Sorption of Heterotrophic and Enteric Bacteria to Glass Surfaces in the Continuous Culture of River Water

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A natural population of heterotrophic bacteria, including enterics, was observed to sorb to glass surfaces and multiply during the continuous culture of river water. An initial rate of attachment equivalent to a doubling time of about 2 h was observed with a corresponding increase in the suspended population. After 24 h both the sorbed and suspended populations stabilized with a mass doubling time approximating 100 h at a dilution rate of 0.012/h. On the basis of respiration and degradative enzymatic data, the sorbed microorganisms appeared to be somewhat more metabolically active than the organisms in suspension.

Of major concern to sanitary bacteriologists today are the factors which affect survival of indicator species both in situ and in collected water samples for analysis. If an indicator organism has been properly chosen, it should respond to environmental conditions in a manner similar to that of any pathogenic bacteria that might be added to the environment along with the indicator species. One of the major factors in the survival of bacteria in aquatic environments is the lack of an adequate nutrient supply, but it is suspected that the sorption of bacteria and nutrients to surfaces in aquatic systems play a major role in the recycling of substrates (4, 13, 23, 36). The nature of this role, however, has not been well defined.

Besides providing a possible role in growth and survival of bacteria in situ, the attachment of aquatic microorganisms to surfaces for the formation of primary films has also been suggested as a necessary requisite for later colonization by higher forms of aquatic life (9, 34). The initial events that lead to slime layer formation, according to Marshall et al. (24), center around a hypothesis of selective and irreversible sorption of small bacterial rods to surfaces. Baier et al. (2) believe that the resulting film alters the critical surface tension of the monolayer and makes it more amenable to further colonization by higher forms of life.

In addition to the theoretical aspects of sorption as they relate to microbial maintenance in situ, sorption of various bacterial species to surfaces is of practical importance to the sanitary bacteriologist. Higher recovery rates of Salmonella in bottom sediments than in surface waters have been reported (13, 33), but perhaps of more significance are the reports of Caldwell and Parr (7) and more recently by Lucking (22) of a progressive coliform reduction in water samples that can not be immediately processed. Although samples should be processed immediately after collection (1), delays of 24 to 48 h are common when the sample location is remote and adequate transportation is not available. Whether these reductions in bacterial numbers are a result of the death of a portion of the population or by some other mechanism is not known, but certainly sorption of part of the bacterial population to the bottle surfaces could also effectively reduce the observed counts.

This study will focus upon both the initial and long term bacterial sorption events and attempt to relate the relative metabolic activity to the maintenance of these populations in the continuous culture of river water.

MATERIALS AND METHODS

Continuous culture system. A continuous culture apparatus similar in design to one previously reported (16) was modified to measure the sorption of bacteria to glass surfaces within the culture vessel. Several glass cover slips (18 mm²) were cemented to a glass rod with Dow-Corning bathroom caulk for suspension beneath the culture surface. The entire apparatus was then cleaned as outlined in the above report and sterilized in the autoclave before each experiment. This system can be used effectively at dilution rates as low as 0.005/h over extended periods of time, but for the studies reported here, the dilution rate was maintained at 0.012/h.

Experimental substrates. River water taken from a site approximately 750 m below a municipal sewage plant on the North Oconee River served as the sole substrate in these studies. Immediately after collec-
tion, the water samples were returned to the laboratory and divided with two samples for sterilization. One portion was autoclaved for 20 min at 121 C, whereas the other was filtered through a washed 0.22 membrane filter (Millipore Corp., Bedford, Mass.) to evaluate the effects of the sterilizing process. All water samples to be used as nutrient sources for the bacterial growth studies were then transferred to sterile flasks and stored at 5 C.

Basal nutrient analyses were made on each sample of water after sterilization. Ammonia nitrogen and orthophosphate were measured by procedures outlined in Standard Methods (1), whereas carbohydrate and protein estimates were made by the anthrone (26) and Folin-Ciocalteau (8) procedures, respectively. Duplicate analyses were made and the means were compared to standard curves prepared with those concentrations observed in situ.

