

EXPLANATION FOR LIMITATION OF POPULATIONS OF *ESCHERICHIA COLI* IN BROTH CULTURES

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ABSTRACT

FRETER, ROLF (Jefferson Medical College, Philadelphia, Pa.), AND ATSUSHI OZAWA. Explanation for limitation of populations of *Escherichia coli* in broth cultures. *J. Bacteriol.* **86**:904-910. 1963.—Veal Infusion Broth surrounding a cellophane bag containing a 24-hr culture of *Escherichia coli* was inhibitory to an inoculum of the same strain when kept under an atmosphere of nitrogen plus CO₂. The inhibition could be abolished by addition of glucose or by aeration. When a small inoculum of a dulcitol-positive *E. coli* strain was introduced into a fully grown (24 hr) static Veal Infusion Broth culture of a dulcitol-negative *E. coli*, no multiplication occurred. However, the inoculum did grow in the presence of a carbon source (dulcitol) which could not be utilized by the static population. The logarithmic growth rate attained by the dulcitol-positive inoculum under these conditions was independent of the dulcitol concentration. In contrast, the maximal population size was a function of the amount of dulcitol present. Similar results were obtained when sucrose or salicin was substituted for the dulcitol. All strains grew well in filtrates of 24-hr broth cultures with growth rates and maximal populations approximating those attained in fresh broth. It was thus concluded that populations of *E. coli* in broth cultures were limited by exhaustion of all those carbon and energy sources which could be utilized under the prevailing highly reduced conditions. No evidence of inhibitory metabolic end products was found except in broth supplemented with 1% glucose. Partial inhibition of multiplication was noted in the latter medium even when the pH was maintained between 6.9 and 7.2.

It is well known that bacterial populations in liquid cultures reach a certain maximal level

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which is characteristic of the strain and the conditions of culture, such as composition of the medium and degree of aeration. The reasons for cessation of bacterial multiplication in populations of maximal size have never been fully established. Most writers who discuss this problem mention several or all of the following possibilities: exhaustion of a limiting nutrient, accumulation of toxic substances, lack of available oxygen, and Bail's (1929) hypothesis of limited "biological space." The pertinent literature was discussed by Hinshelwood (1946), Van Niel (1949), Monod (1949), Dagley, Dawes and Morrison (1951), Wilson and Miles (1955), and Sinclair and Stokes (1962).

In the case of static natural broth cultures, an acceptable theory must account for the following well-known facts. (i) When a fully grown broth culture is filtered and the medium reinoculated with the same strain, growth occurs at a rate approaching that in the original medium. This rules out the possibility of exhaustion of nutrients and also the presence of all but extremely unstable metabolic inhibitors. (ii) The growth rate of many facultative anaerobes in static broth cultures in contact with air is similar to that under a nitrogen atmosphere. Consequently, lack of available oxygen *per se* does not necessarily affect growth. It was this set of observations which originally had prompted Bail (1929) to reject other theories and to formulate his concept of biological space.

Most recent studies of this problem have been carried out with simple defined media, usually containing glucose and ammonium sulfate as the only carbon and nitrogen sources. The studies of Dagley, Dawes, and Foster (1953), Sinclair and Stokes (1962), and Ecker and Lockhardt (1961) showed that growth and multiplication of *Escherichia coli* in static (un-aerated) media of this nature may terminate at a time when adequate concentrations of nutrients are still present. Dagley and co-workers (1953) noted that such cultures became reduced and slightly acidic as

the population approached its maximal level. They explained the cessation of growth on the basis of accumulation of toxic metabolites, such as formic acid, which would prevent bacterial multiplication under these conditions, but not in the presence of oxygen or at neutral pH. Such a mechanism explains the observation that filtrates of fully grown *E. coli* cultures may support growth of a second crop, since the medium becomes reoxidized in the process of filtration (and often is also neutralized before introduction of the second inoculum).

