bacteria in the deep sea. The data were used to evaluate how barophilic bacteria may have been missed in many investigations because of warming of the cells during sample retrieval from the sea or during cultivation in the laboratory. The evaluation revealed the need for temperature and pressure data during retrieval of samples and cultivation in the laboratory. Most deep-ocean microbiology may be possible with thermally insulated equipment for retrieval from the sea and with high-pressure vessels for laboratory incubations.

The deep-sea bacterial isolate designated CNPT-3 is barophilic at 2°C. The organism originated from a depth of 5,700 m in the central North Pacific Ocean (37). ZoBell and Morita (40, 41) reported the isolation of a slow-growing obligate barophile more than 20 years ago. That barophile was lost from laboratory culture. Recent reviews (12, 13, 20, 22, 24) have shown that the understanding of barophiles has remained limited.

This is a report of a study (A. A. Yayanos and A. S. Dietz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, N60, p. 173) of the thermal inactivation of the colony-forming ability of isolate CNPT-3 exposed to temperatures between 0 and 32°C and pressures between atmospheric and 1,035 bars (1 atm = 1.01325 bars = 1.01325×10^5 N/m^2). The results showed the extent to which isolate CNPT-3 was adapted to the low temperatures and high pressures so characteristic of the deep ocean. Furthermore, the study defined limits on warming, in accord with older estimates (15, 41) yet more restrictive than recently thought (13, 35), that should be imposed for continued successful recovery and cultivation of deep-sea barophilic bacteria. Finally, the data supported the hypothesis of an extremely psychrophilic and barophilic nature of deep-sea bacteria. If sustained, the hypothesis implies how the detection of barophilic bacteria has been elusive for nearly two decades since the work of ZoBell and Morita (40), and the hypothesis suggests that much interesting deep-sea microbiology may have been overlooked.

MATERIALS AND METHODS

Organisms and media. Cultures of isolate CNPT-3 were maintained in type 2216 marine broth (Difco Laboratories, Detroit, Mich.) and in a nutrient medium solidified with silica gel (6) at 2 to 4°C and 580 bars. The nutrient medium, designated TYC or Si-TYC when in silica gel, contained tryptone (5 g/liter), yeast extract (1 g/liter), sodium citrate (1 g/liter), and artificial seawater (ASW). Inoculations of culture media for maintenance and for experiments were done at atmospheric pressure. The manipulations at atmospheric pressure were done as quickly as possible, usually within 15 to 30 min.

An ASW was used as the diluent for the assay of colony-forming ability. The ASW contained (in grams) NaCl, 24; KCl, 0.7; $MgCl_2 \cdot 6H_2O$, 5.3; and $MgSO_4 \cdot 7H_2O$, 7, brought to a total volume of 1,000 ml with water (Nanopure grade; Barnstead Co., Boston, Mass.).

Cell concentration, cell size, and assay for thermal inactivation. The concentration of cells in the absence of thermal inactivation was determined with a model B or a model ZBI Coulter Counter (Coulter Electronics,

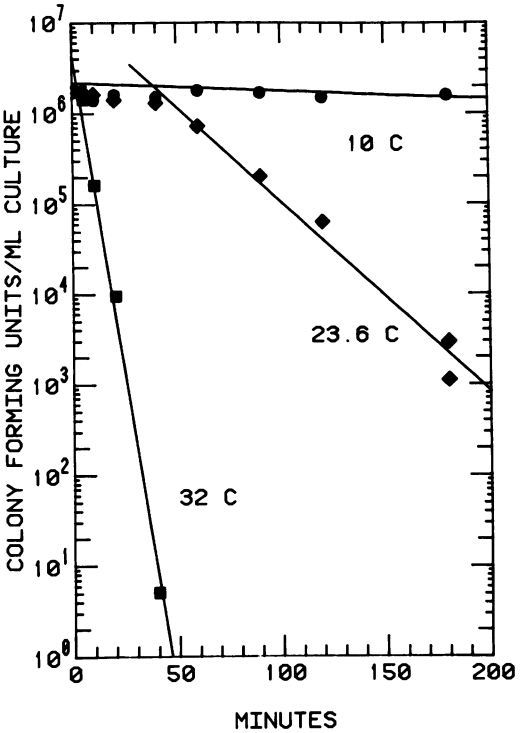

FIG. 1. Kinetics of the thermal inactivation of the colony-forming ability of isolate CNPT-3 at three temperatures, all at atmospheric pressure.

Hialeah, Fla.), using 30-μm-diameter aperture tube orifices. The model ZBI had a Channelizer accessory for determining the distribution of cell sizes in the cultures. Culture samples were diluted with a counting solution (ASW containing 0.05% sodium azide and 0.025% Formalin). The results obtained immediately after dilution with the counting medium were identical to those obtained up to 20 h after dilution. Corrections for coincidence counting by the Coulter Counter were experimentally determined and applied when necessary.

The concentration of cells in the absence of thermal inactivation was also determined from the number of colony-forming units that developed in Si-TYC medium (6, 36). Cell concentrations, determined as colony-forming units, revealed a mean plating efficiency of 94% based on determinations with the Coulter Counter. For example, with six determinations, the mean of the deviations between the number of colony-forming units and particle counts was 7.4%, and the standard deviation was 12%.