**Growth and attachment studies.** Individual experiments were initiated by placing 1.7 liter of fresh, unsterilized river water into the chemostat to provide a bacterial population for the growth studies. In separate experiments, either autoclaved or filtered sterilized river water was used as the nutrient source and fed into the chemostat at a dilution rate of 0.012/h. The incubation temperature of the continuous culture system was maintained at 30 C in all experiments. All experiments have been done repeatedly and the results of a typical experimental series are reported here.

Samples consisting of a portion of culture suspension and a glass cover slip were removed from the chemostat at intervals and subjected to both viable plate and direct counting procedures. A Petroff-Hauser counting chamber was used for making the direct estimates, and the culture suspension was counted without further treatment. The cover slips, after removal from the chemostat, were first rinsed in sterile deionized water to remove any unattached bacteria according to the procedure of Marshall et al. (24). The cover slips were then crushed in a sterile glass tube containing 10 ml of sterile deionized water. This suspension was agitated with a Vortex mixer for 1 min and allowed to set for about 30 s for the glass particles to sediment, and then a portion was removed for counting purposes. Viable plate counts were made on both the culture suspension and the cover-slip preparation using plate count agar (Difco) and MacConkey agar (Difco) by techniques outlined in Standard Methods (1), and incubated at 35 C for 18 h for the enumeration of both heterotrophic and coliform bacterial populations.

**Data calculation.** The growth rates observed in this study were calculated from experimentally observed washout rates according to published procedures (16,17,20). Herbert et al. (17) and Jannasch (20) have observed that a population \( x \) changes during the transient state in a chemostat by the relationship:

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

or

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

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

\[
\mu = D + \frac{1}{t} \ln \frac{x}{x_0}
\]

If

\[
\frac{1}{t} \ln \frac{x}{x_0} = A
\]

where \( A \) equals the washout rate. Therefore:

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

Culture mass doubling time \( G = 1/\mu \) was used where growth measurements were calculated from viable cell counts, but the cell mass doubling relationship \( t_d = \ln 2/\mu \) was used for the microscopic data.

According to Jannasch (20), four distinctly different types of constant washout rates are conceivable in continuous culture studies when these relationships are considered. These include: (1) the population doubles in one retention time and a steady state results; (2) the population grows at a rate greater than the washout rate; (3) the population does not grow and is washed out of the culture device at a rate equal to the washout rate; (4) the population disappears at a rate faster than the washout rate (defined as death of the population).

**Enzymatic and respiration analyses.** After 10 days of incubation, sorbed and suspended bacterial populations within the chemostat were collected for degradative enzyme analyses and respiration determinations. Cells in suspension and those sorbed to 25 to 30 glass cover slips, which had been ground for 30 s in a Waring blender, were harvested by centrifugation at 18,300 relative centrifugal force in Servall RC-2B (GSA Head) for 15 min at 4 C and washed twice in the appropriate river water substrate. A portion of the cells were for respiration determinations, whereas the rest were broken by sonification with a Sonifer Cell disruptor (Heat systems - Ultrasonics Inc., Plainview, N.Y.) at a setting of 85 W for three 15-s periods and then used directly in the enzymatic and protein analysis.

