APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1976, p. 168-172

Vol. 31, No. 2
Printed in U.S.A.

Effect of Dissolved Oxygen and Eh on *Bacteroides fragilis* During Continuous Culture

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Received for publication 18 August 1975

Bacteroides fragilis subsp. *fragilis* was maintained in a chemostat modified for anaerobic conditions to test the effects of dissolved oxygen and Eh on growth. Using a defined medium containing glucose and a dilution rate of 0.16 h^{-1}, a stable population of 3×10^9 colony-forming units/ml was present. At this steady state, the pH was 5.6, the Eh was −50 mV, and the dissolved oxygen concentration was 0% atmospheric saturation. The Eh was then adjusted to +300 mV by adding potassium ferricyanide while oxygen was excluded; in this system there were no demonstrable changes from the steady state in viable cells, pH, glucose concentration, or volatile fatty acid production. In other experiments oxygen was introduced into the original steady state at a dissolved oxygen concentration of 10% atmospheric saturation for a period of 6 to 8 h. During O_2 exposure, the viable cell count decreased at a rate comparable to the theoretical washout rate for a static bacterial culture. Similar results were obtained with a dissolved oxygen concentration of 25 and 100%. Other effects of O_2 exposure included an increase in Eh from −50 to +250 mV, a decrease in glucose consumption, and a decrease in volatile fatty acid production. These results suggest that dissolved oxygen has a bacteriostatic effect on *B. fragilis* in continuous culture, which may be independent of changes in Eh alone.

Several investigators have documented the inhibitory effect of molecular oxygen on anaerobic bacteria (11, 14, 17). There are, however, several questions regarding oxygen inhibition that remain unanswered. For example, it has not been determined whether oxygen is bactericidal or simply inhibits growth of anaerobes. The role of the oxidation-reduction potential (Eh) in suppressing growth of anaerobes has also been difficult to define because of the interrelationship between changes in oxygen levels and Eh.

Techniques have been developed in recent years for the continuous culture of anaerobes (2, 8, 10). Such procedures permit independent alterations of Eh and dissolved oxygen concentration while monitoring growth curves. The availability of minimal media for *Bacteroides fragilis* has made it possible to study this organism under defined growth conditions (18). We have employed these techniques in an effort to determine the effect of oxygen and Eh on *B. fragilis* during continuous culture.

MATERIALS AND METHODS

Microorganism. A strain of *B. fragilis* subsp. *fragilis* SAL 121 (*B. fragilis* 121) was used for all experiments. This isolate was originally cultured from clinical material processed in the Anaerobic Research Laboratory, Veterans Administration Hospital, Sepulveda, Calif. The organism was identified by procedures outlined previously (9). *B. fragilis* 121 was maintained in the frozen state during the course of these investigations. Prior to each experiment, a vial of the stock culture was inoculated into brain heart infusion broth supplemented with hemin and menadione, subcultured for purity, and transferred serially every 24 h in a minimal medium for several days.

Minimal medium. A minimal essential medium (BMM) (18) containing glucose as a fermentable carbon source was used in the continuous culture experiments. BMM was prepared by dissolving all materials except glucose, cysteine-hydrochloride, and Na_2CO_3 in distilled water. After steam sterilization, the solution was cooled under 100% N_2, and filtered aliquots of glucose, cysteine-hydrochloride, and Na_2CO_3 were added to the appropriate concentrations of 0.5, 0.05, and 0.4% (wt/vol), respectively. The final product was equilibrated with 99.9% CO_2, which resulted in a pH of 6.5 ± 0.1.

Continuous culture. Chemostat cultures of *B. fragilis* 121 were grown in BMM using a model C-30 Bioflo fermentor (New Brunswick Scientific Co.) modified to maintain an anaerobic environment. The fermentor was equipped with a peristaltic nutrient pump and flow controller, magnetically coupled agitator, automatic temperature control, and electrodes for measuring Eh, pH, and dissolved oxy-

gen concentrations. The fermentation vessel and nutrient reservoir were kept under a constant flow (40 cm^3/min) of 99.9% CO_2 to insure anaerobic conditions. The pH was monitored with a Corning potentiometer (model 109) after standardization of the electrode with pH 7.0 phosphate buffer at 37 C. Eh measurements were obtained with a platinum electrode and calomel half cell coupled to a Corning potentiometer standardized against a quinhydrone reference solution (15) at pH 4.0. Dissolved oxygen concentrations were assayed with a galvanic electrode (New Brunswick Scientific Co.). The electrode was standardized prior to use with distilled water saturated with atmospheric O_2 at 37 C by agitation. All electrodes were inserted through a rubber septum fitted to the top of the fermentation vessel. Samples for glucose determinations, volatile fatty acid assay, and bacterial counts were obtained by withdrawing aliquots from a side arm sampler extending into the fermentation vessel.