Thermal inactivation was quantified by the loss of the ability to form colonies in Si-TYC medium.

Experimental protocols. The procedure for thermal inactivation at atmospheric pressure was as follows. Cells were grown to late log phase in type 2216 marine broth at 587 bars and 3°C. The culture was decompressed and diluted 100-fold with ASW at 0°C. The diluted suspension was incubated at 0, 10, 23.6, and 32°C. The suspensions were sampled as a function of time, with the samples going into ASW at 0°C for quenching the thermal inactivation and for serial dilutions. A 0.1-ml sample of the serial dilutions was mixed with Si-TYC medium at 0°C and incubated at 10°C and 587 bars for 10 days. The vessels were then decompressed, and the number of colonies was determined with a stereoscopic microscope.

The protocol for inactivation at high pressure was as follows. Cells were diluted, as above, 100-fold into cold ASW. The diluted suspension was placed in test tubes, covered with parafilm, and incubated at 1, 311, 622, and 1,035 bars and at 27°C. Samples were taken at noted times by decompressing the pressure vessels, removing a test tube, placing it into a bath at 0°C, and recompressing the vessels to resume the incubation. The samples were assayed for colony-forming units as above. This procedure allowed for an evaluatin of the magnitude of the effect of pressure on thermal inactivation.

Unless otherwise noted, temperatures were accurate to within 0.5°C.

Phase microscopy. The cultures were routinely examined with a Zeiss Universal microscope, with phase optics at magnifications as great as ×2,000.

Scanning electron microscopy. Culture samples (0.2 ml) were added to ASW (at 0°C) containing 1% glutaraldehyde—an electron microscope grade purchased from Polysciences, Inc. (Warrington, Pa.) The fixed samples were processed over polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.; 13-mm diameter, 0.2-μm pore diameter). The cells were rinsed successively with 100% ASW, ASW diluted with water, water, ethanol-water mixtures, ethanol, Freon (113)-ethanol mixtures, and Freon (113). The samples were dried in a critical point drier and coated with a gold-palladium layer by placing the sample in a sputter coater (Hummer II model; Technics, Inc., Alexandria, Va.) for 30 min.

The samples were examined with a scanning transmission electron microscope (Hitachi model H-500; Hitachi, Ltd., Tokyo, Japan) operated in the scanning mode.

RESULTS

Data on the loss of colony-forming ability at atmospheric pressure as a function of the duration at various temperatures are shown in Fig. 1.

TABLE 1. First-order rate constants for thermal inactivation

Temp (°C)	Pressure (bars)[a]	Rate constant (min^{-1})
0	1	8.89×10^{-5}
10	1	1.09×10^{-3}
20	1	1.36×10^{-2}
22	1	1.20×10^{-2}
23.6	1	4.83×10^{-2}
27	1	7.85×10^{-2}
27	1	9.24×10^{-2}
27	1	7.50×10^{-2}
27	311	6.31×10^{-2}
27	622	5.28×10^{-2}
27	1,035	7.65×10^{-2}
32	1	3.91×10^{-1}

[a] One atm = 1.01325 bars = 1.01325×10^5 N/m^2.

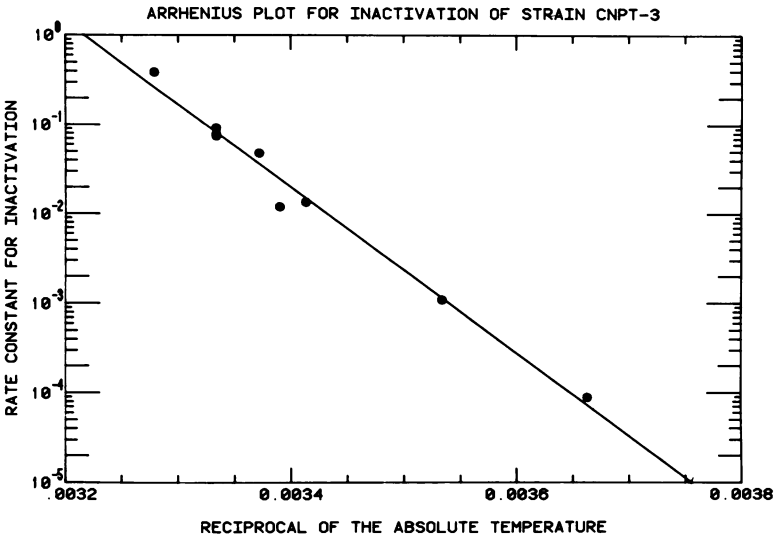

FIG. 2. First-order rate constants for thermal inactivation at atmospheric pressure are shown on an Arrhenius plot.

