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Mechanisms That Control Bacterial Populations in Continuous-Flow Culture Models of Mouse Large Intestinal Flora

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A previous study had established that anaerobic continuous-flow (CF) cultures of conventional mouse cecal flora were able to maintain the in vivo ecological balance among the indigenous bacterial species tested. This paper describes experiments designed to determine the mechanisms which control the population sizes of these species in such CF cultures. One strain each of *Escherichia coli*, *Fusobacterium* sp., and *Eubacterium* sp. were studied. Growth of these strains in filtrates of CF cultures was considerably more rapid than in the CF cultures themselves, indicating that the inhibitory activity had been lost in the process of filtration. Growth rates to match those in CF cultures could be obtained, however, by restoring the original levels of H_2S in the culture filtrates. The inhibitory effect of H_2S in filtrates and in dialysates of CF cultures could be abolished by adding glucose or pyruvate, but not formate or lactate. The fatty acids present in CF cultures matched those in the cecum of conventional mice in both quality and concentration. These acids could not account for the slow rates of growth of the tested strains in CF cultures, but they did cause a marked increase in the initial lag phase of *E. coli* growth. The results obtained are compatible with the hypothesis that the populations of most indigenous intestinal bacteria are controlled by one or a few nutritional substrates which a given strain can utilize most efficiently in the presence of H_2S and at the prevailing conditions of pH and anaerobiosis. This hypothesis consequently implies that the populations of enterobacteria, such as the *E. coli* strain tested, and those of the predominant anaerobes are controlled by analogous mechanisms.

The indigenous microflora plays an important role in health and disease of humans and animals. For example, all indigenous bacteria, those of the mouth and especially those of the intestine, regularly and continuously invade the tissues and can be isolated from regional lymph nodes or the blood. Some of the literature has recently been summarized by Berg (4) for intestinal bacteria. Additional observations demonstrating passage of indigenous bacteria or colloidal particles through the intestinal epithelium have been published by Verzar (26) and Volkheimer and Schulz (27) and have been summarized by McMinn and Crawford (17) for oral flora. Apparently, there exists a constant dynamic equilibrium between the indigenous bacteria entering the body and the systemic defense mechanisms (phagocytosis, etc.) eliminating these invaders. If this equilibrium shifts, systemic infections result in various organs. This constant penetration of indigenous flora also has important beneficial effects on the host, in that it gives rise to the so-called "normal" antibodies (28), which are probably among the most important markers for non-self-recognition in phagocytosis (23). The composition of the indigenous microflora thus controls to a considerable extent the spectrum of microbes which the body can eliminate efficiently via phagocytosis.

Perhaps the most important function of the indigenous microflora is its ability to inhibit the implantation of invading microorganisms (pathogenic or other) on those body surfaces which are normally inhabited by indigenous microflora (20). The flora shares this ability to reject invaders with all other ecosystems in the climax stage. It is probably not much of an exaggeration to say that, before bacterial pathogens can infect humans, there must be first a break in the activity of the indigenous microflora.

There can be no reasonable doubt, then, that it would be of incalculable benefit to clinical medicine if the composition of the indigenous microflora of man could be manipulated in such

a way that bacterial species which are responsible for the beneficial effects of that flora are retained, whereas those which cause detrimental effects are eliminated. This would only be possible, however, if the mechanisms which control the implantation and population size of microorganisms in the indigenous microflora of the body were understood. As discussed in an accompanying paper (14), the lack of a suitable in vitro model of bacterial interactions is largely responsible for this shortcoming. As reviewed elsewhere (9), most of the methods used in the past for studying bacterial interactions were developed many decades ago. These include the determination of inhibitory substances produced in liquid medium or on solid medium (cross-streaks or inhibition zones around macrocolonies), "staled" agar, dialysis bags with liquid culture immersed in a flask with another liquid culture, mixed continuous-flow (CF) cultures of two species, sterile filtrate from one CF culture feeding a second CF culture, and gnotobiotic animals. Such studies have led to the definition of a number of mechanisms by which bacterial populations in the gut are controlled, such as the following: changes in oxidation-reduction potential or in pH; inhibitory substances such as bacteriocins, fatty acids, deconjugated bile salts, H_2O_2 + peroxidase; competition for substrates; competition for adhesion sites; and local immunity. Nevertheless, these data have not yet led to an understanding of bacterial interactions as they actually occur in the normal flora. For this reason, even a competent and knowledgeable writer who reviewed the subject recently was reduced to little more than a listing of suspected mechanisms (19).

The finding that anaerobic CF cultures can reproduce a large variety of bacterial interactions that occur in the mouse large intestine makes it likely that the mechanisms which regulate bacterial populations in vivo are reproduced in this culture system (14). This paper, therefore, describes a study of the mechanisms of bacterial interactions in CF cultures representing the entire complex flora present in the mouse cecum. While it might have appeared more straightforward to study less complex populations, one must consider that the mechanisms of interaction among bacteria in simplified systems are unlikely to resemble those operating among the entire flora (9). There are obvious technical difficulties in trying to isolate and manipulate mechanisms resulting from the interaction of several hundred different strains of bacteria growing in a dynamic system. These were overcome to a considerable extent by the use of diffusion chambers that were in contact with the complex flora via a cellophane membrane. This experimental design allowed the determination of the metabolic influences of the whole complex flora on single strains that were maintained within these chambers.