Continuous cultures were initiated after steam sterilization of the fermentor by adding 5 ml of an 18-h BMM culture of *B. fragilis* 121 to the fermentation vessel. This culture was allowed to grow for 24 h at 37 C with agitation. A flow of sterile medium was then started to give a dilution rate (Dc) of 0.16 h^{-1} (2). The dilution rate was calculated from the formula: Dc = flow rate (milliliters/hour)/fermentor volume (milliliters). Experimental studies were initiated after determining that a stable population of viable cells was established.

Glucose determination. Culture supernatants were obtained by aspirating 2.0 ml from the chemostat. The sample was immediately placed in an ice bath and centrifuged (4 C) at 10,000 × *g* for 15 min, and the supernatant was removed for assay. Duplicate 1-μl samples were injected into a Beckman glucose analyzer standardized with solutions containing known amounts of glucose.

Volatile fatty acid determinations. The concentrations of short-chain fatty acids in the culture supernatant were assayed by gas-liquid chromatography. Volatile fatty acids were extracted over ice from 0.1 ml of an acidified aqueous specimen with 0.25 ml of diethyl ether. A Packard 419 dual-flame chromatograph equipped with a Honeywell recorder was employed. Chromatographic separations were obtained by injecting duplicate 2-μl samples into silanized Pyrex glass columns (183 cm by 2 mm) containing 6% carbowax-20 M terephthalic acid on 80- to 100-mesh Gas-Chrom Z (Applied Science). The chromatographic operating conditions were as follows: detector, 200 C; injection port, 150 C; column oven, 120 C; and N_2 flow at 300 ml/min. Peak areas were interpreted as microvolt seconds on a Shimadzu ITG-2A electronic digital integrator. An internal standard of 100 μg of valeric acid per ml was added to all specimens for quantitation.

Bacteriology. Viable cell density was determined by removing a 1.0-ml aliquot from the fermentor and placing it immediately into an anaerobic chamber. Serial 10-fold dilutions were made in VP1 dilution salts (9); duplicate 0.1-ml aliquots were plated on Brucella agar base containing 6% sheep's blood and menadione. The plates were incubated for 48 h at 37 C within the anaerobic chamber. Colonies were enumerated, and the results were recorded as the $\log_{10}$ colony-forming units per milliliter. In addition, blood agar plates were inoculated periodically and incubated at 37 C with increased CO_2 to check for contamination.

Adjustment of Eh. The Eh within the fermentation vessel was adjusted with a sterile aqueous solution of 0.1 M potassium ferricyanide. The solution was added dropwise to the vessel until the desired Eh had been achieved. Positive Eh potentials were maintained by continuous addition of $K_3Fe(CN)_6$ during the experiment, not exceeding a 0.003 M concentration in the fermentor. (We had established that this concentration did not effect cell growth or viability.)

Adjustment of dissolved oxygen. The amount of dissolved oxygen in the fermentor was adjusted by regulating the flow of O_2 gas entering the fermentation vessel through a small cannula in the top of the vessel by means of an adjustable flow valve. It was determined that the best results were obtained by slowly adjusting the flow rate of O_2 (1 to 2 cm^3/min) while continuously monitoring the dissolved oxygen present via the dissolved oxygen electrode.