Recent studies from this laboratory (Freter, 1962) indicated that multiplication of *Shigella flexneri* was inhibited in a fully grown static Veal Infusion Broth culture of *E. coli*. This inhibition was due to reduction of the medium by *E. coli* and exhaustion of all carbon or energy sources that could be utilized by *Shigella* under these conditions. There was no evidence of toxic metabolites, since growth of the *Shigella* resumed when either oxygen or glucose was made available. Thus, it appeared that cessation of multiplication of *E. coli* in broth cultures might be due to the same mechanism. The present paper presents evidence for this assumption, indicating that the mechanism of inhibition in synthetic media with a single carbon source may be fundamentally different from those operating in natural broth cultures.

MATERIALS AND METHODS

All strains of *E. coli* were obtained from human sources. Strain C25 is streptomycin-resistant and dulcitol-negative (i.e., incapable of producing acid or gas in dulcitol broth). It is the same strain used in earlier studies (Freter, 1962). Strain 81 is streptomycin-sensitive and dulcitol-negative, and strain 784 is dulcitol-positive and streptomycin-resistant.

Bacterial populations were estimated by spreading suitable saline dilutions of broth culture on Desoxycholate Agar (Difco or BBL) plates. To detect small numbers of strain 784 in a large population of strain 81, the agar was supplemented with 1 mg/ml of streptomycin. All cultures were grown in Veal Infusion Broth (Difco or BBL). Incubation temperature in all experiments was 37 C.

Inhibition across cellophane membranes was tested in Pyrex cylinders (1.5 in. inner diameter) closed with rubber stoppers. A cellophane bag of

1½ in. diameter (No. 4465-A2; Arthur H. Thomas Co., Philadelphia, Pa.) filled with 80 ml of broth was inserted into the cylinder and attached to a glass tube leading through the rubber stopper. The tube was closed with a cotton plug. The space outside the bag was filled with 40 ml of broth. Nitrogen plus CO₂ or air was bubbled through the outside medium by means of a glass tube extended through the rubber stopper. The gas was exhausted through a third tube which led into an oil bath to prevent back-diffusion of air. In some experiments, the cellophane bag was fitted with a string leading through the gas exhaust tube. The bag could then be lifted above the level of the outside broth at any time during the experiment without contaminating the gas atmosphere in the cylinder. The nitrogen used was prepurified grade and the CO₂ was bone dry grade; both were obtained from the Matheson Co., Inc., East Rutherford, N.J. The nitrogen plus CO₂ mixture employed in all experiments was supplied at the rate of 0.25 ft³ per hr of nitrogen plus 0.01 ft³ per hr of CO₂. For the purposes of inoculation and sampling of outside broth, the rubber stopper was lifted briefly to allow introduction of a pipette. During this time, the flow of N₂ was increased to five times the normal rate.

RESULTS

In the first type of experiment, a culture of *E. coli* 25 was grown inside a cellophane bag containing Veal Infusion Broth. The sterile broth outside the bag was agitated by bubbling N₂ plus CO₂ through it. The culture inside the bag reached its stationary phase after about 8 hr with a concentration of 5×10^8 to 10×10^8 cells per ml. After 24 hr, the broth outside the bag was inoculated with 1 ml of saline suspension containing *E. coli* C25 from a 24-hr culture grown on Veal Infusion Agar. This inoculum was diluted to give a final concentration of about 1000 cells per ml of outside broth. The results obtained are shown in Fig. 1. There was a lag phase of about 8 hr followed by extremely slow growth with a generation time which varied between 120 and 180 min in different experiments. When the inoculum for the outside medium was taken from the C25 culture inside the bag (rather than from a Veal Infusion Agar culture), the lag was eliminated, but the growth rate in the outside medium

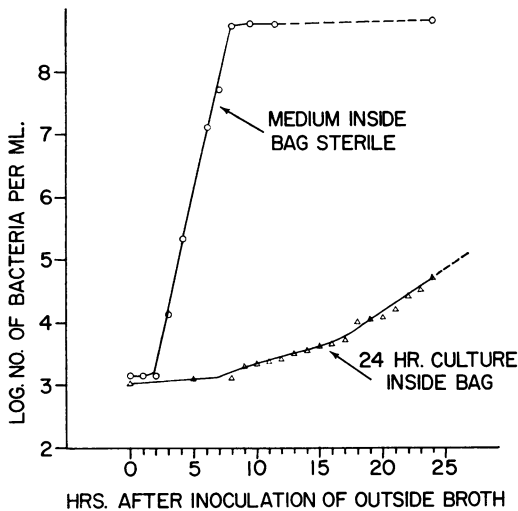


FIG. 1. Growth of *Escherichia coli* C25 in broth surrounding a cellophane bag containing a 24-hr broth culture of the same strain. Nitrogen plus CO_2 was bubbling through the outside medium.

remained unchanged with a generation time of 120 min.