Some of these data have been presented earlier in preliminary form (9, 10, 12).

MATERIALS AND METHODS

CF cultures. CF cultures were maintained in an anaerobic chamber, essentially as described in an accompanying paper (14), except that 200 ml growth tubes were used in experiments involving diffusion chambers. The flow rate was 1/6 h^{-1}. The conventional mouse cecal flora from BALB/cwm mice was inoculated as described in an accompanying paper (14).

The effluent from CF cultures for use in subsequent experiments was collected in a flask kept at freezing temperature by means of a thermoelectric cold plate to prevent changes due to bacterial metabolism occurring after the material had left the growth tube. In other experiments, filtrates were prepared from culture fluid removed directly from the growth tube, limiting the volume withdrawn to 50% or less of the culture volume. There was no discernible difference between these two preparations.

Restoration of dissolved H_2S. All procedures for restoration of dissolved H_2S were carried out in the anaerobic chamber (2). Readjustment of pH levels of culture filtrates after reaction with H_2S was unreliable because of the volatility of this substance. For this reason, the amount of H_2SO_4 required to achieve the desired final pH (as determined in preliminary experiments) was added first. The filtrate was then shaken by hand in a flask containing H_2S gas at a predetermined pressure (usually 5 lb/in^2) for 5 min to obtain the desired concentration of dissolved H_2S. The filtrate was then inoculated with the *Escherichia coli* strain under test. To prevent subsequent loss of H_2S, the filtrates were dispensed into several replicate sterile test tubes. The tubes were filled to the top and closed with sterile rubber stoppers. The stoppers were held down tightly by placing the test tubes into wider tubes into which the atmosphere of the anaerobic chambers was pumped to a pressure of 8 lb/in^2. Growth curves were constructed by opening one of these tubes for each data point (i.e., once opened, a tube was discarded).

All additives (e.g., glucose solution, distilled water, etc.) used in these experiments were kept in the anaerobic chamber for at least 2 days before use.

Dialysis chambers. The principle of the dialysis chambers is illustrated in Fig. 3. Two types were used.

(i) A plexiglass chamber of 5- by 1.8-cm cross section and 1.8 cm deep was bisected by a thick cellophane membrane (Bel Art Products; no. 299), to form two chambers. The first chamber, accessible through a vertical port, was filled with culture filtrate. Inoculation and subsequent withdrawal of samples was done via this port. The second chamber had inlet and outlet ports through which the contents of the CF culture were slowly circulated by means of a peristaltic pump, taking care to avoid turbulences or splashing that might cause the loss of volatile substances.

(ii) The second type of dialysis chamber was a single chamber constructed of Teflon and stainless steel (1.8 by 1.2 by 6.5 cm) with one large side formed

by a cellophane membrane. The chamber was accessible through a vertical port and immersed directly into the CF culture. Two such chambers could be immersed simultaneously into the same CF culture. This second design was used mainly to verify results obtained with the first, i.e., to rule out the possibility that the continuous circulation of the CF culture through the dialysis chamber might introduce artifacts. None was detected. All experiments involving dialysis chambers discussed in this paper were carried out at least 3 times each in each of the two types of chambers.

Sulfide determination. An Orion no. 94-16 silver/sulfide ion electrode was used in conjunction with an Orion no. 90-02 sleeve-type double-junction reference electrode and a Corning Digital 112 pH meter. Samples were diluted in an equal volume of buffer of the following composition (per liter of working solution): 40 g of sodium hydroxide, 160 g of sodium salicylate, and 36 g of ascorbic acid. The buffer was stored at 2× concentration and diluted daily to the working strength, according to the electrode manufacturer's instructions. The electrode was calibrated against serial dilutions of a standard solution of Na_2S which had been titrated against lead acetate shortly before use, with the electrode as an endpoint detector (3). Preliminary titrations established that this method of titration gave results similar to the standard iodine-thiosulfate method (1).

Determination of fatty acids. Methyl esters, prepared by the method of Sheppared and Iverson (21), were extracted with chloroform and analyzed in a gas-liquid chromatograph. Molarities of fatty acids in cecal contents were calculated after subtracting the dry weight from the sample volume.

