Enteric Bacterial Growth Rates in River Water

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Enteric bacteria, including stocked strains of pathogenic species and organisms naturally present in the stream, were capable of growth in a chemostat with autoclaved river water taken 750 m below a sewage outfall. Maximal specific growth rates for all organisms occurred at 30 C, whereas culture generation times ranged between 33.3 and 116 hr. Of the six laboratory strains of enteric species used, Escherichia coli and Enterobacter aerogenes grew at generation times of 34.5 and 33.3 hr, respectively, while the remaining Proteus, Arizona, Salmonella, and Shigella spp. reproduced at a rate two to three times slower than the coliforms. Little or no growth occurred in the water at incubation temperatures of 20 and 5 C, and death was observed for Salmonella senftenberg at 20 and 5 C and for E. aerogenes and Proteus rettgeri at 5 C. When enteric bacteria naturally present in the river water were employed in similar experiments, coliform bacteria demonstrated a generation time of approximately 116 hr, whereas fecal coliforms failed to grow. Growth of the bacteria from the river demonstrated a periodicity of approximately 100 hr, which suggests that much of the growth of these organisms in the chemostat may be on the glass surfaces. This phenomenon, however, was not observed with any of the stocked enteric species. Neither the stock cultures nor the aquatic strains were capable of growth in autoclaved river water taken above the sewage outfall at the three temperatures tested.

Enteric bacteria are assumed to exist in fresh water lakes and streams under starving conditions with growth being limited primarily by the nonavailability of a suitable carbon source. Although this assumption is the basis for use of selected enteric bacteria as fecal pollution indicators, there is evidence to indicate that limited enteric bacterial growth may occur in particular fresh water environments. For some time it has been known that terrestrial bacterial species can grow in extremely dilute concentrations of laboratory media (3, 7, 12), but recent studies have demonstrated that enteric bacteria can grow in mineral salts solutions with glucose concentrations that could be found in some natural aquatic systems (22) and also can metabolize substrates found in river water and extracts of bottom sediments (6).

Continuous culture provides a useful technique in which the growth of cells on natural substrates can be measured with ease, and in which various environmental parameters such as pH, oxygen tension, nutrient concentration, and metabolic waste products can be maintained constant or, if desired, varied independently to suit the requirements of the experiment. In both theory and practice, the rate at which a culture grows within the chemostat, up to some maximal level, can be governed by the rate at which a limiting nutrient is added to the culture. Once a particular dilution rate has been established, and providing that this rate is less than the maximum growth rate of the culture and that the population has the ability to grow at some rate less than the maximum, then the culture can achieve a steady state at a rate equal to the dilution rate (18).

There is also evidence to suggest that there are definite minimum steady state growth rates that can be achieved in chemostat experiments when using the carbon source as the rate-limiting nutrient (14, 15). In later studies by Tempest et al. (24), steady states for Enterobacter aerogenes have been achieved at dilution rates between 0.24/hr and 0.004/hr with culture doubling times ranging from about 7 to 173 hr. When water from the natural environment is used as a source of substrates for continuous culture experiments, often the concentration of unknown rate-limiting nutrients is not sufficient to establish a steady state population at a particular dilution rate. This requires altering the dilution rate to
values that will maintain the population at the low substrate concentrations. Except for heavily contaminated inshore sea water, Jannasch (10) has observed that steady state populations often cannot be maintained at dilution rates less than 0.042/hr when seawater was used as a nutrient source. Although such data cannot directly be used in calculating growth rates, Jannasch has provided a useful indirect procedure by which specific growth rates can be estimated by the rate at which a population is washed out of the chemostat.

In the present study, river water above and below a sewage plant effluent was used as a nutrient source for selected pathogenic and nonpathogenic enteric bacteria. These cultures were grown continuously in a stirred, glass chemostat similar in design to that described by Herbert et al. (9), and growth rates were calculated either directly from the steady state dilution rate or by the indirect procedure of Jannasch (10). In the present study, growth due to attachment on the glass walls of the culture device was disregarded for data calculation purposes.

MATERIALS AND METHODS

Study sites. River water was collected for investigation from the North Oconee River, a typical stream of the northern Georgia piedmont in Clarke County, near Athens, Ga. Three sites were selected for this study: one above the city where the water was free from urban contamination (site 1), another within the corporate limits but below the center of the city (site 2), and a third located about 750 m below the municipal sewage plant whose effluent contributed a significant biological oxygen demand to the stream (site 3).