As a control, a similar experiment was carried out, but the medium inside the bag was not inoculated and was kept sterile throughout the experiment. Under these conditions, *E. coli* C25 grew with a generation time of 18 min after a lag period of about 2.5 hr (Fig. 1). The growth rate was the same whether or not a N_2 plus CO_2 mixture of air was bubbled through the outside broth.

In the second type of experiment, the bag containing the primary culture of *E. coli* C25 was kept immersed in the outside broth for 24 hr as in the first procedure. Nitrogen plus CO_2 was bubbled through the sterile outside medium. At the end of the 24-hr period, the outside medium was inoculated with *E. coli* C25 from a 24-hr culture on Veal Infusion Agar. At 0.5 hr after inoculation, the cellophane bag was lifted up so that it was suspended above and no longer in contact with the outside broth. In various experiments, growth in the outside medium was then determined under three different conditions. (i) The inoculum was introduced in 1 ml of saline, and N_2 plus CO_2 was kept bubbling through the medium. (ii) The inoculum was introduced in saline containing sufficient glucose to make a final concentration of 0.2% in the outside medium, and N_2 plus CO_2 was supplied as before.

(iii) The inoculum was suspended in saline, and air was substituted at the time of inoculation for the N_2 plus CO_2 gas mixture.

Figure 2 presents the results obtained. In most experiments, there was no growth at all during the 8-hr observation period when the nitrogen atmosphere was maintained in the absence of glucose. In a few experiments, very slow multiplication occurred, possibly because a small amount of air had been introduced in the process of inoculation and of taking samples for quantitative plate cultures (lower three curves in Fig. 2). In the presence of glucose under N_2 plus CO_2 atmosphere, or in the aerated medium without glucose, the bacteria multiplied readily with a generation time of 30 min. The maximal population attained under the latter two conditions approximated that in fresh medium. When both air and 0.2% glucose were supplied to the outside medium, the growth rate (not shown in Fig. 2) was identical with that in fresh broth.

Growth of the inoculum in the outside medium could also be induced by aeration without first removing the cellophane bag. However, the addition of glucose to the outside medium (while maintaining the nitrogen atmosphere) had no effect unless the bag was removed. When the bag was kept in contact with the outside medium, glucose was quickly metabolized by the inside culture, resulting in an increase in the population,

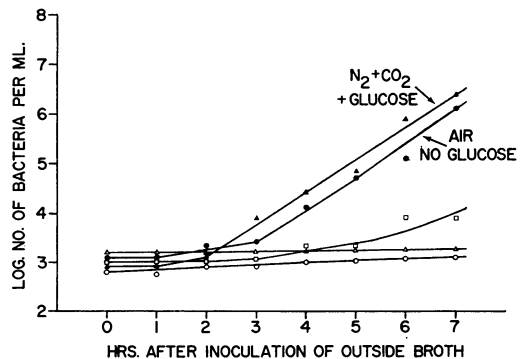


FIG. 2. Growth of *Escherichia coli* C25 in broth which had been in contact for 24 hr with a cellophane bag containing a broth culture of the same strain. The bag was removed after inoculation of the outside broth. The medium was then supplemented with glucose or aerated. Open symbols indicate triplicate experiments of growth without glucose supplement under N_2 plus CO_2 atmosphere.

but there was no effect on the inoculum in the outside broth.