RESULTS

As reported in an accompanying paper (14), a pure CF culture of *E. coli* reaches population levels of approximately 10^9 per ml. In the presence of conventional mouse cecal flora, *E. coli* populations decrease to 10^5 to 10^7 per ml. To study the mechanisms underlying this decrease, the effluent from CF cultures of conventional mouse cecal flora was sterilized by filtration through a membrane filter (0.22-μm pore size; Millipore Corp.) inside the anaerobic chamber. The filtrate was then inoculated with *E. coli* C25 and incubated as a static culture inside the anaerobic chamber. The results (Fig. 1) show that the population of this organism reached levels of 10^6 to 10^7 per ml in a few hours, i.e., a level resembling that of *E. coli* in the original CF culture (Fig. 1). The subsequent cessation of *E. coli* growth could be prevented by adding glucose to the effluent (Fig. 1), in which case growth of *E. coli* resembled that in untreated veal infusion broth, i.e., in the medium used in the CF cultures (Fig. 1). At first glance one might conclude from these data that growth of *E. coli* in the filtrates was limited by low substrate concentration and that therefore growth of *E. coli* in the original CF culture was also controlled by competition for substrate. Closer inspection of Fig. 1 shows, however, that this is not the case.

One of the critical tests of an inhibitory mechanism in this type of experiment is to determine whether it reduces the growth rate of *E. coli* to that found in CF cultures of conventional mouse cecal flora. To form constant (rather than increasing) populations in our CF cultures, which operate at a dilution rate of 1/6 h^{-1}, *E. coli* must multiply with a growth rate constant which cannot be greater than the dilution rate (and which must be even smaller if wall-associated growth is present [13]). In static cultures a growth rate of 1/6 h^{-1} will result in a doubling time (t_d) of the *E. coli* population of $t_d = 6 \ln 2 =$

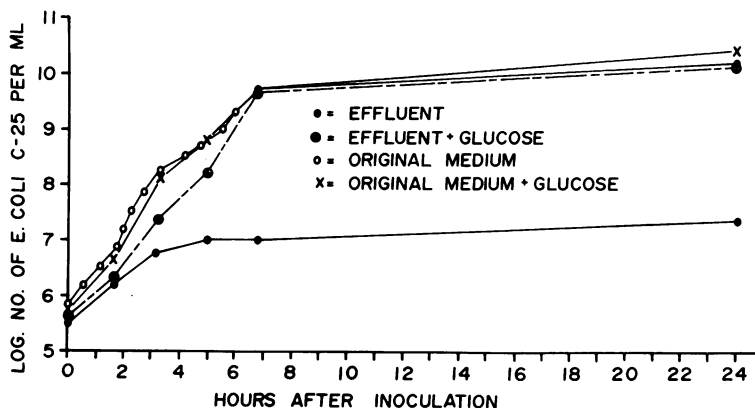

FIG. 1. Anaerobic growth of *E. coli* C25 in filtrates from the effluent of a CF culture of conventional mouse cecal flora: effect of glucose and comparison with growth in the original medium (i.e., the medium which feeds the CF culture).

4.16 h. This, of course, represents a high degree of inhibition when compared with the growth rate which *E. coli* would exhibit if it were growing as a pure culture in the rich medium used in our CF cultures. Consequently, an inhibitory mechanism that does not reduce the doubling time of *E. coli* to 4.16 h or less in static culture cannot be responsible for regulating the growth of *E. coli* in CF cultures of mouse cecal flora. As is evident from the data in Fig. 1, the doubling time of *E. coli* in filtrates of CF culture effluent during the first 4 h of the experiment (approx. 0.8 h) was much shorter than 4.16 h. One must therefore conclude that the mechanism which reduced the growth rate of *E. coli* in the original CF culture had been lost in the process of filtration. The data in Fig. 1 show, however, that the medium in the CF culture (and its filtrate) lacked sufficient substrate to support *E. coli* populations much in excess of those already present. Consequently, if the mechanism controlling the growth rate of *E. coli* in the original CF culture were to become inoperative for some reason, the *E. coli* population would rise only very slightly until it then became limited by the lack of substrate.

Much has been speculated about the role of short-chain fatty acids in the control of intestinal microflora (reviewed in reference 19). Table 1 shows the concentrations of fatty acids found in CF cultures of mouse cecal flora. It is noteworthy that these coincide well with the ranges found in the ceca of conventional mice (11). This is another critical observation pointing to close similarity between CF cultures and mouse large intestine as habitats of the indigenous microflora. The levels of these acids were not changed by filtration and subsequent incubation of the filtrates as practiced in the experiment described in Fig. 1.

TABLE 1. Range of fatty acid concentrations in CF cultures inoculated with *E. coli* C25 and conventional mouse cecal flora[a]

Acid	Molarity	
	Lowest	Highest
Acetic	0.02	0.08
Propionic	Trace[b]	0.03
Butyric	0.003	0.03
Isobutyric	Trace	0.006
Valeric	None	Trace
Isovaleric	Trace	0.014
Succinic	None	0.006
Lactic	None	Trace

[a] Data are based on five weekly specimens each, taken from six CF cultures between 1 and 6 months after inoculation of the cultures.

[b] Trace denotes readings that were significantly above background, but too low for accurate quantitation.