The data were interpreted as first-order kinetics. There was only a slow rate of inactivation at 10°C. The line shown in Fig. 1 for the isotherm at 10°C was determined by least squares from data up to 4,500 min, even though data are shown over only 180 min. The data at 23.6°C showed a delay in the onset of thermal inactivation because of the time taken to equilibrate the culture to that temperature. Table 1 contains values of the first-order rate constants for thermal inactivation at all of the temperatures used. Fig. 2 shows these rates plotted according to the Arrhenius equation. The activation energy was 43 kcal (ca. 179.9 kJ)/mol of colony-forming units compared to 45.5 kcal (ca. 190.3 kJ)/mol for *Vibrio marinus* MP-1 that we calculated from the data of Robison and Morita (28) and to 46.7 kcal (ca. 195.3 kJ)/mol for *Bacillus psychrophilus* that we calculated from the data of Alsobrook et al. (2).

Although colony-forming ability was slowly lost at 10°C and atmospheric pressure (Fig. 1), the cells reproduce at 10°C and 580 bars (A. A. Yayonos, R. Van Boxtel, A. S. Dietz, and K. Jones, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, N59, p. 173). At 27°C, pressure only antagonized thermal inactivation maximally at 622 bars (Fig. 3.)

The thermal inactivation data at atmospheric pressure were summarized with a single equation to allow for objective evaluations of exposures to elevated temperatures during either sampling or cultivating. Heat injury can be reversible (10), but the following analysis does not consider this phenomenon. The equation related the probability of the survival of a colony-forming unit to the duration of exposure to various temperatures. The equation was derived as follows. First, the rate of inactivation of

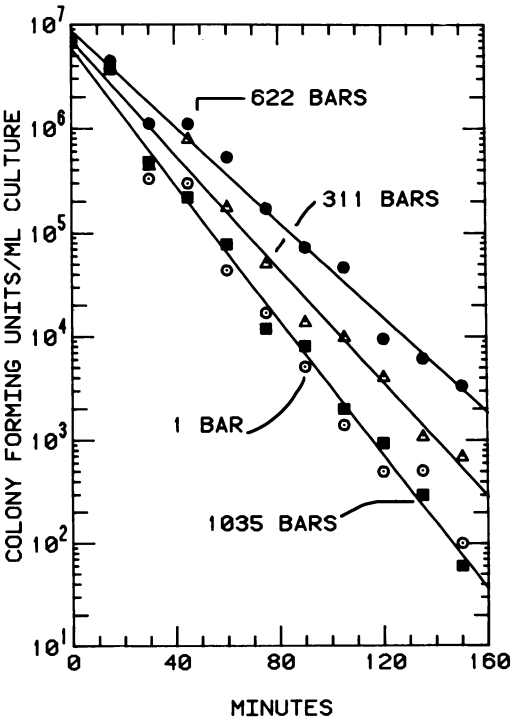

FIG. 3. Kinetics of the thermal inactivation of isolate CNPT-3 at 27°C as a function of pressure.

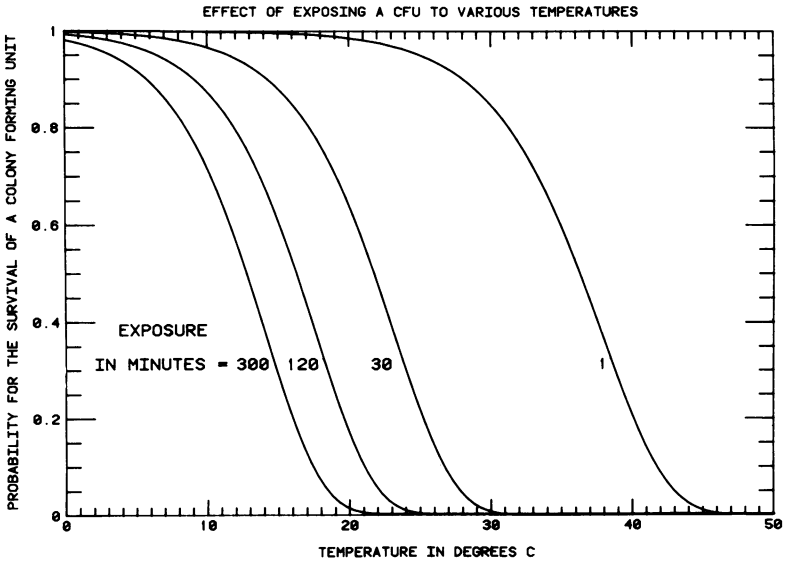

FIG. 4. These curves were generated, using equation 3, for exposures of 300, 120, 30 and 1 min. The latter exposure time was used to assess the effect of using warm liquid agar as a solidifying agent for the cultivation of bacteria. The longer exposure times are what might be encountered during a variety of oceanographic sampling procedures. These curves allow for an easy evaluation of sampling and cultivation procedures for the likelihood of yielding true deep-sea microbes.