Organisms. One strain each of Escherichia coli, ATCC 11775; E. aerogenes (Aerobacter aerogenes), ATCC 12658; Proteus retgeri, Arizona arizonae (Paracolobactrum arizonae), Shigella flexneri A1 (National Center for Disease Control, Atlanta, Ga.); and Salmonella senftenberg (Canadian Communicable Disease Center, Ottawa, Canada) was grown in Trypticase soy broth (BBL) at 30 C for 16 hr. Cells were then harvested by centrifugation (11,000 × g for 10 min), washed three times in sterile, carbon-free, deionized water, incubated at 30 C for 4 hr to expend endogenous metabolism, and rested at 4 C for 18 hr before each experiment.

Bacteria naturally present in the river water from site 3 were also used in some experiments. For these studies, 1.7 liters of water were centrifuged at 8,500 × g for 30 min in a Sorvall RC2-B centrifuge (Norwalk, Conn.). The resulting pellet was resuspended in carbon-free, deionized water and was centrifuged in conical tubes at 2,000 × g for 5 min to sediment the heavier particulates, thus leaving most of the microorganisms free in the supernatant. After aspirating the supernatant into another conical tube, the microorganisms were sedimented at 11,000 × g for 10 min. The organisms were then washed in carbon-free, deionized water and prepared for direct use by the same procedure outlined for the stock cultures.

Experimental substrates. River water was collected from each study site. The river water was immediately sterilized in an autoclave at 121 C for 15 min and then frozen after samples were removed for basal nutrient analysis. No attempt was made to use filter-sterilized water because of the high solid content present. To serve as a control substrate, a dilute glucose-mineral salts medium was prepared of the following composition per liter: K2HPO4, 7.0 mg; KH2PO4, 3.0 mg; (NH4)2SO4, 1.0 mg; MgSO4·7H2O, 0.1 mg; glucose, 10.0 mg. The glucose in this medium was autoclaved separately from the rest of the medium and added aseptically when both the glucose and the salt solutions had cooled to room temperature.

Analyses for basal nutritional constituents were made on the river water from each site as well as the control minimal salts medium by colorimetric procedures. Carbohydrate and protein (compounds with phenol rings) estimates were made by the anethone (3) and Folin-Ciocalteau (4) procedures, respectively, whereas Standard Methods (1) provided techniques for measuring ammonia nitrogen and orthophosphate. Samples were run in duplicate, and the means were compared to curves prepared from the recommended standard at those concentrations observed in situ.

Organic-free water and glassware. Since many prototrophic enteric bacterial species are reported to be capable of growth in basal salts solution without added organic material (2, 17, 22), distilled, deionized water that was carbon-nitrogen free, as determined by C-H-N analyses, was used in all phases of the study. Laboratory glassware was soaked overnight in hot sulfuric acid-dichromate solution, rinsed repeatedly in carbon-free, deionized water, and capped with paper prior to autoclaving. With such precautions, control experiments using the test organism in a basal salts solution (without carbon source) demonstrated no increase in bacterial numbers.

Continuous culture system. A chemostat similar in design to those reported by Herbert et al. (9) was constructed to yield a working volume of 1.7 liters. The dilution rates were controlled by varying the height of the pressure head and the speed of the medium-waste peristaltic pump (Buchler Instruments, Fort Lee, N.J.). The temperature of the chemostat was controlled with a Lauda-Brinkman K-2/R Circulator (Westbury, N.Y.), and back contamination of the medium from the chemostat was prevented by heating a portion of the medium inlet tube with 0.1 ohm/cm nichrome wire connected to a regulated power supply. This system yielded satisfactory dilution rates as low as 0.005/hr over extended periods of time (Fig. 1).

Bacterial growth studies with river water and dilute minimal media. Two different procedures were used to study enteric bacterial growth in dilute nutrient systems. The first series of experiments employed stocked enteric bacterial strains, whereas
later studies utilized organisms that were present in the river. All individual experiments were run in triplicate, and the rate of bacterial decline in numbers was resolved by linear regression (23).

Rested cell suspensions of each stocked bacterial culture were prepared in carbon-free, deionized water and standardized to 0.9 optical density at 540 nm with a Spectronic 20 (Bausch and Lomb, Rochester, N.Y.) colorimeter. In separate experiments, 0.1 ml of each test strain was introduced into chemostats containing river water from each study site. Initial experiments with E. coli were run at varying dilution rates, but ultimately 0.012/hr was used in all subsequent experiments unless otherwise noted. Samples were taken from each chemostat daily, and the organisms present were counted on M-Endo broth (Difco) by the Millipore procedure at 30 C.

