Teflon Chemostat for Studies of Trace Metal Metabolism in *Streptococcus mutans* and Other Bacteria

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A Teflon chemostat constructed for studies of microbial trace metal metabolism is described. The utility of this continuous culture system was demonstrated with *Streptococcus mutans*, in which iron and manganese stimulated growth in ranges of 0.18 to 0.45 and 18 to 54 μM, respectively. This device should facilitate studies of the effects of trace metals on a variety of physiological functions.

Certain trace metals are essential for the growth of microorganisms, and the presence and concentration of other metal ions may have significant effects on microbial metabolism (3, 7, 9, 11). The optimum or effective concentrations of metals may be low. Batch culture procedures may be inadequate for demonstration of a requirement for some metals, because simple attainment of maximum growth (without addition of the test metal) is not a sensitive measure of a requirement (2; C. A. Dawkins and B. R. Byers, unpublished data). Moreover, the possible changes in rates of cell division that may occur after addition of a metal may be difficult to detect in batch cultures. Even when a chemically defined medium (prepared from high-purity ingredients) is used in batch cultures, carry-over of trace metals with (or in) the inoculum may mask an effect caused by addition or deletion of a metal. Additionally, treatment of culture media by some procedure may be necessary to lower trace metal contamination below required levels.

Continuous cultivation in a chemostat (6) should allow definitive measurement of changes in physiology caused by alteration of the concentration of a trace metal. However, all commercially available chemostats have stainless steel and glass components that are in contact with the culture and incoming medium. Leaching of trace elements from these components is an uncontrolled variable. For studies of iron metabolism in continuous cultures of *Neisseria meningitidis*, Archibald and DeVoe (2) modified a commercial fermentor by replacing some of the stainless steel components with glass devices and by coating other stainless steel parts with polyvinyl chloride. They reported response of the organism to an iron concentration of 0.4 μM (22 ng/ml). For batch production of several microbial siderophores (this type of batch production requires cultivation of the organisms in low-iron medium), we have used a 20-liter fermentor (Virtis 43-100) in which stainless steel components were coated with Teflon. Although this device contained a glass culture vessel, good siderophore production was obtained with several organisms (B. R. Byers and J. E. L. Arceneaux, unpublished data).

In the present work, we constructed and tested a chemostat made of Teflon to overcome problems of trace metal contamination from culture vessels and medium reservoirs (Fig. 1 and 2). The only non-Teflon components were the glass pH and dissolved-oxygen probes and short pieces of silicone rubber tubing that necessarily passed through the peristaltic pumps. The culture chamber was specially made from a 1-liter Teflon screw-top jar (Savillex 01-1000) by Savillex Corp., Minnetonka, Minn. Tight-sealing Teflon panel mounts (connectors) of appropriate diameters were installed in the lid for entrance of the probes and the input-output lines. The working volume was 600 ml. Two designs were used to maintain constant volume. For simple overflow, a 0.5-inch (1.27-cm) Teflon connector (with a 0.5-inch Teflon line attached to a collection vessel) was inserted in the side of the vessel at an appropriate height to maintain a 600-ml volume. The overflow configuration may be unsuitable for continuous cultivation of organisms that aggregate excessively, because clumps of cells formed in the overflow lines may block the effluent. Constant volume also was achieved by pump-out, which may overcome the problem of aggregates of cells in overflow lines. For this configuration, a Teflon line was inserted through the lid so that the lower end of the line corresponded to the desired liquid level. A continuously operating peristaltic pump (Pharmacia P3) attached to this line by silicone rubber tubing maintained constant volume.

The pH was monitored and controlled with a sterilizable pH probe (Ingold 465-K9) connected to a pH controller (Chemtrix 45AR). A sterilizable dissolved-oxygen probe (New Brunswick M
FIG. 1. Schematic diagram of the Teflon chemostat (effluent pump-out configuration). 1, Teflon culture chamber; 2, pH control system consisting of sterilizable pH electrode, pH controller-recorder, peristaltic pump, and acid-base reservoir(s); 3, effluent system consisting of peristaltic pump and collection vessel; 4, gas exhaust; 5, gas input consisting of gas source, flowmeter, and 0.22-μm-pore-diameter filter (routinely, a single port was used for both gas input and gas exhaust by inserting the gas delivery tube into the culture through the larger-diameter gas exhaust tube); 6, sampling device; 7, addition port; 8, influent system consisting of Teflon reservoir and peristaltic pump; 9, dissolved-oxygen monitor consisting of sterilizable dissolved-oxygen probe and analyzer-recorder; 10, constant-temperature circulating water bath; 11, Teflon-coated magnetic stirring bar; 12, magnetic stirrer.

1016-02808, without stainless steel holder) also was inserted and connected to a dissolved-oxygen analyzer (New Brunswick DO-50). Medium input was accomplished with a high-performance peristaltic pump (Harvard 1203) from 2-liter Teflon bottles that served as reservoirs. For pH control, acid and base additions (from Teflon bottle reservoirs) were done with peristaltic pumps (Markson R1300 or Sigmamotor AL2E34) activated by the pH controller. Gas was delivered through an in-line 0.22-μm-pore-diameter filter (Millipore Millex-GS); gas flow was adjusted with a flowmeter (Matheson Rotameter). The culture chamber was placed in a constant-temperature water bath (Braun Thermomix 1420 BKU constant-temperature circulator and 1400 plastic tank) that was seated on a magnetic stirrer (Thermolyne S18525). The culture was stirred with a Teflon-coated magnetic bar. For periodic sampling, a closed vacuum sampler was devised from a Teflon column segment (Savillex 504-24). Teflon tubing was used for all gas and liquid transfer. The culture system was sterilized by autoclaving.