RESULTS

Continuous cultures. Preliminary studies using batch cultures of *B. fragilis* 121 grown in BMM indicated that the viable cell density was maximum at 48 h. This was followed by a decrease in viable cell density at 72 h. At 72 h the glucose concentration had decreased to 0. The final pH (5.2) and Eh (−75 mV) did not appear to be limiting factors since we had determined that this organism could initiate growth at pH values of 4.5 and Eh values of −50 mV or higher. The specific growth rate in batch cultures was calculated from the formula: $\mu m = 2.3 \times (|\log P_t - P_{t_o}|/t - t_o)$ where $t - t_o$ is the time interval during which the bacterial population P_{t_o} increased to the value P_t. This value was found to be 0.239, and the mean generation time (g) was found to be 2.9 h ($g = \ln 2/\mu m$). These results indicated that a dilution rate (Dc) of 0.16 h^{-1} would provide conditions suitable for continuous culture (Dc = 1/2 g).

Effect of Eh during continuous culture. Initial experiments established the steady-state conditions of the chemostat. It was found that a stable bacterial population of 10^9 colony-forming units/ml developed under these conditions and that an Eh of −75 mV and pH of 5.6 were held constant during culture at a Dc of 0.16 h^{-1}.

The Eh was altered by the addition of potassium ferricyanide to the fermentation vessel. This manipulation resulted in Eh values of +300 mV. The elevated Eh could only be maintained by continued addition of potassium ferricyanide to the fermentor with constant moni-

toring of the Eh. Cessation of this addition procedure was followed by a rapid decrease of the Eh to pretreatment levels. The results of this experiment indicated that no detectable change in viable cell density occurred either during or after adjustment of the Eh (Fig. 1). Neither the pH (5.6) nor dissolved O_2 concentration (0%) was changed as a consequence of adding potassium ferricyanide.

Effect of dissolved oxygen during continuous culture. The effect of dissolved oxygen on the growth of *B. fragilis* 121 was studied by introducing O_2 into the fermentation vessel after establishment of the steady state. Increasing the dissolved oxygen from 0% (atmospheric saturation) to 10 to 12% resulted in a steady decline in the viable cell density (Fig. 2). The decrease in viable cell density corresponded to the theoretical washout calculated for a static bacterial culture at a Dc of 0.16 h^{-1}. This result suggested that the presence of 10% dissolved oxygen in the fermentation vessel caused a net bacteriostatic effect. It was also found that the pH changed only slightly from 5.6 to 5.8, whereas the Eh increased from −75 to +250 mV. These findings were confirmed in additional experiments employing 25 and 100% dissolved oxygen in which similar results were obtained.

Changes in the level of glucose and short-chain fatty acid end products of fermentation during exposure to oxygen were monitored. The results (Table 1) indicate that acetic acid was the major short-chain fatty acid present in the culture supernatant. Small amounts of propionic and succinic acids were also detected. It was found that there was a decrease in the acetic acid concentration during 10% O_2 exposure for 6 h; however, an increase in concentration was noted during the first 2 h. In addition, the concentration of glucose increased during oxygen exposure from 160 to 350 mg/100 ml. A Dc of 0.16 h^{-1} results in a medium replacement every 6.25 h in the fermentation vessel. Thus, a complete metabolic shutdown of *B. fragilis* should produce a decrease in short-chain fatty acid concentrations and an increase of glucose concentration during this time. The change in the measured values for acetic acid and glucose corresponded to this prediction.

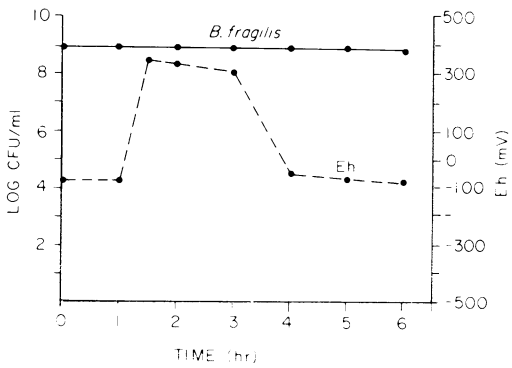

FIG. 1. *Effect of Eh on growth of B. fragilis during continuous culture. The Eh was adjusted by the addition of potassium ferricyanide solution. CFU, Colony-forming units.*

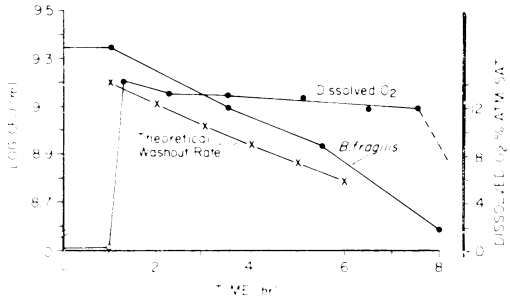

FIG. 2. *Effect of dissolved oxygen on B. fragilis during continuous culture. CFU, Colony-forming units.*