It seemed desirable to demonstrate the effect of an added carbon source on the growth of an inoculum which was in direct contact with a stationary maximal population, rather than separated by a cellophane membrane. To permit counting of a small inoculum in a large stationary population, the streptomycin-resistant dulcitol-positive *E. coli* 784 was introduced into a population of the streptomycin-sensitive dulcitol-negative strain 81. Strain 81 was grown in 50 ml of Veal Infusion Broth in a Pyrex cylinder closed with a cotton stopper. After 24 hr, a small inoculum of strain 784 from a 24-hr culture on Brain Heart Infusion Agar, suspended in 1 ml of saline, was introduced. In different experiments, the following were added to the inoculum of strain 784: (i) 1 ml of saline, (ii) 1 ml of saline containing dulcitol to make a final concentration of 0.2% in the medium, or (iii) 1 ml of saline containing glucose to a final concentration of 0.2%. Care was taken to avoid agitation of the medium, except at the time of inoculation of strain 784, when mixing was accomplished by shaking the cylinder gently for a few seconds.

The results of Fig. 3 indicate that the inoculum of strain 784 grew under these conditions only in the presence of dulcitol, but not with glucose or in the absence of added carbohydrate. The generation time of strain 784 in the presence of dulcitol was 60 min, i.e., about twice as long as

that in fresh broth (25 min). There was a remarkably long lag phase of about 10 to 11 hr (Fig. 3). With larger inocula (10^6 cells per ml), the lag was reduced to 6 hr. The lag was eliminated when the inoculum of 784 was already adapted, i.e., was taken from a fully grown culture of strain 81 supplemented with dulcitol, in which strain 784 had been growing for 18 to 24 hr. The history of the inoculum (i.e., adapted or nonadapted) had no influence on the logarithmic growth rate. Within the range tested (0.67 to 0.002%), the growth rate was independent of the dulcitol concentration. In contrast, maximal population size of strain 784 was a direct function of dulcitol concentration (Fig. 3). Experiments analogous to those described above were carried out also with sucrose or salicin, with the appropriate combinations of sucrose- or salicin-positive and -negative *E. coli* strains. The results obtained were similar to those shown for dulcitol in Fig. 3.

To test for inhibitory metabolites resulting from the fermentation of glucose, the following experiment was carried out. A 24-hr static broth culture of strain 81 was inoculated with strain 784 as usual. The inoculum was already adapted, i.e., taken from a growing, dulcitol-supplemented culture of strain 784 in a maximal population of strain 81. Together with the inoculum, dulcitol and glucose were supplied to a final concentration of 0.2 and 1%, respectively. The air over the culture was then replaced with pure nitrogen, and the medium was slowly stirred by means of a magnetic stirrer. The cylinder containing the culture was closed with a rubber stopper which admitted a calomel and a glass electrode. The pH was maintained between 6.9 and 7.2 by addition of 3.3 N NaOH for 5 hr. After this time, the pH turned alkaline and was maintained within the above range by addition of 1.0 N HCl. A similar control culture was inoculated simultaneously. This was supplemented with dulcitol only; the glucose was omitted. This culture remained at pH 7.0 to 7.5 throughout the experiment, with no adjustment of the pH required. The results are shown in Fig. 4. There was a definite depression in the growth rate of strain 784 when the culture medium contained dulcitol and glucose, indicating that fermentation of glucose by strain 81 resulted in the production of inhibitors which were active even at optimal pH. Upon addition of glucose, the stationary population of strain 81 resumed growth for about

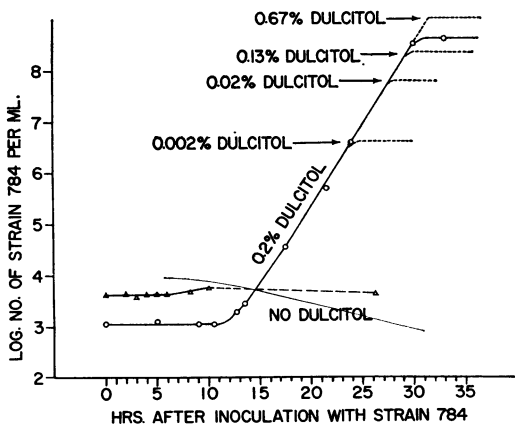


FIG. 3. Growth of dulcitol-positive strain 784 in a maximal population of dulcitol-negative strain 81. Dulcitol (0.2%) was added with the inoculum of strain 784. Broken lines indicate the maximal population size attained by strain 784 in the presence of various dulcitol concentrations.