The Teflon chemostat was used to measure trace metal requirements of Streptococcus mutans. This organism was selected because of its
suspected association with dental caries and because of the recognized cariogenic or cariostatic properties of certain trace elements (other than fluoride) (5). The culture medium was a modification of the chemically defined medium previously designated as FMC medium (10). To lower trace metal contamination, all ingredients except the amino acids, vitamins, and nucleic acid bases were treated by previously described methods (1) with Chelex-100 (Bio-Rad Laboratories). The sodium carbonate was deleted and replaced with continuous CO₂ input (35 ml/min), which maintained less than 1% dissolved oxygen in the cultures. These procedures lowered the concentrations of magnesium, manganese, and iron below the levels detectable by atomic absorption spectrophotometry (0.3, 0.4, and 0.7 μM, respectively). The medium was sterilized by filtration, and the previously listed metals were added as filter-sterilized solutions of high-purity sulfate salts (Johnson-Matthey) at desired concentrations. Water used for preparation of reagents and the medium was purified by reverse osmosis (Millipore RO-40) and a charcoal-demineralizer column system (Millipore Milli-Q). Growth of S. mutans was determined by quintuplicate dry-weight determinations in which 3-ml aliquots of culture were filtered through previously dried and weighed 0.45-μm-

FIG. 3. Effect of manganese on growth of S. mutans BHT in the Teflon chemostat. The culture first was grown as a batch culture in the chemostat at 35°C in modified FMC medium (pH 6.5) containing 420 μM magnesium, 180 μM manganese, and 18 μM iron, with CO₂ input at 35 ml/min. At 11 h, medium input was started at a dilution rate of 0.23/h; at 33 h, the manganese was removed from the incoming medium. Beginning at 58 h, stepwise increases in manganese concentration were made at indicated concentrations and times. Growth was determined by dry-weight measurements.

FIG. 4. Effect of iron on growth of S. mutans BHT in the Teflon chemostat. The culture first was grown in the chemostat as a batch culture at 35°C in modified FMC medium (pH 6.5) containing 420 μM magnesium, 54 μM manganese, and no added iron, with CO₂ input at 35 ml/min. At 11 h, medium input was started at a dilution rate of 0.23/h. Beginning at 52 h, stepwise increases in iron concentration were made at indicated concentrations and times. Growth was determined by dry-weight measurements.

pore-diameter filters. The filters then were washed with an equal volume of water, dried at 110°C for 1 h, cooled, and reweighed to determine the dry weight of the organisms.

The data in Fig. 3 illustrate one of the strategies that may be used to determine trace metal requirements. S. mutans strain BHT (obtained from G. D. Shockman) required addition of both magnesium and manganese for growth in the modified FMC medium (data not shown). To determine the specific level of the manganese requirement, the culture first was grown as a batch culture in the chemostat vessel. The medium contained added manganese (180 μM), magnesium (420 μM), and iron (18 μM). At 11 h, medium input was started. When steady-state (equilibrium) growth was reached, input medium was changed to medium without manganese, and the concentration of manganese in the culture vessel was decreased by dilution with incoming medium. When the dry weight of the culture had decreased (probably due to manganese starvation), the effect of various concentrations of manganese on growth was tested. The manganese concentration was increased in stepwise fashion by simultaneous addition to both the culture vessel and medium reservoir. Manganese concentrations of 18 and 36 μM produced marked stimulation of growth (Fig. 3). As shown by decreased dry weight, however, 72 μM manganese was inhibitory. Inhibition by concentrations of manganese above 54 μM probably accounted for the low dry weight of the
initial steady-state culture in medium containing 180 μM manganese.

A second type of experimental approach was used to determine the iron requirement of *S. mutans* BHT in the modified FMC medium (Fig. 4). In medium containing magnesium and manganese additions of 420 and 180 μM, respectively, the organism could be grown at steady state without addition of iron. However, addition of 0.18 μM iron stimulated growth, and 0.45 μM (25 ng/ml) was near the optimum iron concentration. Higher concentrations of iron were less effective than 0.45 μM iron.

The Teflon continuous culture system can be used for other bacteria. *Bacillus subtilis* produces the siderophore 2,3-dihydroxybenzoic acid (DHB) when grown in low-iron medium (4). In a Chelex-100-treated, sucrose-mineral salts medium (1) (containing trace metal additions of 0.0001 μM iron, 823 μM magnesium, and 36 μM manganese), cultures maintained at steady state (medium input at a dilution rate of 0.18/h; incubation at 37°C; air input at 50 ml/min) in the Teflon chemostat produced 38 to 41 μg of DHB per ml. Equilibrium growth of these cultures, expressed as absorbance at 600 nm, was 0.16. Addition of iron to give a concentration of 36 μM repressed synthesis of DHB and increased the level of equilibrium growth (absorbance at 600 nm, 0.27).

To our knowledge, a Teflon chemostat has not been used previously for studies of trace metal physics. A Teflon culture vessel has been employed for suspension cultures of animal cells to prevent adherence of the cells to the culture vessel (8). Use of the Teflon continuous culture system described here, in conjunction with methods to lower trace metal contamination (use of high-purity water and culture medium ingredients and appropriate treatment of culture medium), should overcome several of the inherent problems encountered in bacterial trace metal studies. This system may demonstrate previous-ly unknown effects of trace metals on a variety of physiological functions. Essential components of trace metal metabolism (e.g., transport of the metal) also may be studied during steady-state growth in a controlled trace metal environment.

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**LITERATURE CITED**