colony-forming ability was interpreted as a first-order process,

$$-d(CFU)_t/dt = k(CFU)_t \quad (1)$$

in which $(CFU)_t$ is the number of colony-forming units at time t, and k is the first-order rate constant for the thermally induced inactivation. The experimentally determined rate constants were summarized with the Arrhenius equation (Fig. 2),

$$k = A \cdot \exp(-E_a/RT) \quad (2)$$

in which R is the gas constant, T is the absolute temperature, A is a preexponential constant, and E_a is the activation energy for the inactivation process. From a linear least-squares procedure (Fig. 2), we found that E_a = 42,695 cal (ca. 178.6 kJ)/mol and $A = 6.59 \times 10^{29}$/min. By combining equations 1 and 2,

$$(CFU)_t/(CFU)_0 = \exp(-A \cdot t \cdot \exp[-E_a/RT]) \quad (3)$$

in which $(CFU)_0$ is the number of colony-forming units initially present (at t = 0 min), and $(CFU)_t$ is the number of colony-forming units at time t, which is the duration of the exposure to the temperature T. We interpreted the ratio on the left-hand side of equation 3 as being the probability that a colony-forming unit would survive an exposure to a temperature, T, for t minutes. Equation 3 was used to generate the curves seen in Fig. 4. These clearly show that colony-forming ability of autochthonous deep-sea bacteria such as isolate CNPT-3 cannot be expected if samples are exposed to high temperatures.

Table 2 shows a sampling schedule used to obtain evidence of morphological changes caused by exposures to various temperatures. At 0 min, a culture of strain CNPT-3 at 2°C was decompressed from 587 bars and placed in a 0°C bath. Figure 5A shows cells with a normal appearance after 105 min at 0°C and at atmospheric pressure. This was expected, since strain CNPT-3 grew at atmospheric pressure (37; Yayanos et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, N59, p. 173), albeit slowly. Figure 5B shows cells exposed to 22°C for 75 min. The exposure to 22°C was actually less than 75 min since the warming of the culture occurred over a 30-min period. We estimated, on the basis of the thermal inactivation kinetics, that no more than 50% of the cells seen in Fig. 5B could have been colony-forming units. Thus, thermal inactivation preceded gross morphological changes.

TABLE 2. Sampling scheme for SEM[a] study

Temp. (°C)	Min of exposure of sample:		
	A	B	C
0	105	0	0
22	0	75	55
32	0	0	32

[a] SEM, Scanning electron microscope.