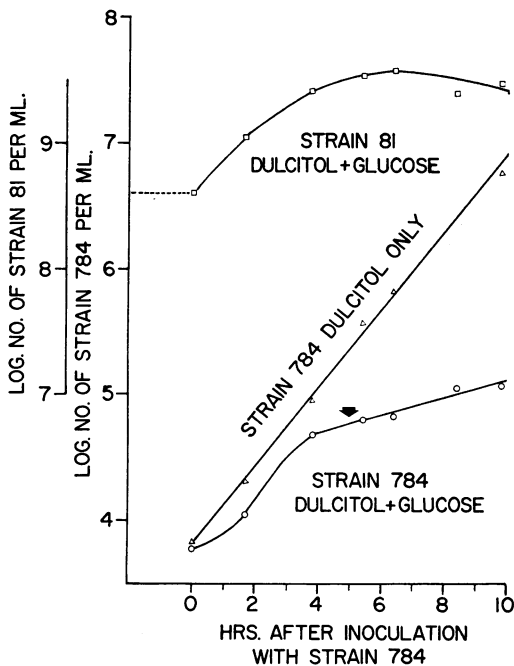


FIG. 4. Growth of dulcitol-positive strain 784 in a maximal population of dulcitol-negative strain 81. Glucose or dulcitol, or both, were added with the inoculum of strain 784, and pH was maintained between 6.9 and 7.2. Arrow indicates the time at which acid production from glucose was completed.

5 hr (Fig. 4), i.e., about as long as acid production occurred.

Growth of strains 81 and 784 in culture filtrates was determined as follows. A 24-hr broth culture of strain 81 was filtered through a Seitz pad. Portions of the sterile filtrate were then placed in cotton-stoppered test tubes and inoculated with either strain 81 or 784 to give an initial concentration in the medium of about 1000 cells per ml. The generation times in the logarithmic growth phase under these conditions were 21 min for strain 81 and 30 min for strain 784. This compares with generation times of 20 min and 25 min, respectively, for these organisms in fresh broth. The maximal populations attained by these strains in the culture filtrate were in the order of 5×10^8 to 5×10^9 cells per ml, i.e., similar to those found in fresh broth. Identical results were obtained when the first crop of strain 81 or 784 was removed by centrifugation, rather than by filtration. This experiment, reported originally by Bail (1929), rules out the possibility that any inhibitory substances might

have been removed by adsorption to the filter material.

DISCUSSION

The results described in the first part of this paper indicate that the inhibitory activity which prevented further multiplication of *E. coli* 25 in the stationary growth phase was transmissible through cellophane membranes. Growth resumed in the sterile inhibitory medium outside the cellophane bag when either glucose or air was supplied. One must, therefore, conclude that the inhibition was due to exhaustion of all those carbon or energy sources which could be utilized under the highly reduced conditions prevailing in stationary cultures of *E. coli* (Hewitt, 1950; Freter, 1962).

One possible objection to the above experiments might be based on the fact that inhibition in the outside medium was not complete, since an inoculum of *E. coli* 25 was able to grow with the extremely slow generation time of 120 to 180 min (Fig. 1). It is, of course, well known for many bacterial species that the stationary phase of growth may represent a dynamic equilibrium in which the actual growth rate equals the rate of death. It is conceivable that the slow multiplication found in the outside medium (Fig. 1) represented the actual growth rate of a stationary *E. coli* culture, which could be observed under the experimental conditions because autolytic enzymes (often responsible for the death in stationary cultures) were held back by the cellophane membrane.