To quantitate difference in relative metabolic activity between the sorbed and suspended bacterial populations, respiration studies were carried out with the prepared cells with the use of a Biological Oxygen Monitoring System (Cole-Parmer, Chicago, Ill.) in which 1 ml of substrate (m-Plate Count broth, Difco) and 1 ml of cells were placed into the monitor and oxygen uptake was measured for 15 min at 30 C. Respiration was calculated as nmol of oxygen consumed per hour per microgram of protein after correcting for endogenous activity in buffer. Alkaline phosphatase activity was measured by a modification of the Torriani (32) procedure in which 0.5 ml of 1.5 M 2-amino-2-methyl-1-propanol buffer (pH 10.3) and 0.5 ml of 0.01 M p-nitrophenyl phosphate substrate were incubated with 1.0 ml of a cell suspension at 25 C. The procedure of Craven et al. (10) was used to measure \( \beta \)-galactosidase. One milliliter of 0.014 M \( \beta \)-nitrophenyl, \( \beta \)-D-galactopyranoside substrate and 1 ml of 0.3 M sodium phosphate buffer containing 0.003
M MgCl₂ (pH 7.3) were incubated with 1 ml of the cell suspension at 30°C. Arylsulfatase activity was measured by mixing 0.5 ml of 0.05 M of p-nitrophenyl sulfate in 0.19 M tri(hydroxymethyl)aminomethane-acetate buffer (pH 8.3) with 0.5 ml of a cell suspension according to the procedure Milazzo and Fitzgerald (25). Control determinations consisting of the appropriate nitrophenyl derivative and buffer system without the cell preparation was run with each experiment, and all reactions were run in duplicate and terminated with the addition of 4 ml of 0.2 M NaOH after 24 h of incubation as described by Fitzgerald and Milazzo (12) when total enzyme activity is found to be low. Activity was determined by spectrophotometric measurement of the nitrophenol liberated at 400 nm.

RESULTS

A natural population of heterotrophic bacteria was observed to sorb to glass surfaces and multiply during the continuous culture of sterilized river water. Associated with the glass cover slips were what appeared to be two separate layers of material similar to what Marshall et al. (24) have observed in batch cultures of sea water. The first layer was easily removed whereas the remaining organisms tightly adhered to the slide. Those organisms remaining on the glass after washing in a running stream of water are considered here and elsewhere (24) to be irreversibly sorbed to the glass. No difference in the basal nutrient content of either the filtered or autoclaved river water substrates was observed (Table 1), but sufficient nutrient was present to allow for a biochemical oxygen demand (BOD₅) of 2.70 mg/liter. When autoclaved river water was used in the continuous culture of microorganisms normally present in the stream at the point of collection, it was observed (Fig. 1) that both the heterotroph and enteric bacterial populations attached to surfaces within the first 2 h of the experiment. After an initial rapid rate of attachment that was equivalent to a doubling time of about 2 h, both populations continued to sorb over the 24-h period. A corresponding increase

in the number of suspended organisms in both populations was also observed, and it is of interest to note that the number of suspended organisms did not appear depressed during the sorption process with the possible exception of the first 2 h of the experiment. These organisms demonstrated an overall culture mass doubling time of approximately 4.7 h during the initial transient phase.

When autoclaved river water was cultured for extended periods, the initial rapid increase in bacterial numbers of both the suspended and attached population was observed to plateau at 24 h and remain relatively constant until the experiment was terminated (Fig. 2). In this particular system, those organisms enumerated by direct microscopy reached and maintained levels approximately 100-fold greater than the corresponding viable counts of both the attached and suspended heterotrophs, which suggests that these bacteria were capable of growth in the continuous culture system, but not on the heterotrophic media used to enumerate the heterotrophic and the enteric populations. Whereas no attempt was made to clearly determine whether the majority of these organisms were alive or dead, mass doubling times, calculated to compensate for the dead cells counted along with the living, suggest that this population is growing at about the same rate observed for the heterotrophic bacteria including coliforms (Table 2).

Figure 3 contains the results of an experiment similar in design but utilized filter-sterilized river water as a nutrient source. Initial popula-

| Nutrient assayed       | River water (mg/liter) |
|------------------------|------------------------|
| Ammonia nitrogen       | 0.65                   |
| Folin protein          | 6.30                   |
| Carbohydrate           | 4.00                   |
| Biological oxygen demand | 2.70                |
| Orthophosphate         | 0.75                   |
| pH                     | 6.90                   |
tions developing in filter-sterilized river water were similar to those observed with the autoclaved substrate, and little difference was noted in the general growth patterns, including washout. These observations were confirmed by the culture doubling times (Table 2) calculated from the 10-day cultures. No significant difference was detectable between the substrate sterilization process but prolonged culture doubling times were observed for those organisms in suspension that were counted with the aid of the microscope. When these calculations were corrected for that portion of the population representing dead bacteria, the resulting cell doubling times fell into typical values that were obtained for the heterotroph and enteric bacteria. Both the autoclaved and filter-sterilized river water substrates allowed for maximal sorbed populations of about $3 \times 10^6$ cells/ml to be maintained over the 10-day period and this represented about one cell for every 10 $\mu$m of available space on the slides.