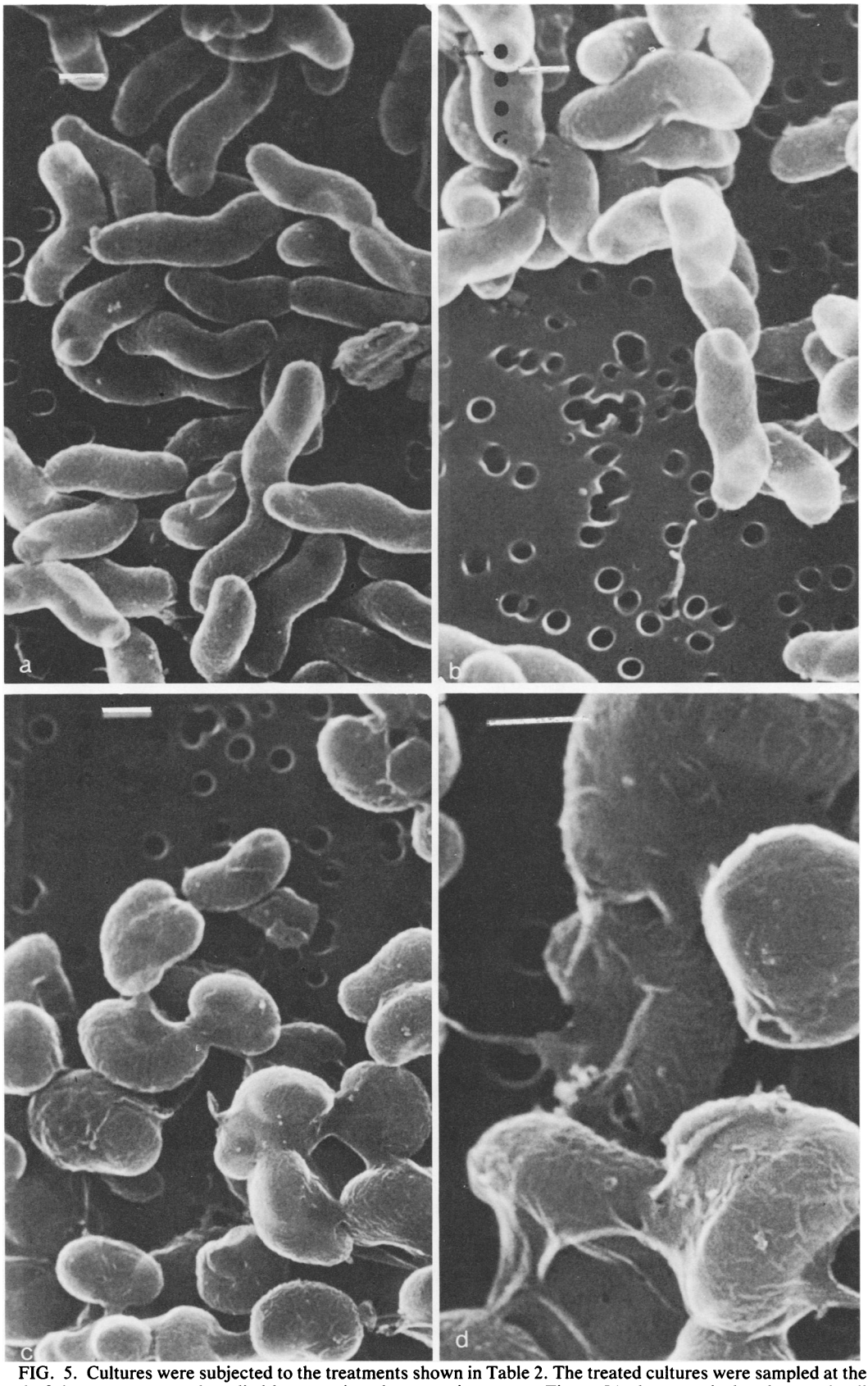

FIG. 5. Cultures were subjected to the treatments shown in Table 2. The treated cultures were sampled at the end of the treatments and studied by scanning electron microscopy. Figure 5A shows typical and normal cell morphology after 105 min at 0°C. Bar, 0.5 μm. Figure 5B shows cells almost normal in appearance after a 75-min exposure to 22°C. Bar, 0.5 μm. Figures 5C and D reveal drastically modified cell morphologies after an 87-min treatment (22°C for 55 min and 32°C for 32 min). Bars, 0.5 μm.

The latter are seen in Fig. 5C and D, which show cells from a culture exposed to 22°C for 55 min and then to 32°C for 17 min (see Table 2). Less than 1% of the cells in these cultures could have been colony-forming units as judged from the thermal inactivation kinetics.

These profound morphological changes were first noted by phase-contrast microscopy, which revealed the formation of partly transparent spheres. That is, the cells appeared to be curled up along the inside of a sphere having mostly poor phase contrast. This morphology was preserved with glutaraldehyde fixation (Fig. 5C and D). The cells appeared to be detached from their walls. Also, some substance seemed to be cross-linking several cells (Fig. 5D).

Both *Vibrio* and *Spirillum* species are known to transform into spherical cells in old cultures (9, 16). Further studies will be needed to determine the range of conditions leading to spherical cells in cultures of isolate CNPT-3.

DISCUSSION

Psychrophilic bacteria as defined by Morita (23) are those growing at temperatures within the range of 0 to 20°C, with the temperature for optimum growth at less than 15°C. Baross and Morita (4) have provided a tabulation of data on 20 marine psychrophilic bacteria (isolated from shallow portions of the deep sea). Only one has an upper temperature limit of less than 10°C (*Vibrio* AP-2-24, isolated [23] from Antarctic waters, grows at 9°C or less). Most of the tabulated psychrophilic bacteria can grow above 15°C. Thus strain CNPT-3, which was inactivated at 10°C at atmospheric pressure, ranked among the more psychrophilic marine bacteria known. We wish to find out if psychrophily is a general characteristic of the indigenous deep-sea bacteria (living in ca. 2°C waters).

Autochthonous or indigenous (as defined in reference 1, p. 9–10) bacteria in the ocean at 2°C and at depths greater than 2,000 m may thus be psychrophilic (13, 41) and barophilic (5, 37, 38). Those that are heterotrophs may additionally have a reasonably rapid generation time (about 10 to 30 h) (5, 37, 38) at deep-sea pressures and at 2°C when in nutrient-rich media. If proven universal in deep seas of 2°C, then these properties of true deep-sea microorganisms might allow for the design of experiments to determine the relative importance and the abundance of two classes (1) of microorganisms: (i) the indigenous deep-sea microbes (autochthonous species) and (ii) the invaders (allochthonous species). The latter could originate from shallow water, possibly from deeper water loci (e.g., hadal forms [38] entering abyssal regions), perhaps from warm deep seas (the Red Sea, the Mediterranean Sea, or the Sulu Sea), from warm water near hydrothermal vents, and from terrestrial sources. Research has clearly established the presence of shallow water forms in the deep sea (19).

There are four major possibilities regarding the nature of strain CNPT-3. First, the organism may be rare and atypical (21) of most deep-sea microorganisms. We do not conceive that this is the case since strain CNPT-3 grows best (37; Yayanos et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, N59, p. 173) at deep-ocean temperatures and pressures. The growth rate of strain CNPT-3 is sufficiently better at deep-ocean conditions of temperature and pressure than that of previously described bacterial isolates from depths comparable to 5,700 m, that it could easily competitively exclude such organisms as well as avoid displacement by microorganisms invading from shallower depths. The second possibility is that the nature of isolate CNPT-3 may be typical only of microbes living in nutrient-rich environments such as those found in the guts of animals. We do not know if strain CNPT-3 originated from the gut of an amphipod or from the decomposition processes ensuing from the death of cold, pressurized amphipods (37). We cannot imagine how the barophilic and psychrophilic qualities of strain CNPT-3 are linked to these niches. Some attempts of others to find barophilic bacteria associated with deep-sea animals have failed (27, 31, 36), but others have succeeded (5, 37, 38). This suggests that some aspect of the procedures used to retrieve the samples from the deep sea or of methods used to cultivate bacteria may have eliminated the true deep-sea bacteria sought. The third possibility is that the phenotypes of barophily and psychrophily may not be due to a genotype that confers these characteristics to all of the components of a cell. Marquis and Bender (21) have shown that pressure-tolerant variants of *Streptococcus faecalis* can be obtained by transferring the cultures over a period of time to increasingly higher pressures. Olsen and Metcalf (26) were able to make a psychrophile from a mesophile by transduction. We have begun experiments with isolate CNPT-3 to determine whether its phenotype is stable under maintenance at pressures lower than its optimum pressure of 400 bars. The ensuing discussion needs to be tempered with caution until the results of adaptation experiments are obtained. Nevertheless, the fourth possibility is that strain CNPT-3 is typical of most deep-sea bacteria. Then, how have bacteria with similar characteristics eluded cultivation in more than one laboratory (40, 41) until recently (5, 37, 38)? How have experiments done in situ (12, 13) or with pressure-retaining instruments (15) failed to find activities (such as uptake of nutrients) that are greater in situ than