It is well known that the acidity of the medium affects the inhibitory activity of fatty acids on *E. coli* and other bacteria (15, 16). Accordingly, the growth of *E. coli* C25 was determined in static cultures of CF culture filtrate or in fresh enriched veal infusion broth. Where indicated in Fig. 2, the latter medium was supplemented with fatty acids to resemble those present in the particular CF culture from which the filtrate had been prepared (i.e., to make final concentrations of 0.0285 M butyric, 0.023 M propionic, 0.065 M acetic, 0.0055 M isobutyric, and 0.012 M isovaleric acids). Filtrates were prepared as usual without exposure to air in the anaerobic chamber. Fresh medium was allowed to prereduce in the anaerobic chamber for 3 days before use. Where indicated in Fig. 2, a small amount of palladium black was added to bring about a high degree of reduction of the medium during preincubation in the hydrogen-containing atmosphere of the anaerobic chamber (2).

The data in Fig. 2 indicate that the fatty acids added to fresh medium (or already present in the filtrates) reduced the growth rate of *E. coli* only at the very low pH of 6.0 to 6.2, which is considerably more acid than the pH range of 6.8 to 7.2 found in CF cultures of conventional mouse cecal flora. In no instance was *E. coli* growth reduced to a doubling time of approximately 4 h or less (Fig. 2), which would correspond to its growth in the original CF culture of conventional mouse cecal flora. It should be noted that under conditions where fatty acids were most inhibitory, they caused a pronounced increase in the lag phase before *E. coli* growth commenced, even when the subsequent inhibition of the growth rate was insufficient to explain the slow growth of *E. coli* in the CF cultures (Fig. 2). The possible significance of this will be discussed in a subsequent paper (13).

It became obvious at this point that two mechanisms of control of *E. coli* populations were present in the CF cultures (i.e., low substrate concentration and fatty acids), but that neither could account for the actual growth rate of *E. coli* observed. The actual inhibitory principle appeared to be unstable in that it was lost upon filtration. To study the postulated inhibitor apart from the mixed bacterial populations of the CF culture, the dialysis cell shown schematically in Fig. 3 was used. A static pure culture of *E. coli* (in filtrate of effluent from the CF culture) was kept in contact with the growing CF culture of conventional mouse cecal flora through a thick cellophane membrane. The design of this apparatus, and especially the choice of a membrane, required considerable effort, because the most commonly used membranes and ultrafilters

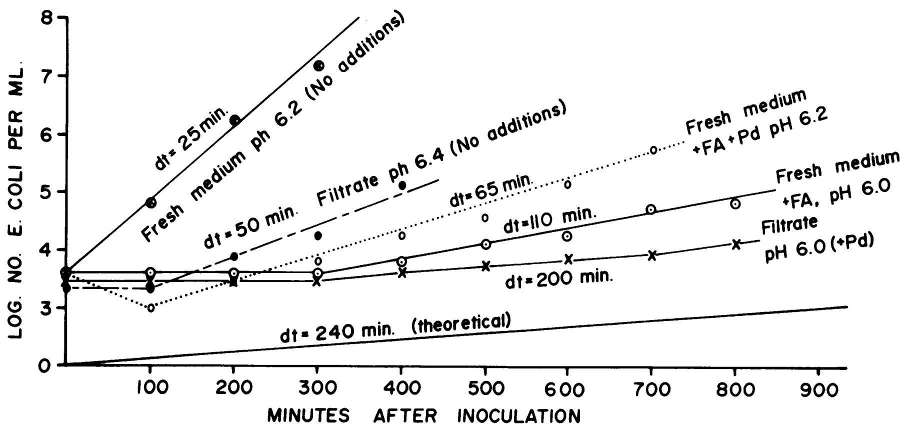

FIG. 2. Anaerobic growth of *E. coli* C25 in fresh medium (the same medium which feeds the CF cultures) or in filtrates from the effluent of a CF culture of conventional mouse cecal flora. Additives were as indicated: FA, short chain fatty acids to resemble qualitatively and quantitatively those present in the filtrate; Pd, palladium black, which served to reduce the medium beyond the level that establishes spontaneously in the anaerobic chamber. dt, Doubling time of the *E. coli* population.

(even those designed to retain substances of molecular weights as low as 20,000) have sufficient imperfections to allow the passage of one or a few bacteria from the CF culture within 2 or 3 days. This frequently resulted in contamination by bacteria of the filtrate on the sterile side of the membrane during the 2 days required for equilibration of the system. Only thick cellophane resisted penetration by bacteria over long periods of time.

When *E. coli* C25 was inoculated into the sterile filtrate of the dialysis cell (Fig. 3), there was a long lag phase (often several days) followed by growth within the range of the theoretical rate, i.e., with a doubling time of approximately 4 h or less. When glucose solution was added to the static side of the dialysis cell, growth of *E. coli* resumed at a high rate (Fig. 4). In control experiments, the medium on the static side of the cell was merely mixed (to simulate mixing of the glucose in the actual experiment) by pipetting it up and down three times. This resulted in a transient burst of growth of *E. coli* (Fig. 4). This observation suggested that *E. coli* was inhibited by a volatile factor which was removed from the medium by agitation, but which could diffuse through cellophane membranes. When prereduced glucose solution (or distilled water in the controls) was added to the *E. coli* culture in the dialysis cell without mixing, growth occurred only in the glucose-supplemented dialysis cell and not in the controls (Fig. 5). This effect could be obtained with final concentrations of glucose ranging from 1 to 0.01%. Pyruvate, but not formate or lactate, supported *E. coli* growth in this type of experiment in the same manner as glucose.