Whereas minor differences in specific activity of various degradative enzymes were observed between the autoclaved and filtered river water substrates (Table 3), the attached population appeared to be somewhat more metabolically active than the suspended organisms in terms of alkaline phosphatase activity. No significant difference was noted in the $\beta$-galactosidase nor

aryl sulfatase activity, but the attached population demonstrated a somewhat elevated respiration rate.

The results reported here are typical within standard counting error and represent a 10-day experiment with a sample of river water collected at one time. Both higher and lower bacterial growth rates and levels of enzymatic activity have been noted and these observations correlate exceptionally well with basal nutrient levels in the river.

**DISCUSSION**

There has in recent years been a developing interest in the use of continuous culture to aid in the understanding of problems in microbial ecology and water pollution (16,18-20). Deviations from theoretical predictions in chemostat studies are especially noticeable when nutrient concentrations are high (17,21,29), but the effect of incomplete mixing, liquid entrainment, or in this case, wall growth below the culture surface while using low nutrient substrates, has yet to be evaluated beyond the theoretical basis at Topiwala and Hamer (31). These authors

### Table 2. Bacterial growth in autoclaved and filtered river water

| Organisms          | Wash-out rate per h | Growth rate per h | Culture doubling time $^a$ (h) |
|--------------------|---------------------|-------------------|-------------------------------|
| **Autoclaved water** |                     |                   |                               |
| Attached bacteria  |                     |                   |                               |
| Direct counts      | 0.000               | 0.012             | 83.4$^a$                      |
| Heterotrophic bacteria | 0.001           | 0.011             | 90.9                         |
| Enteric bacteria   | 0.001               | 0.011             | 90.9                         |
| Suspended bacteria |                     |                   |                               |
| Direct counts      | 0.005               | 0.007             | 98.8$^a$                      |
| Heterotrophic bacteria | 0.001           | 0.011             | 90.9                         |
| Enteric bacteria   | 0.001               | 0.011             | 90.9                         |
| **Filtered river water** |                 |                   |                               |
| Attached bacteria  |                     |                   |                               |
| Direct counts      | 0.002               | 0.010             | 100.0                        |
| Heterotrophic bacteria | 0.003           | 0.009             | 111.1                        |
| Enteric bacteria   | 0.001               | 0.011             | 90.9                         |
| Suspended bacteria |                     |                   |                               |
| Direct counts      | 0.006               | 0.006             | 115.5$^a$                     |
| Heterotrophic bacteria | 0.001           | 0.011             | 90.9                         |
| Enteric bacteria   | 0.003               | 0.009             | 111.1                        |

$^a$ Dilution rate = 0.012/h.

$^*$ Culture mass doubling time calculated by $G = 1/\mu$.

$^\ast$ Cell mass doubling time calculated by $t_d = \ln 2/\mu$. 
predict that the sorbed population would have a profound effect on extending the operational range of the continuous culture especially when nutrient concentrations are low, and the culture would not wash out until higher dilution rates were used. It has been previously shown in this laboratory (16) that heterotrophic bacterial populations are capable of growth in the local river water only over a narrow range of dilution rates of 0.005 to 0.027/h with an abrupt washout at the higher rate, and this study has shown that a significant portion of the total bacterial population within the culture device are sorbed to surfaces. Although the sorbed population appeared somewhat more metabolically active than those organisms in suspension, one cannot discount the possibility that the sorbed population may be very different from the suspended population in terms of component species.