TABLE 3. Evaluation of some typical procedures in deep-sea microbiology for the probability of yielding psychrophiles

Type of sample	Location	Water depth (m)	In situ conditions		Retrieval conditions[a]				Incubation conditions		Survival (P)[b]	Reference no.
			Temp at depth (°C)	Pressure at depth (bars)	T_{max} (°C)	Time at T_{max}	P_{min} (bars)	Time at P_{min}	Temp (°C)	Pressure (bars)		
Sediments from gravity and piston corers	Central North Pacific Ocean	1,707–5,942	ca. 2.5	173–601			1	Always		1	0	25
Sediments from cores and grabs	Philippine Trench	1,023–10,462	ca. 2.5	104–1,060			1	Hours to always	2.5 and 30	1 and 1,013	0–1	39
Water and sediments	Pacific Ocean	To over 5,000	20–2.5	A few bars to over 500	>22	Always	1	Always	22	1	0	17
Water taken with a Niskin bag sampler	Indian Ocean	To 2,000	20–2	Up to 200	>20	Always	1	Always	25	1	0–1	8
Sediment from a core	Western Atlantic Ocean	4,490	ca. 3	500			1	Always	4	1	0–1	30
Sediments from gravity corers	Puerto Rico Trench	8,130 and 7,750	ca. 2.5	825 and 786	ca. 8		1		3	826 and 785	0–1	29
Gut contents of an amphipod	Aleutian Trench	7,050	ca. 1.5	715			1		3	760	0–1	31
Sediments and Mn nodules from dredges and cores	Atlantic and Pacific Oceans	396–5,182	ca. 2.5	4–525			1	Always	14–18	1	0	3, 7
Water under pressure	Atlantic Ocean	3,550–5,225	ca. 2.5	360–529	3	Always	360 and 529	Always	3	360 and 529	1	34
Amphipods under pressure and at atmospheric pressure	Philippine Trench	9,600	ca. 2.5	960	28	30 min	1	30–60 min	2	960	0	[c]
Amphipods in a pressure-retaining trap	Central North Pacific Ocean	5,700	ca. 2.5	580	<10	30 min	580	5 months	3	580	1	37

[a] Maximum temperature (T_{max}) and minimum pressure (P_{min}) at which samples were retrieved.
[b] Probability of survival was estimated from equation 3 or from Fig. 5.
[c] Unpublished data from one of our own cruises in 1975.

in controls done at atmospheric pressure?

These two questions may be answered almost completely with only one assumption about the nature of the bacteria indigenous to the 2°C regions of the deep sea: that they are as psychrophilic as strain CNPT-3 is. This assumption remains to be proven and, in fact, is a testable hypothesis for future studies. Table 3 was compiled from a few typical deep-sea microbiological studies to address the first of the above two questions. The studies remain as valuable data on the microbiology of the deep sea. The time of exposure to temperatures other than in situ ones was estimated from the data or methods described in each paper and the consequences of the exposure evaluated using equation 3 or Fig. 4. Microbes in both samples and incubations were in many cases exposed long enough to elevated temperatures to have greatly reduced the probability of the survival of colony-forming units as psychrophilic as isolate CNPT-3 is. Thus, it may be that some of the studies in Table 3 were of the allochthonous deep-sea microbes. Table 3 shows, furthermore, that data of long-recognized importance (15, 41) on the thermal and pressure history of samples are often lacking in reports of deep-sea microbiology.

Barophilic responses were not seen in several experiments conducted in situ (12, 13) and with undecompressed water samples (12, 14). A rigorous analysis of these studies is difficult because the studies were with natural populations and dealt with possible patchy distributions of microbes in the ocean. The following analysis is offered as one line of reasoning that may in part account for the apparent difference between those results and ours with respect to the occurrence of barophily. We noted the following uncertainties with some of the work on natural populations. (i) The values of increased temperature and the duration of exposure to them during sampling are often not given. (ii) Sometimes the pressurized metal samplers were deployed without any thermal insulation (see Fig. 4 in reference 12; means of achieving insulation with this sampler have been discussed in reference 15) in regions in which even the 10°C isotherm is at a depth greater than 800 m. We hope that the future use of these samplers will be accompanied with data on their thermal history during recovery and shipboard handling. Only then can an objective, rather than a conjectural, analysis be done. (iii) Samples for "controls" incubated at atmospheric pressure were often taken with a Niskin bag sampler (14). This device, in our experience, results in the warming of sampled cold deep-sea water to temperatures close to those of the surface waters. Results from these controls at atmospheric pressure may be a function of the duration and extent of warming before incubation at atmospheric pressure and at 2°C (4). Even without warming, the incubation at atmospheric pressure of a deep-sea sample (14, 32) containing an unknown proportion of autochthonous and allochthonous microbes cannot serve as a control without recognition of the changes imposed by the decompression. The two classes of microorganisms present in unknown proportion will respond differentially to decompression in a manner dependent on the species composition of the community, on the depth of origin of the sample, and on a host of other factors.