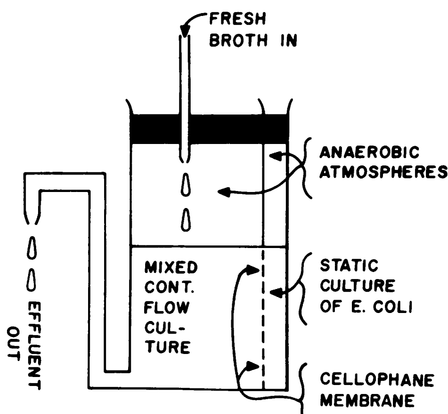

FIG. 3. Schematic representation of the dialysis cells which we use to study growth inhibition of *E. coli*. The cells are in contact through a cellophane membrane with the CF culture of conventional mouse intestinal flora.

The possibility that *E. coli* was inhibited by a volatile factor also became apparent in attempts to remove culture filtrate from the sterile side of the dialysis cell (Fig. 3) after 2 to 3 days of equilibration. When such filtrate was transferred into sterile screw cap tubes and inoculated (in the anaerobic chamber) with *E. coli*, rapid growth of *E. coli* resumed quickly (Fig. 6). However, when the filtrate was carefully removed from the dialysis cell, inoculated with *E. coli* without undue agitation, and subsequently stored in pressurized, rubber-stoppered tubes as described above, inhibition of *E. coli* growth was the same as if the filtrate had not been

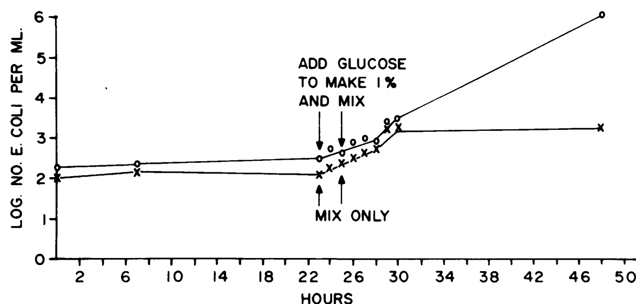

FIG. 4. Effect of glucose and of agitation on the anaerobic growth of *E. coli* C25 in dialysates that were in continuous contact with a CF culture of conventional mouse cecal flora.

removed from the dialysis cell (Fig. 6). However, agitation of the filtrate before filling the rubber-stoppered, pressurized tubes resulted in rapid growth of the *E. coli* (data not shown).

The above experiments have been repeated many times with the same results. One must therefore conclude that growth of *E. coli* in the CF cultures was reduced to a low rate by a volatile factor that can penetrate cellophane membranes and that is lost when medium is agitated, filtered, or stored in partially filled screw-cap tubes. Moreover, the effect of this inhibitor can be overcome by the addition of glucose. To detect such volatile substances, the gas phase accumulating above the growth tube of CF cultures of conventional mouse cecal flora was drawn into glass vials and analyzed by mass spectroscopy. Peaks in the resulting spectrogram were compatible with the presence of mercaptoethanol and H_2S. Subsequent experiments with mercaptoethanol indicated that reintroduction of this gas did not restore inhibitory activity for *E. coli* growth to filtrates of CF cultures of conventional mouse cecal flora. Similar experiments with H_2S were more successful and are reported below.

The concentration of H_2S in CF cultures of conventional mouse cecal flora ranged from 7×10^{-4} to 3.5×10^{-5} M (14 specimens) with a mean of 3.1×10^{-4}. In the cecum of conventional mice H_2S concentrations ranged from 2.5×10^{-4} to 1.74×10^{-4} M (nine specimens) with a mean of 2.2×10^{-4} M. In the experiments shown in Fig. 7, filtrates of CF cultures of conventional mouse cecal flora were prepared in the usual manner in the anaerobic chamber. Glucose (0.01%) was added to portions as indicated in the figure. The filtrates were then shaken with gaseous H_2S in a glass-stoppered flask until this gas was dissolved to a concentration of approximately 3×10^{-4} M. The pH of various portions of these filtrates was adjusted as indicated in Fig. 7. All of these and subsequent manipulations, were carried out in the anaerobic chamber. The various preparations were then inoculated with *E. coli* C25 and incubated in pressurized tubes. Analysis at the end of the experiments indicated that the concentra-