In experiments to determine specific growth rates of enteric bacteria in river water from the three study sites, bacteria present in 1.7 liters of river water from site 3 were recovered by centrifugation and prepared for use by procedures outlined earlier. These organisms were then introduced into chemostats in which minimal medium and river water from each study site were used as nutrient sources. A dilution rate of 0.012/hr was maintained in these experiments and the incubation temperature was 30.0 C. Samples also were taken from these chemostats on a daily basis, but organisms present were counted on M-Plate count broth and M-Endo broth (Difco) by the Millipore procedure at both 30.0 and 44.5 C.

Data calculation. The procedure used to calculate growth rates obtained in this study is essentially that of Herbert et al. (8) and Jannasch (10). (These references should be consulted for additional information.) It was observed that a population (x) changes during the transient state in a chemostat by the relationship:

\[
\frac{dx}{dt} = \mu x - Dx
\]

or

\[
x = x_0 e^{\mu t - D t}
\]

where \(x_0\) is initial population \((t = 0)\), \(\mu\) is growth rate, \(D\) is dilution rate, and \(t\) is time. By solving equation 2 for the growth rate \(\mu\):

\[
\mu = D + 1/t (\ln x/x_0)
\]

If

\[
1/(\ln x/x_0) = A
\]

where \(A\) equals the washout rate.

Therefore:

\[
\mu = D + (A)
\]

The culture generation time \((G = 1/\mu)\) was used in the following experiments rather than the cell generation time \((t_d = 1/2/\mu)\) because the growth measurements were calculated from viable cell counts.

By using these relationships, Jannasch (10) has postulated that four distinctly different cases of constant washout rates are conceivable in continuous culture studies. These are as follows: (i) the population doubled in one retention time and a steady state results. (ii) The population grows at a rate greater than the washout rate. (iii) The population does not grow and is washed out of the culture device at a rate equal to the washout rate. (iv) The population disappears at a rate faster than the washout rate (defined as death of the population).

RESULTS

The basal nutrient concentration of the autoclaved river water from the three study locations and the dilute minimal salts-glucose medium is presented in Table 1. These concentrations were found to increase gradually as the river water flowed down stream (sites 1 and 2) and to reach a maximum below the sewage effluent (site 3). The carbohydrate and protein colorimetric procedures used allow for the detection of a variety of compounds, some of which may not be readily degradable by some enteric bacteria. Morris (13) has reported that, besides glucose, anthrone (in the presence of acid) will give a blue color with other monosaccharides (both hexoses and pentoses) and by oligo- and polysaccharides including glycogen, starch, and cellulose. Likewise, the Folin-Ciocalteau protein procedure detects those compounds containing a phenol ring, including
TABLE 1. Mean basal nutrient concentration of autoclaved river water and dilute mineral salts-glucose medium

| Assay          | Source | Concen (mg/liter) |
|----------------|--------|-------------------|
| Ammonia nitrogen | Site 1 | 1.1               |
|                | Site 2 | 1.5               |
|                | Site 3 | 3.0               |
|                | Minimal medium | 0.5   |
| Folin-protein   | Site 1 | 2.9               |
|                | Site 2 | 3.2               |
|                | Site 3 | 5.1               |
|                | Minimal medium | 0.0   |
| Carbohydrate    | Site 1 | 2.1               |
|                | Site 2 | 2.4               |
|                | Site 3 | 2.8               |
|                | Minimal medium | 10.0  |
| Orthophosphate  | Site 1 | 1.3               |
|                | Site 2 | 1.6               |
|                | Site 3 | 3.2               |
|                | Minimal medium | 5.0   |
| pH             | Site 1 | 7.0               |
|                | Site 2 | 6.9               |
|                | Site 3 | 6.9               |
|                | Minimal medium | 7.0   |

most phenols (except nitrophenol), tyrosine, tryptophan, uric acid, quanine, and xanthine, but not adenosine (4).

Figure 2 contains results of numerous experiments to determine the optimal dilution rate for E. coli in river water from the three study sites. Results of these experiments indicated that water from sites 1 and 2 were incapable of supporting growth of the organism, whereas dilution rates of less than 0.027/hr would allow for growth in water from site 3.

Mean specific growth rates obtained at 30, 20, and 5 C for the selected enteric bacterial strains in river water from site 3 are shown in Table 2. All six organisms demonstrated positive specific growth rates at 30 C, but growth was slight or nonexistent at the two lower temperatures.

