A Pressurized Chemostat for the Study of Marine Barophilic and Oligotrophic Bacteria†

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A continuous culture system that allows bacteria to be grown in steady-state populations under pressures of up to 700 atm (71 MPa) was constructed and tested. With readily available or slightly modified high-pressure chromatography equipment, a continuous flow of sterile medium is pressurized and passed through a 500-ml nylon-coated titanium reactor at flow rates of 0.01 to 10 ml min⁻¹. The pressure in the reactor is controlled by a backpressure regulator with greater than 1% accuracy. In test experiments, a culture of a psychro- and barophilic marine isolate from a depth of 4,900 m (strain F1-A, identified as a *Shewanella* sp.) was grown at 1, 300, and 450 atm (0.1, 30.4, and 40.5 MPa) and dilution rates of 60 and 90% of the organism's maximum growth rate (determined at 1 atm) in the required complex medium at levels of 3.3 and 0.33 mg of dissolved organic carbon per liter in the reservoir. Growth limitation by carbon was assured by an appropriate C/N/P ratio of the medium. The data indicate that barophilic growth characteristics in steady-state cultures of this psychro- and barophilic deep-sea isolate were positively affected by a decreasing growth rate at the higher of two substrate concentrations in the reservoir. After a 10-fold lowering of the substrate concentration, the effect was reversed. Under these conditions, the cell viability increased significantly, especially at the higher of the two pressures tested. The basic design of the system can principally also be used for growth studies on hyperthermophilic bacteria and archaea.

The need for a pressurized continuous-culture system arises from the fact that the assumed oligotrophy or oligocarbophily of barophilic deep-sea bacteria cannot be studied in batch culture. Measuring growth rates in relation to an extremely low initial concentration (S) of the specific growth-limiting nutrient meets with the difficulty that two artificial growth phases, lag and stationary phase, may overlap and thereby obscure the exponential rate of cell number increase (10, 12). In contrast, steady-state cultures, as attainable in a chemostat, allow the growth of a cell population to be measured in the presence of extremely low concentrations of the growth-limiting substrate, expressed by the difference between S_o (the concentration of the limiting substrate in the reservoir) and s (the substrate concentration in the chemostat at steady state):

$$P = (S_o - s)y \quad (1)$$

where P is the product of substrate conversion, e.g., population density, and y is a conversion or yield constant. The substrate level at steady state (s) may be unmeasurably low or attain a threshold concentration below which growth ceases (7, 10). It is determined by the dilution rate of the experiment (D) and the organism's growth parameters, maximum growth rate, $\mu_{\max}$, and substrate saturation constant, K_s :

$$s = K_s (D/\mu_m - D) \quad (2)$$

This simple theoretical model (3) has proven to be useful in a number of ecological studies on microbial growth response to environmental factors (9, 10) in which pure cultures and single-substrate growth limitation were prerequisites. Technical problems in controlling this limitation at decreasing substrate concentrations and dilution rates, often leading to premature washout, have been discussed earlier (6, 10).

A specific problem in working with the available psychro- and barophilic deep-sea isolates is their general requirement for a complex substrate and its adjustment to ensure carbon limitation.

While incubating a chemostat culture at low temperature in a water bath posed no problem, the pressurizing and depressurizing of medium at a low and constant flow rate at up to 700 atm (71 MPa) was more difficult. In the following, we describe a pressurizable chemostat and the results of a series of growth experiments with a psychro- and barophilic deep-sea isolate, strain F-1A (11). Our choice not to go beyond pressures of 700 atm (71 MPa) was based on economic and ecological considerations. Less than 1% of the ocean's volume lies below a depth of 7,000 m (15).

MATERIALS AND METHODS

Design and construction of a pressurized chemostat. The requirements for a pressurized continuous-culture system are the pressurization of sterile medium during constant flow at low rates, the maintenance of constant pressure in the well-mixed reaction vessel, and decompression and collection of spent medium.

Besides the medium reservoir and receiving vessel, the seven major parts of the system (Fig. 1) are the titanium pump head (P), programmable drive module (DM), pressure module including a pulsation damper (PM), injection valve (V; optional; detached in Fig. 2), chemostat vessel (CV), detachable subsampling chamber (SC), and backpressure valve (BV). The first three components, made by Rainin Instrument Co. (part numbers are given in parentheses), introduce the culture medium at the programmed pressure and flow rate (range, 0.01 to 10 ml min⁻¹) into the culture vessel. The pressure module (PM; 48-805) includes a built-in transducer for pressure reading and a pulsation damper to mitigate pressure spikes from the pump (P; 48-010-WTI) and the drive module (DM; HPXL). Standard high-pressure liquid chromatography (HPLC) tubing is used with taper seal valves (High Pressure Equipment Co.). An optional ALTEX autoclavable injection valve (ALTEX Division of Beckman Instruments, Inc.; 210A) with loop volumes of 5 to 2,000 μ l is placed near the culture vessel inlet port.