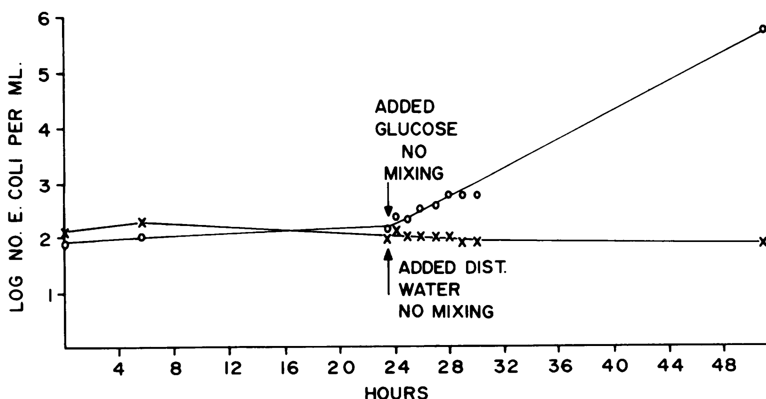

FIG. 5. Effect of glucose on the anaerobic growth of *E. coli* C25 in dialysates that were in continuous contact with a CF culture of conventional mouse cecal flora.

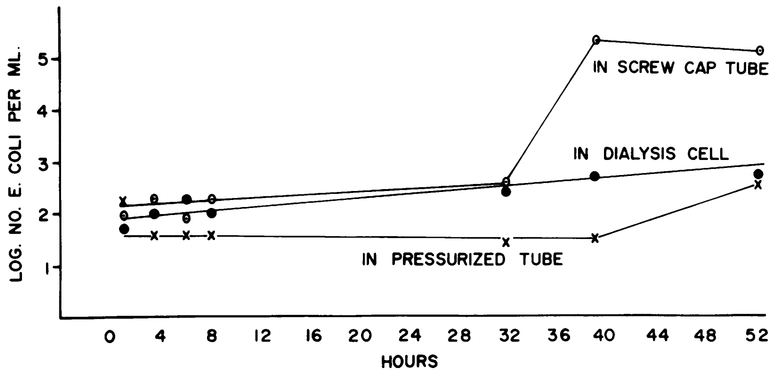

FIG. 6. Anaerobic growth of *E. coli* C25 in dialysates from a CF culture of conventional mouse cecal flora. After an initial 2-day period of equilibration, the dialysates were kept in uninterrupted contact through the cellophane membrane with the CF culture (●), in a partially filled screw-cap tube (○), or in a rubber-stoppered pressurized tube filled completely so as to leave no gas space inside (×).

tion of H_2S had decreased only slightly (by approximately 10^{-4} M), possibly by reaction of this gas with media components.

The results are shown in Fig. 7. Restoration of H_2S-levels to those found in the original CF cultures restored the inhibitory activity of the filtrates at neutrality, i.e., at the pH of CF cultures of conventional mouse cecal flora (Fig. 7). The presence of glucose in the filtrates allowed the *E. coli* to multiply. In many experiments of this type, growth of *E. coli* in glucose-supplemented filtrates commenced only after an extended lag phase (Fig. 7). Thus, the restored filtrates reacted in the same manner as dialysates of CF cultures, in that they contained H_2S as a volatile inhibitor, the action of which could be reversed by the presence of glucose. The inhibitory activity of H_2S was less pronounced at more alkaline pH (Fig. 7).

To test the mechanisms that control the populations of the strictly anaerobic indigenous microflora, the experiments shown in Fig. 5 were repeated with one strain each of *Fusobacterium* sp. (strain 102) and *Eubacterium* sp. (strain 53). In a typical experiment of this type (Fig. 8), *Fusobacterium* sp. was added to the static sides

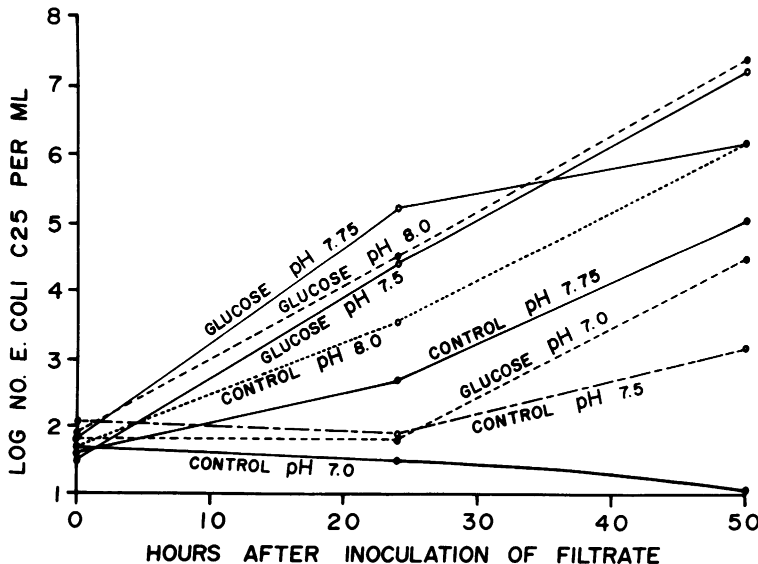

FIG. 7. Anaerobic growth of *E. coli* in filtrates from the effluent of a CF culture of conventional mouse cecal flora. All filtrates had been replenished to contain approximately 3×10^{-4} M H_2S (control). Glucose was added to H_2S-containing filtrates in some experiments, as indicated.