Be this as it may, the above objection does not apply to the experiments described in the second part of this paper, in which the inoculum was mixed directly with a stationary culture. In this case, the inoculum did not multiply at all unless a source of carbon was made available to it. The carbon source was effective only when it could be utilized by the inoculum, but not by the stationary culture. Theoretically, addition of a fermentable carbon source such as glucose, which is available to both stationary culture and to the inoculum, should have a similar effect. It was not possible to demonstrate this, however, since the large stationary population metabolized the glucose before any effect on the small inoculum became apparent.

In view of the above results, the concept that growth of *E. coli* in broth cultures is limited by

accumulation of toxic substances appears to be no longer tenable. To still maintain such a view, one would have to postulate the presence of growth inhibitors which are oxygen-labile and whose action is directly and specifically reversed in the presence of carbohydrates such as glucose, sucrose, dulcitol, and salicin. However, for reasons detailed below, the action of these carbohydrates in the present experiments was almost certainly that of carbon and energy sources. (i) As shown in Fig. 3, only the maximal population size was a function of carbohydrate concentration, whereas the growth rate remained constant over the entire concentration range tested. These relations are characteristic of a limiting nutrient (Hinshelwood, 1946; Monod, 1949). If the function of the carbohydrate had been one of neutralizing a growth inhibitor, one would expect partial neutralization at low carbohydrate concentrations, resulting in a decreased growth rate. (ii) Strains which were already adapted to a given carbohydrate showed immediate growth in a mixed, carbohydrate-supplemented culture, whereas a nonadapted inoculum exhibited a long lag phase. This phenomenon would be hard to explain if the carbohydrate were neutralizing a toxic metabolite. (iii) Growth in mixed cultures was supported only by those carbohydrates which could be metabolized by the strain involved. This, again, would be an unusual requirement for a specific neutralizing substance. (iv) Growth in mixed cultures was supported by such diverse substances as sucrose, salicin, dulcitol, and glucose, a finding which makes it rather unlikely that any direct specific neutralization of metabolic inhibitors was involved.

One must, therefore, conclude that the cessation of growth in maximal populations was due to exhaustion of all energy sources which could be utilized under the prevailing highly reduced conditions. This mechanism accounts for the common finding that a second crop of *E. coli* can be grown in broth cultures from which the first crop has been removed by either centrifugation or filtration. Both of these procedures involve a considerable degree of aeration, with consequent reoxidation of the medium. Also explained is the well-known fact, shown again in the present study (Fig. 4), that stationary broth cultures of *E. coli* resume growth after addition of glucose.

Our results with broth cultures differ from the findings with simple defined media, discussed in

the introduction above, where reproduction appears to stop because of accumulation of toxic metabolites. It seems reasonable to assume that broth, in contrast to synthetic media, contains a variety of different fermentable carbon sources which conceivably yield a variety of different metabolic end products, such that no one of these reaches an inhibitory concentration. The data in Fig. 4 suggest, however, that broth supplemented with a relatively high concentration of a single carbon source (in this case, 1% glucose) may give results similar to those obtained with synthetic media. The considerable reduction in growth rate found under these conditions (Fig. 4) is indicative of the accumulation of inhibitors.

It should be emphasized that the oxidation-reduction potential in stationary broth cultures of *E. coli* is considerably lower than that found under conditions commonly referred to as "anaerobic." As pointed out earlier (Freter, 1962), this degree of reduction cannot be achieved artificially, even with such agents as thioglycolate. As shown in Fig. 1, simple anaerobic conditions, i.e., replacement of oxygen with nitrogen, had no effect on the growth rate of *E. coli* in broth, whereas the reduced conditions in a dulcitol-supplemented maximal population of strain 81 were most likely responsible for increasing the generation time of strain 784 to 60 min, as compared with 30 min for the same strain in culture filtrate.

It is interesting to note that both reduction (or absence of available oxygen) and exhaustion of nutrients have been mentioned frequently in the literature as factors responsible for the stationary phase of the growth cycle. As the present results show, neither of these alone was sufficient to account for the termination of growth in broth cultures of *E. coli*. As far as we could determine, the present finding that a combination of these two factors operates in the stationary phase of *E. coli* broth cultures has been mentioned in the literature only once (Wilson and Miles, 1955) as one of several hypothetical mechanisms capable of explaining the then known facts.

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