TABLE 1. *Acetic acid and glucose concentrations during continuous culture of B. fragilis 121*

O_2 exposure (h)	Acetic acid	Glucose (mg/100 ml)
0	0.072[a]	160
2	0.110	250
3	0.075	275
5	ND[b]	325
6	0.040	350
Sterile BMM[c]	0	450

[a] Acetic acid microvolt seconds/valeric acid microvolt seconds.
[b] ND, Not done.
[c] BMM, Minimal medium.

DISCUSSION

Recent studies (17) indicate that many strains of *B. fragilis* are relatively aerotolerant. Loesche (11) showed that *B. fragilis* could survive 6 h of air exposure without a decrease in viable cell population. Similarly, Tally et al. (17) noted that clinical isolates of several anaerobic genera were able to survive varying degrees of oxygen exposure.

Our experiments suggest that concentrations of 10 to 100% dissolved oxygen introduced during continuous culture result in a net bacteriostatic effect on *B. fragilis*. There was an in-

crease in glucose and a decrease in short-chain fatty acid concentrations, suggesting that a decline in metabolic activity occurred concomitant with oxygen exposure. When the effect of Eh alone was monitored, it was found that alterations of Eh by chemical means did not influence cell growth within the fermentor.

Previous investigators (3, 6) have observed a bacteriostatic effect when cultures of anaerobes were adjusted to Eh values of 150 mV or more. Isolates of *Clostridium perfringens* and *B. fragilis* subsp. *vulgatus* would not grow at Eh values of 150 and 140 mV, respectively (pH 7.0). However, these studies were performed with cultures in which the Eh was either electrically poised or was altered by the addition of O_2. Studies by Hanke and Katz (7) utilized electrolyzing currents to poise the Eh in cultures of *B. vulgatus (B. fragilis* subsp. *vulgatus)* and *C. sporogenes*. These investigators also found that Eh values of 150 mV or greater were inhibitory for initiation of growth. In other experiments, the Eh was maintained at a low level (−150 mV). Growth occurred in these cultures even when a flow of oxygen was introduced. Unfortunately, the criteria for growth were not quantitated, the dissolved oxygen concentration was not monitored, and the pH proved difficult to control during oxygen exposure. Since we used chemostat cultures and were not measuring the ability of *B. fragilis* to initiate growth, it is difficult to compare these studies with our own.

The mechanism of oxygen-mediated inhibition of *B. fragilis* is not clear. McCord et al. (12) have proposed that oxygen toxicity is a result of the generation of superoxide radicals and this reaction may occur in the culture medium. These radicals can be generated by a wide variety of reactions, including oxidation of catecholamines (1), autooxidation of thiols in the presence of metals (13), and photochemical oxidation of riboflavine (5). The array of biological sources of superoxide radicals makes it impossible to exclude their presence during introduction of oxygen into a bacterial system. Although it has been assumed that superoxide radicals are lethal, in our studies of *B. fragilis* the effect of oxygen was bacteriostatic rather than bactericidal. The explanation for this apparent paradox may come from recent investigations in our laboratory (16) which have shown that strains of *B. fragilis* subsp. *fragilis* contain significant concentrations of superoxide dismutase. This enzyme may protect the organism from death, but it is not known whether oxygen-generated superoxide radicals are responsible for growth inhibition. Further studies are required on the biochemical effects of oxygen exposure to arrive at more conclusive evidence regarding the mechanism of oxygen-mediated growth inhibition.

Several investigators have observed a spectrum of oxygen sensitivities for anaerobic bacteria. It is clear from recent reports (4) that only certain members of the indigenous microflora of humans are associated with clinical infections. Since many of the clinical isolates, including *B. fragilis*, can be classified as "moderate anaerobes," the actual effect of oxygen on these microorganisms is of interest. The procedures of continuous culture in a defined medium should prove valuable in discerning both bactericidal and bacteriostatic effects of oxygen on anaerobes. Information derived from such studies may help define the elusive effect of molecular oxygen on anaerobic microorganisms.

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