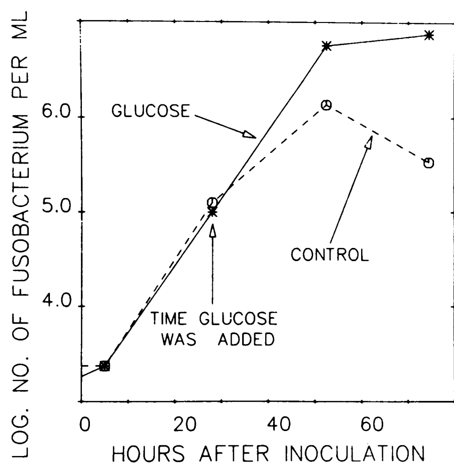

FIG. 8. Effect of glucose on the growth of a strain of *Fusobacterium* sp. in dialysates that were in continuous contact with a CF culture of conventional mouse cecal flora.

of two dialysis cells that were attached to the same CF culture of conventional mouse cecal flora. Prereduced glucose solution was added to the static side of one cell to make a final concentration of 1%; an equivalent amount of prereduced distilled water was added to the other. The data in Fig. 8. indicate that the growth of this bacterium before the addition of glucose proceeded at precisely the theoretically expected rate (doubling time, 4.19 h). The addition of glucose at 28 h increased the growth significantly. Similar results were also obtained with the strain of *Eubacterium* sp. tested. These data and those in Fig. 9 show that rapid death of these strict anaerobes occured when an initially rapid rate of growth ceased because of the lack of nutrients. This phenomenon was observed in most experiments of this type.

In the experiment shown in Fig. 9 filtrates were prepared in the anaerobic chamber from the contents of a CF culture of conventional mouse cecal flora. These were replenished with H_2S to a concentration of approximately 3×10^{-4} M, adjusted to neutrality, and inoculated with a strain of *Eubacterium* sp. Two portions were filled into rubber-stoppered pressurized tubes; one set of tubes received glucose solution to make a final concentration of 0.8%, and the other tubes received an equivalent volume of distilled water as a control. A third portion was placed into screw-cap tubes of sufficient size to leave a headspace. The data shown in Fig. 9 are typical for this type of experiment. The microorganism was inhibited in its growth in the H_2S-supplemented filtrate, and this inhibition was abolished in the presence of glucose and in the screw-cap tubes where H_2S could escape into the head space. Similar results were obtained with the *Fusobacterium* sp.

The data in Fig. 8 and 9 show that, in every aspect tested, the two strains of *Eubacterium* sp. and *Fusobacterium* sp. reacted in the same manner as *E. coli* C25. Consequently, regulation of the population sizes of these three strains in CF cultures of conventional mouse cecal flora appears to be mediated by similar mechanisms.

DISCUSSION

Terms such as bacterial antagonism (8), bacterial interference (6, 22), and colonization resistance (25) have been used to collectively describe two related but not necessarily identical phenomena: (i) the fact that the indigenous microflora of the body considerably restricts the population sizes of its various component species below the maximum that they could establish in monoassociated gnotobiotic animals; and (ii) the fact that implantation of new strains or species into an established undisturbed microflora is usually difficult or impossible to accomplish. Discussions in the literature of the possible mechanisms involved (19) have often concentrated on the presence of inhibitors of bacterial growth, such as fatty acids. However, it is theoretically impossible to account for the presence of constant low populations of one sensitive species (e.g., *E. coli*) in the indigenous microflora on the basis of the production of

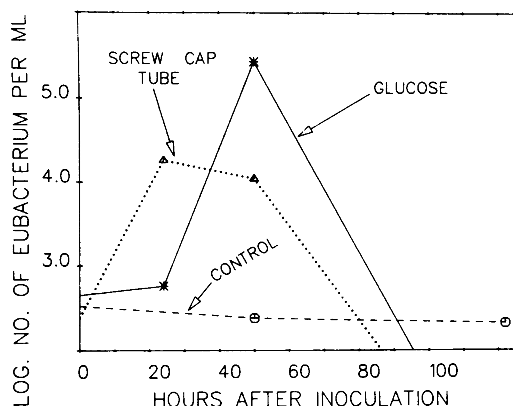

FIG. 9. Anaerobic growth of a strain of *Eubacterium* sp. in filtrates from the effluent of a CF culture of conventional mouse cecal flora. All filtrates had been replenished to contain approximately 3×10^{-4} M H_2S at neutral pH. Glucose solution was added to one portion to make a final concentration of 0.8%; an equivalent amount of distilled water was added to the controls. Both portions were incubated in completely filled, pressurized, rubber-stoppered tubes. A third portion (without added glucose) was incubated in a screw-cap tube with ample headspace above the filtrate.