Typical results of experiments using natural heterotrophic bacterial populations from site 3 in dilute minimal salts medium and in river water recovered below the sewage plant (site 3) are shown in Fig. 3 and 4, respectively. Heterotrophic, enteric, and coliform bacterial counts were made daily from cultures incubated at 30.0 and 44.5 C to estimate coliforms and fecal coliforms. Table 3 shows the mean specific growth rates of the bacterial populations in Oconee River water (site 3) and in dilute minimal medium. No growth was observed with these organisms in water from sites 1 and 2.

DISCUSSION

For some time it has been known that various bacterial species are capable of growth and reproduction in extremely dilute nutrient concentrations of laboratory media (3, 7, 12), but most of these organisms are not involved in the pathogenesis of man or higher animals. Enterobacteriaceae, however, not only contains bacteria which serve as indicators of fecal pollution, but also contains others, such as Salmonella, Shigella, and Arizona, that can produce serious intestinal disease. Data obtained in this study (Table 1) indicate that the basal nutrients for the test organisms were in short supply. River water taken below the sewage plant effluent (site 3) appeared slightly higher in nitrogen, Folin-protein, carbohydrate, and phosphate content but contained less carbohydrate and phosphate than did the dilute mineral salts-glucose medium.

There is, however, some difficulty in attempting to compare directly basal nutrient data from one site with that from another, or even that from distant localities. Even though the colorimetric procedures used in this study are capable of detecting a wide range of possible nutrients, it is conceivable that other compounds could be present in the water that could either stimulate or inhibit growth and yet still remain undetected. However, on the basis of the growth studies, the enteric bacteria

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**Fig. 2. Washout rate versus dilution rate for Escherichia coli in Oconee River water from sites 1, 2, and 3 at 30 C. E. coli was capable of growth when the dilution rate minus washout rate equals a positive growth rate (μ). 1/μ = culture generation time.**
employed in this study were able to grow in river water from below the sewage plant, which implies that conditions were more favorable in the water from this site than the other two.

Early experiments with *E. coli* in Oconee River water from the three study sites (Fig. 2) indicated that a linear relationship existed between the dilution rate and the washout rate for this organism in river water from sites 1 and 2. Similar results were observed for water from site 3 at dilution rates greater than 0.027/hr. By using the relationship \( \mu = D + A \), these results indicate that *E. coli* is incapable of growth in river water from sites 1 and 2 and in site 3 water at dilution rates greater than 0.027/hr. Growth of the test organisms, however, can take place in water from site 3 at dilution rates of at least 0.027/hr to 0.005/hr. A dilution rate midway between these two extremes and equal to 0.012/hr was chosen for all subsequent experiments.

Results shown in Fig. 2, which demonstrated the lack of growth of *E. coli* in river water from sites 1 and 2, were confirmed with the remaining five stock cultures. Growth, however, was observed for all six cultures, including pathogens, in water from site 3 (Table 2). Maximal specific growth rates were observed in site 3 water at the 30 C incubation temperature for all organisms tested, whereas culture generation times ranged between 33.3 and 90.0 hr at this temperature. *E. coli* and *E. aerogenes* demonstrated generation times of 34.5 and 33.3 hr, respectively, while the remaining *Proteus, Arizona, Salmonella* and *Shigella* spp. reproduced at a rate two to three times longer than the coliforms. Generation times of the six test organisms were extended at 20 and 5 C, and death was observed for *S. senftenberg* at 20 and 5 C and for *E. aerogenes* and *P. rettgeri* at 5 C. These results are consistent with those of Postgate et al. (16) who observed generation times of approximately 100 hr (\( D = 0.0101/hr \) at 37 C) for *E. aerogenes* in media where the carbon source was rate limiting. In later studies, however, generation times of 38.9 hr (\( D = 0.0145 \) at 37 C) were also observed (17).