The syringe (S), as depicted in Fig. 2, serves for sterilizing and priming the pump head. The peristaltic pump (PP) and the distilled-water reservoir (DW) are required for preventing salt build-up in the titanium pump head (P). This separate rinsing cycle ensures the continuous running of the pressure pump. As these auxiliary items (S, PP, and DW) are not part of the chemostat's actual medium flow system, they are not included in the simplified scheme in Fig. 1.

The culture vessel is machined from a solid billet of Ti 6AL-4V ELI titanium

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FIG. 1. Scheme of pressurized chemostat. R, medium reservoir; P, titanium pump head; DM, HPLC drive module; PM, pressure module; V, injection valve (5 μ l to 2 ml); W, constant-temperature water bath; CV, titanium culture vessel (500 ml); M, magnetic stirrer; SC, subsampling chamber; BV, backpressure regulating valve; RV, receiving vessel; heavy line, pressurized part of the system (modified from reference 11).

alloy. This material was chosen for its superior strength and corrosion resistance. The top cap of the culture vessel is sealed to the bottom part by a single O-ring-type radial T seal and fastened by eight cap screws. The entire inside surface of the culture vessel is nylon coated to prevent potentially toxic metal ions from entering the medium. The culture vessel was designed for an operating pressure of 700 atm (71 MPa) and has been proof tested at 1,000 atm (101.2 MPa). During operation, the culture vessel (CV), containing a stirring bar, is immersed in a constant-temperature water bath (W) over a magnetic stirrer (M).

In order to set and maintain constant pressure in the system, a mechanical backpressure regulating valve (BV; Tescom Corp.; 26-1761-99-160) is connected to the tubing near the outlet of the culture vessel. This regulator is a hand-adjustable, spring-operated, piston-sensing device controlling the valve's inlet pressure. The valve opens when the pressure is slightly above and closes when the pressure is slightly below the set value. Adjustable from 1 to 700 atm (71 MPa), the pressure setting is maintained at $\pm 0.5\%$.

The autoclavable subsampling chamber (SC) can be attached to the culture vessel via an outlet valve for the removal of up to 10 ml of culture medium. This unit was machined from the same material as the culture vessel and is a cylinder

with a screw-operated piston. A T-handle allows the operator to extract precisely a known quantity of liquid by counting the number of turns. During subsampling, the flow rate is increased, the stirring is stopped, and the sample is taken slowly in order to prevent a drop in pressure.

The entire chemostat system was completed during the summer of 1994 and has been in operation since that time.

Organism. The psychro- and barophilic isolate F1-A was originally obtained from a pressure-retaining water sample collected at a depth of 4,900 m in the North Atlantic ocean (11). According to 16S RNA sequencing data, the isolate belongs to the genus *Shewanella* (1a). Stock cultures are maintained in Difco 2216 marine broth at 1 and 300 atm (30.4 MPa) (pressure for optimum growth) and in liquid nitrogen.

Growth medium. The medium for these chemostat experiments consists of artificial seawater (ASW) with the following composition (in grams per liter): NaCl, 20.0; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 3.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.0; $(\text{NH}_4)_2\text{SO}_4$, 1.0; NaHCO_3 , 0.2; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3; KCl, 0.5; and KH_2PO_4 , 0.015, plus trace element solution (SL-8), 1 ml (16), vitamin solution, 5.0 ml (1), and distilled water, 1,000 ml. The phosphate, trace element solution, and vitamins were added from filter-sterilized solutions to the autoclaved basal medium after cooling. The final pH was 7.3. Bacto yeast extract (Difco) was added from an autoclaved stock solution to render concentrations of 10 or 1.0 mg/liter in the final medium. These concentrations correspond to 3.3 and 0.33 mg of C per liter, respectively (1b, 4). To ensure growth limitation by a source of organic carbon, the C/N/P ratio of the yeast extract medium was set at 10:630:10 for the higher and 10:6,300:100 for the lower concentration. The specific limiting source of carbon in the yeast extract medium is not known.

Inoculum and culture. A culture of strain F1-A was pregrown in 100 mg of yeast extract per liter of ASW at 3°C and 1 atm. Approximately 3 ml of this culture was inoculated into the sterile pre-filled (500 ml) chemostat vessel to give an initial cell density of about 2×10^4 cells per ml. The chemostat was then pressurized, and the cells were allowed to grow as a batch culture for 48 h before the flow was turned on. The flow rate was set at 0.534 or 0.355 ml/min, to result in dilution rates equivalent to 90 and 60%, respectively, of the maximum growth rate determined earlier in batch culture at 1 atm and 3°C, namely, $D = 0.066$ and 0.044 h^{-1} . A circulating water bath kept the temperature of the stirred chemostat culture constant at 3°C. The pressure was kept constant by the backpressure regulating valve.