growth inhibitors, such as fatty acids, that are produced by other species (such as the predominant anaerobes). If this were the case, either there would be too much inhibitor present, causing elimination of the sensitive species, or there would be too little of it, and the population of the sensitive species would increase until it became limited by some other mechanism (such as lack of nutrients). Constant populations of the sensitive species could be maintained only at one precise inhibitor concentration, where the growth rate of the sensitive species equals the flow rate of the system. Such precise control of inhibitor concentrations is not likely to occur in natural systems. This view is consistent with the mathematical analysis of Freitas and Fredrickson (7) of the effects of various types of inhibitors on the population dynamics in mixed chemostat cultures (their case IV). As described in an accompanying paper (13), the effects of bacterial adhesion to the wall of the gut or CF culture will broaden somewhat the range of inhibitor concentrations at which coexistence is possible.

Our current hypothesis, which is consistent with the findings reported in the present paper, holds therefore that the populations of most indigenous intestinal bacteria are controlled by substrate competition, i.e., that each species is more efficient than the rest in utilizing one or a few particular substrates and that the population level of that species is controlled by the concentration of these few limiting substrates. This view is also consistent with current mathematical theory of bacterial competition in chemostats which holds that *n* competing populations should be able to coexist as long as at least *n* limiting nutrients are present (reviewed in reference 6a). Considering the large number of breakdown products produced by the microflora from the large number of primary dietary or host-derived substrates in the gut, and considering the number of substances that are synthesized by the microflora itself, one must assume that a rather enormous multitude of potential substrates for bacterial growth are present in the gut, each of these probably in a low concentration. Our hypothesis further holds that the function of this system is modified by the presence of H_2S (and possibly other inhibitors of similar activity). The experiments illustrated in Fig. 4 through 8 indicate that there were nutritional substrates present in CF cultures of conventional mouse cecal flora which supported rapid growth of the tested bacterial strains only in the absence of the normally present H_2S concentrations. There obviously must exist other substrates in the CF cultures which support growth even in the presence of normal H_2S concentrations, as did glucose and pyruvate which were tested in the present study. Apparently, then, H_2S restricts the range of substrates which a given bacterial strain can efficiently utilize for anaerobic growth, but does not necessarily suppress growth altogether. In this regard its effect is basically different from that of general growth inhibitors, such as short-chain fatty acids. Most likely, the nature and range of substrates that can be utilized in the presence of H_2S differ for different bacterial strains and species. Our hypothesis postulates, therefore, that each of the several hundred bacterial species which comprise the intestinal ecosystem is controlled by one or a few nutritional substrates which this strain can utilize most efficiently in the presence of H_2S and at the conditions of pH and anaerobiosis prevailing in the intestine (i.e., by achieving a higher rate of multiplication than all other strains at low concentrations of this particular substrate). If, according to this hypothesis, some strains are removed from the indigenous microflora, the limiting nutrients that normally supported these strains will then increase in concentration and, at this higher concentration, will be able to support other bacteria. This prediction of our hypothesis is supported by the finding that intestinal *E. coli* populations in gnotobiotic mice equilibrated at levels that were inversely proportional to the complexity of the coexisting flora (24). An accompanying paper (13) extends this hypothesis by showing in a mathematical model that 2 or more strains which compete for the same limiting nutrient, are able to coexist, if the metabolically less efficient strain is able to adhere to the wall of the CF culture or the gut.

As mentioned above, a number of substances have been discussed in the recent literature as possible inhibitors of the in vivo growth of intestinal bacteria, but H_2S is not among them. It is therefore interesting to note that, as early as 1941, Bergheim et al. had studied the growth-inhibiting effect of H_2S and fatty acids on intestinal bacteria. They concluded that H_2S levels found in the gut of rats could account for inhibition of Candida but not of *E. coli* (5). Their failure to recognize the inhibitory effect of H_2S on *E. coli* might be due to the presence in their media (maltose broth) of nutrients that *E. coli* can utilize even in the presence of H_2S, or it might have been due to the inadequate anaerobiosis achieved by these early workers.

As discussed above, much study and speculation has been devoted in the past to the mechanisms that might control *E. coli* and other enterobacterial species (including the classical enteric pathogens) in the gut. In contrast, the mechanisms that regulate the population sizes of the predominant anaerobes have received little attention. The present finding that the popula-

tions of *E. coli* and of the two strains of anaerobes tested were controlled by similar mechanisms is therefore noteworthy. If confirmed by future studies, our current hypothesis might conceivably evolve (with suitable modifications and extensions, to be sure) into the general theory of the indigenous microbiota which Rosebury identified 20 years ago as the goal to be contemplated by students of the ecology of indigenous microflora (18).

The above discussion is concerned exclusively with mechanisms that control population levels within an established microflora. An accompanying paper (13) is concerned with additional factors such as adhesion and length of the lag phase, especially as these determine whether a bacterium that is newly introduced into an established intestinal ecosystem will be able to implant in this habitat.

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