Thus, studies on populations observed in situ or with undecompressed water samples may best be interpreted absolutely with reference perhaps only to other similarly done studies as a function of depth of origin, locale, or treatment to poisons in replicate samples. As knowledge of the characteristics of the autochthonous deep-sea bacteria emerges, then treatments of samples to, for example, heat, may allow for the selective elimination of the activities of the autochthonous bacteria. Reincubation of a heat-treated sample at deep-sea pressures could then provide one means of evaluating the relative roles of autochthonous and allochthonous bacteria.

The molecular basis of thermophily has been extensively studied (33). A similarly detailed understanding of barophiles remains to be achieved. The results of this study and two recent ones (37, 38) led to the hypothesis that autochthonous deep-sea bacteria are extremely psychrophilic and barophilic. These two characteristics may prove to be of great taxonomic importance for marine bacteria. These results further showed that the isolation of these bacteria does not require overcoming "enormous technical problems" (18). Pressure-retaining samples will continue to have an important place in oceanography; but the majority of marine microbiology is approachable with samplers thermally insulated and with cultivations at high pressures. Recognition of these significant conclusions will greatly accelerate the acqusition of understanding of the long-acknowledged (11, 18, 39) importance of high pressures and low temperatures in deep-sea microbiology.

ACKNOWLEDGMENTS

We are greatly indebted to Kristin Jones for skillful electron microscopy.

This work was supported by grant OCE79-08972 from the National Science Foundation and by a contract from Sandia National Laboratories, a prime contractor for the U.S. Department of Energy.

LITERATURE CITED

1. **Alexander, M.** 1971. Microbial ecology. John Wiley & Sons, Inc., New York.