Subsampling. After a medium flow for a minimum of at least six retention times, a sample of 5 to 6 ml was taken with the sterilized subsampling device for

FIG. 2. Photo of chemostat system excluding the water bath. The symbols are the same as in Fig. 1 plus S, syringe, used for the chemical sterilization of the pump head and priming of the medium flow; PP, peristaltic pump; and DW, sterile distilled-water reservoir, both needed for the continuous flushing of the pump head.

FIG. 3. Steady-state population densities of the psychro- and barophilic deep-sea isolate F1-A as measured by direct (microscopic) count (shaded bars) and viable (plate) count (solid bars), grown at 3°C with two limiting concentrations of yeast extract (YE), at two dilution rates (D), and at three pressures. (A) YE, 10 mg/liter (3.3 mg of carbon per liter), $D = 0.066 \text{ h}^{-1}$. (B) YE, 10 mg/liter, $D = 0.044 \text{ h}^{-1}$. (C) YE, 1 mg/liter (0.33 mg of carbon per liter), $D = 0.066 \text{ h}^{-1}$. (D) YE, 1 mg/liter, $D = 0.044 \text{ h}^{-1}$.

cell counting to determine the establishment of steady states of the population and the ratio of viable to nonviable cells. Following the sampling at one steady state, the flow rate or pressure was changed, and the culture was allowed to establish a new steady state.

Growth measurements. Growth was measured by cell counts over time only. In the subsamples removed from the chemostat, cell densities were too low for determinations of dry weight, optical density (OD), or protein. The total cell number was determined by epifluorescence microscopy with acridine orange (5). Counts of viable cells recoverable after exposure of the suspension to pressure were determined from duplicate plates streaked by dilutions that resulted in 10 to 300 colonies per plate. The medium used was Difco 2216 marine agar, and the plates were incubated at 3°C.

RESULTS

Functioning of the chemostat. During its operation over more than a year, the pressurized chemostat worked extremely well and no major problems were encountered. Changing the substrate concentration in the reservoir between experiments requires dismantling and reautoclaving. A Teflon coating inside the culture vessel started to peel off after the first several pressure cycles. When a second coating resulted in the same problem, we switched to a Rilsan nylon coating, which was less expensive and is performing well. After 6 months of operation, the Vespel seat (a polyamide high-performance plastic from Dupont) in the backpressure regulating valve had to be replaced. This is considered routine maintenance. By upgrading the seat material to sapphire, the number of operating cycles could be increased.

Data. As shown in Fig. 3, the variables in the series of four growth experiments were pressure, substrate concentration, and dilution rate (imposed growth rate). Optimum growth at 300 atm, as originally determined in batch culture experiments (11), remained the same in chemostat cultures. Barophily, as defined here by the difference between the population densities at 1 and 300 atm, increased when the growth rate (imposed dilution rate) was lowered at the higher substrate concentration (10 mg of yeast extract per liter) (Fig. 3A and B). It

decreased, however, at the same lower growth rates when supplied with a 10-fold-lower substrate concentration (Fig. 3C and D). Cell viability, defined by the recovery of cells on agar plates compared with microscopic counts, increased significantly at the lower of the two substrate concentrations even beyond the pressure for optimum growth (450 atm) (Fig. 3C and D).

DISCUSSION

The construction and operation of a pressurized chemostat appeared to be easier and less expensive than expected. The availability of off-the-shelf items, developed primarily for high-pressure liquid chromatography, greatly reduced the costs calculated earlier for the development of the necessary pump system. The same is true for the backpressure regulating valve. The whole setup is a relatively small benchtop item, which contributes to its usefulness. In spite of its use at fairly high pressures, the lack of a gas phase eliminates cumbersome safety measures.

The argument may be made that the amounts of dissolved organic carbon used in these experiments (0.33 and 3.3 mg/liter [0.027 and 0.27 mM]) still somewhat exceed the range of concentrations reported for the pelagic and deep ocean, namely, 0.03 to 0.2 mM (2, 14, 16) or, in earlier measurements, 0.029 to 0.058 mM (13). However, this point disregards the fact that the quantity of dissolved organic carbon in seawater does not theoretically correspond to the concentration of growth-limiting substrate in the reservoir of the chemostat, S_o in equation 1, but to the amount that is used for growth in steady-state populations, i.e., $S_o - s$ (8). Not yet knowing and being unable to measure the specific growth-limiting substrate remains a drawback of these studies. On the other hand, a seemingly better-suited isolate, growing on—but also limited to the use of—a clearly defined and measurable substrate might not be representative of typical deep-sea bacteria.

Although the described system was conceived for studies on oligocarbophilic bacteria, which are mostly, if not exclusively, psychrophiles, it can principally and with little modification also be applied to the study of hyperthermophilic microorganisms. Here, at temperatures higher than 100°C, pressurization has the purpose of keeping water liquid. For the presently known maximum growth temperatures of ca. 113°C in a sand bath for studying steady-state growth responses, the system should work as is. In contrast to the noncorrosiveness of the titanium reactor, the possible effect of hydrogen sulfide, produced by many of the hyperthermophilic archaea and bacteria, on the backpressure regulating valve has not yet been tested.

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