Organisms present in the Oconee River only demonstrated the capacity for growth in river water from site 3 (Fig. 4). The mean specific growth rate for the coliform bacteria was 0.006/hr (Table 3) with an equivalent generation time of approximately 166 hr. Although the fecal coliform bacteria, as estimated by the 44.5 C population, demonstrated a negative growth rate which can be defined as death of the population (17), both the 30 and 44.5 C

### Table 2. Mean specific growth rates* of selected enteric bacteria in Oconee River water from site 3

| Organism                | Temp (C) | Washout rate/hr | Growth rate/hr | Culture generation time (hr) |
|-------------------------|----------|-----------------|----------------|-----------------------------|
| *Escherichia coli*      | 30       | 0.017           | 0.029          | 34.5                        |
|                         | 20       | -0.009          | 0.003          | 333.3                       |
|                         | 5        | -0.011          | 0.001          | 1,000.0                     |
| *Enterobacter aerogenes*| 30       | 0.018           | 0.030          | 33.3                        |
|                         | 20       | -0.006          | 0.006          | 166.6                       |
|                         | 5        | -0.018          | D*             |                             |
| *Proteus rettgeri*      | 30       | -0.002          | 0.012          | 83.3                        |
|                         | 20       | -0.001          | 0.010          | 100.0                       |
|                         | 5        | -0.017          | D              |                             |
| *Arizona arizonae*      | 30       | -0.001          | 0.011          | 90.0                        |
|                         | 20       | -0.011          | 0.001          | 1,060.0                     |
|                         | 5        | -0.011          | 0.001          | 1,000.0                     |
| *Salmonella senftenberg*| 30       | 0.001           | 0.013          | 76.9                        |
|                         | 20       | -0.020          | D              |                             |
|                         | 5        | -0.032          | D              |                             |
| *Shigella flexneri*     | 30       | 0.001           | 0.013          | 76.9                        |
|                         | 20       | -0.007          | 0.006          | 200.0                       |
|                         | 5        | -0.010          | 0.002          | 500.0                       |

* Dilution rate was 0.012/hr in each experiment.  
* Death of the population.
population of heterotrophic bacteria were capable of growth in the water. When these data were compared with those obtained with dilute minimal medium, the heterotrophs, as well as the enteric and coliform bacteria, were capable of growth.

In all chemostat experiments using bacteria recovered from the river and as shown in Fig. 3 and 4, bacterial numbers were observed to oscillate with a periodicity of approximately 100 hr. Although such growth has been termed "cryptic" by other investigators (17, 19), it is doubtful whether sufficient energy could be derived from the dead and lysed cells to allow for replenishment of the loss in population, especially in a chemostat where wastes are being constantly removed. A more plausible explanation could involve the slime layer that is produced on the glass walls of the chemostat. Previous studies (11, 21) have shown that mixed bacterial cultures capable of growing in water or dilute nutrient systems can produce slime layers on bare artificial surfaces, and Sanders (21) has shown that the slime layer can slough off when the underlying layers become sufficiently anaerobic to alter the metabolism of the bacteria present. Since slime production would be a function of time, it is conceivable that the slime could be sloughed at 100-hr intervals, and this process contributes not only bacteria but also added nutrients for metabolism by the suspended populations. Such a hypothesis is consistent with previous reports of bacterial and nutrient sorption to negatively charged surfaces (6, 24).
TABLE 3. Mean specific growth rates* of native bacterial populations in site 3 Oconeé River water and dilute minimal medium

| Nutrient source | Bacterial group | Growth rate/hr |
|-----------------|-----------------|---------------|
|                 |                 | 30 C pop. | 44.5 C pop. |
| River water (site 3) | Heterotrophs | 0.007 | 0.013 |
|                 | Enterics | 0.007 | D* |
|                 | Coliforms | 0.006 | D |
| Minimal medium  | Heterotrophs | 0.017 | 0.019 |
|                 | Enterics | 0.018 | 0.015 |
|                 | Coliforms | 0.019 | 0.012 |

* Dilution rate was 0.012/hr in each experiment.
* Death of the population.

Data reported in this study certainly cannot be extrapolated directly to the natural aquatic environment where one could say unequivocally that enteric bacteria, including pathogens, are capable of growth in fresh water. There is, however, data to indicate that these organisms are able to grow in situ under very stringent nutrient conditions (7), and this study has demonstrated that there are sufficient nutrients in autoclaved river water that was taken below a sewage outfall to support limited bacterial growth. Although the factors governing enteric bacterial growth in fresh water constitute a complex phenomenon, investigations in this area can help explain apparent inconsistencies such as the recovery of Salmonella in high quality mountain river water (5) and how high mountain watersheds that are closed to recreational activities can exhibit elevated coliform and ion determinations as compared to those of open areas (20). Observations such as these demonstrate the need for the development of procedures which assay the aquatic system’s ability to support and maintain particular bacterial populations rather than having to rely on indirect procedures which can only estimate the presence of a particular bacterial group or species.

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