2. **Alsobrook, D., J. M. Larkin, and M. W. Sega.** 1972. Effect of temperature on the cellular integrity of *Bacillus psychrophilus*. Can. J. Microbiol. **18**:1671–1678.
3. **Arcuri, E. U., and H. L. Ehrlich.** 1977. Influence of hydrostatic pressure on the effects of the heavy metal cations of manganese, copper, cobalt, and nickel on the growth of three deep-sea bacterial isolates. Appl. Environ. Microbiol. **33**:282–288.
4. **Baross, J. A., and R. Y. Morita.** 1978. Microbial life at low temperatures: ecological aspects, p. 9–71. *In* D. J. Kushner (ed.), Microbial life in extreme environments. Academic Press, Inc., New York.
5. **Deming, J. W., P. S. Tabor, and R. R. Colwell.** 1981. Barophilic growth of bacteria from intestinal tracts of deep-sea invertebrates. Microb. Ecol. **7**:85–94.
6. **Dietz, A. S., and A. A. Yayanos.** 1978. Silica gel media for isolating and studying bacteria under hydrostatic pressure. Appl. Environ. Microbiol. **36**:966–968.
7. **Ehrlich, H. L., W. C. Ghiorse, and G. L. Johnson.** Distribution of microbes in manganese modules from the Atlantic and Pacific oceans. 1972. Dev. Ind. Microbiol. **13**:57–65.
8. **Fell, J. W.** 1967. Distribution of yeasts in the Indian Ocean. Bull. Mar. Sci. **17**:454–470.
9. **Felter, R. A., R. R. Colwell, and G. B. Chapman.** 1969. Morphology and round body formation in *Vibrio marinus*. J. Bacteriol. **99**:326–335.
10. **Griffiths, R. P., and R. D. Haight.** 1973. Reversible heat injury in the marine psychrophilic bacterium *Vibrio marinus* MP-1. Can. J. Microbiol. **19**:557–561.
11. **Inniss, W. E.** 1975. Interaction of temperature and psychrophilic microorganisms. Annu. Rev. Microbiol. **29**:445–463.
12. **Jannasch, H. W.** 1979. Microbial turnover of organic matter in the deep sea. Bioscience **29**:228–232.
13. **Jannasch, H. W., and C. O. Wirsen.** 1977. Microbial life in the deep sea. Sci. Am. **236**:42–52.
14. **Jannasch, H. W., C. O. Wirsen, and C. D. Taylor.** 1976. Undecompressed microbial populations from the deep sea. Appl. Environ. Microbiol. **32**:360–367.
15. **Jannasch, H. W., C. O. Wirsen, and C. L. Winget.** 1973. A bacteriological pressure-retaining deep-sea sampler and culture vessel. Deep-Sea Res. **20**:661–664.
16. **Krieg, N. R.** 1976. Biology of the chemoheterotrophic spirilla. Bacteriol. Rev. **40**:55–115.
17. **Kriss, A. E., I. E. Mishustina, N. Mitskevich, and E. V. Zemtsova.** 1967. Microbial population of oceans and seas. St. Martin's Press, New York.
18. **MacLeod, R. A.** 1965. The question of the existence of specific marine bacteria. Bacteriol. Rev. **29**:9–21.
19. **Malone, T. C., C. Garside, R. Anderson, and O. A. Roels.** 1973. The possible occurrence of photosynthetic microorganisms in deep-sea sediments of the North Atlantic. J. Phycol. **9**:482–488.
20. **Marquis, R. E.** 1976. High-pressure microbial physiology, p. 159–241. *In* A. H. Rose and D. W. Tempest (ed.), Advances in microbial physiology, vol. 14. Academic Press, Inc., New York.
21. **Marquis, R. E., and G. R. Bender.** 1980. Isolation of a variant of *Streptococcus faecalis* with enhanced barotolerance. Can. J. Microbiol. **26**:371–376.
22. **Marquis, R. E., and P. Matsumura.** 1978. Microbial life under pressure, p. 105–157. *In* D. J. Kushner (ed.), Microbial life in extreme environments. Academic Press, Inc., London.
23. **Morita, R. Y.** 1975. Psychrophilic bacteria. Bacteriol. Rev. **39**:144–167.
24. **Morita, R. Y.** 1980. Microbial life in the deep sea. Can. J. Microbiol. **26**:1375–1385.
25. **Morita, R. Y., and C. E. Zobell.** 1955. Occurrence of bacteria in pelagic sediments collected during the mid-pacific expedition. Deep-Sea Res. **3**:66–78.
26. **Olsen, R. H., and E. S. Metcalf.** 1968. Conversion of mesophilic to psychrophilic bacteria. Science **162**:1288–1289.
27. **Pope, D. H., W. P. Smith, M. A. Orgrinc, and J. V. Landau.** 1976. Protein synthesis at 680 atm: is it related to environmental origin, physiological type, or taxonomic group? Appl. Environ. Microbiol. **31**:1001–1002.
28. **Robison, S. M., and R. Y. Morita.** 1966. The effect of moderate temperature on the respiration and viability of *Vibrio marinus*. Z. Allg. Mikrobiol. **6**:181–187.
29. **Schwarz, J. R., and R. R. Colwell.** 1975. Heterotrophic activity of deep-sea sediment bacteria. Appl. Microbiol. **30**:639–649.
30. **Schwarz, J. R., J. D. Walker, and R. R. Colwell.** 1975. Deep-sea bacteria: growth and utilization of n-hexadecane at *in situ* temperature and pressure. Can. J. Microbiol. **21**:682–687.
31. **Schwarz, J. R., A. A. Yayanos, and R. R. Colwell.** 1976. Metabolic activities of the intestinal microflora of a deep-sea invertebrate. Appl. Environ. Microbiol. **31**:46–48.
32. **Seki, H.** The role of microorganisms in the marine food chain with reference to organic aggregate. 1972. Proceedings of the IBP-UNESCO Symposium on Detritus and its role in aquatic ecosystems. Mem. Ist. Ital. Idrobiol. Dott Marco de Marchi Pallanza Italy **29**(Suppl.):245–259.
33. **Singleton, R., Jr., and R. E. Amelunxen.** 1973. Proteins from thermophilic microorganisms. Bacteriol. Rev. **37**:320–342.
34. **Tabor, P. S., J. W. Deming, K. Ohwada, H. Davis, M. Waxman, and R. R. Colwell.** 1981. A pressure-retaining deep ocean sampler and transfer system for measurement of microbial activity in the deep sea. Microb. Ecol. **7**:51–65.
35. **Wirsen, C. O., and H. W. Jannasch.** 1975. Activity of marine psychrophilic bacteria at elevated hydrostatic pressures and low temperatures. Mar. Biol. **31**:201–208.
36. **Yayanos, A. A.** 1980. Measurement and instrument needs identified in a case history of deep-sea amphipod research, p. 307–318. *In* F. D. Diemer, F. J. Vernberg, and D. Z. Mirkes (ed.), Advanced concepts in ocean measurements for marine biology. University of South Carolina Press, Columbia, South Carolina.
37. **Yayanos, A. A., A. S. Dietz, and R. Van Boxtel.** 1979. Isolation of a deep-sea barophilic bacterium and some of its growth characteristics. Science **205**:808–810.
38. **Yayanos, A. A., A. S. Dietz, and R. Van Boxtel.** 1981. Obligately barophilic bacterium from the Mariana Trench. Proc. Natl. Acad. Sci., U.S.A. **78**:5212–5215.
39. **ZoBell, C. E.** 1952. Bacterial life at the bottom of the Philippine Trench. Science **115**:507–508.
40. **ZoBell, C. E., and R. Y. Morita.** 1957. Barophilic bacteria in some deep-sea sediments. J. Bacteriol. **73**:563–568.
41. **ZoBell, C. E., and R. Y. Morita.** 1959. Deep-sea bacteria. Galathea Rep. Copenhagen **1**:139–154.