Within the confines of these general remarks, irreversible sorption of a portion of the attached bacterial population is of consequence for both practical and theoretical reasons. Current procedures for the bacteriological analysis of water samples, as outlined in Standard Methods (1), calls for the immediate processing but does allow for mail transit of the sample providing the elapsed time period between collection and examination does not exceed 30 h. Certainly the results of this study demonstrate that significant numbers of enteric bacteria, as well as other heterotrophs, will sorb to surfaces and are not removed under a stream of flowing water. Previous reports of substantial reductions in bacterial counts are common (7,11,22), and the data reported suggest that a portion of the collected sample population can sorb to the container walls and in some cases increase in numbers if the basal nutrient concentration is elevated. It has been our general experience that 30 to 40% reductions in the enteric population are common after 24 h of storage at refrigerator temperatures. Although the reduction may not be especially significant when there are several thousand organisms initially present in the sample, a substantial reduction in the count may make the difference in rejection or acceptance of the sample when high quality water is being processed.

The role of irreversible sorption of bacteria to surfaces in the natural environment is unclear, but at the present time freely suspended heterotrophic bacteria are assumed to exist under starving conditions in most natural waters (20,27), and the survival of these organisms in situ largely depends on their ability to reproduce at a rate which will overcome losses in population due to natural causes (5). For some

### Table 3. Respiration and various enzymatic activities of bacteria in suspension or attached to surfaces after continuous culture in autoclaved and filtered river water

| Bacterial population | River water | Respiration* | Enzyme activity* |
|----------------------|-------------|--------------|------------------|
|                      |             |              | Alkaline phosphatase | β-Galactosidase | Aryl sulfatase |
| Suspended            | Autoclaved  | 0.20         | 7.1              | 1.0            | 0.7           |
|                      | Filtered    | 0.20         | 5.2              | 1.0            | 0.3           |
| Attached             | Autoclaved  | 0.22         | 19.0             | 1.1            | 0.6           |
|                      | Filtered    | 0.22         | 13.7             | 0.7            | 0.2           |

* Oxygen (nmol) consumed per hour per microgram of protein.

* Nitrophenol (μg) liberated per 24 h per mg of protein.
time it has been known that many terrestrial bacterial species can grow in waters extremely dilute in nutrient concentration (6,14,20), but the derived doubling times of approximately 100 h seem hardly sufficient to allow for maintenance of the organism in situ (19). It is possible, however, that the average doubling time observed for the freely suspended population in the previous studies may not represent a valid estimate of growth since Bott and Brock (3) have observed bacterial doubling times on surfaces in situ ranging between 2 to 8 h before the competition for space and nutrients become critical. Although it is not possible here to differentiate between sorption to the glass surfaces by organisms from the suspended population and increase in numbers due to growth, data in the present study (Fig. 1) can be resolved into culture doubling times of 1.5 to 3.0 h for the initial stages of attachment. Sanders (28) has shown that slime layers break down when the underlying layers become sufficiently anaerobic to alter the metabolism of the bacteria present, which suggests that a fall in the attached population should result in a concomitant rise in the suspended population. Data in Fig. 2 and 3 do not demonstrate the periodicity observed by Sanders (28) but generally a rise in the attached population resulted in a rise also in the suspended population of bacteria and suggest that after the film becomes stabilized, organisms could be continually sloughed off into the suspended portion of the culture. Such a hypothesis is especially significant because the numbers of bacteria remain constant after the initial rise, and any progeny of these organisms must be released into the culture.

Whereas it is recognized that the chemostat and other laboratory procedures are not exceptionally good models of the natural environment, there is justification in using the device to study natural phenomena especially where low nutrient substrates are used. Wuhrmann (35) has compared a river to a continuous fermentation system similar in many respects to a plug flow type where part of the biomass is in suspension and part is living attached to surfaces, and of which reaches may be considered as a single batch reaction. The application of continuous culture in this study, by virtue of the extremely low dilution rate, does model a controllable continuous system in which the culture volume is replaced approximately once every 3.4 days. Such a system not only allows for the growth of indicator bacteria, but could conceivably be used in bioassay studies to measure stimulatory or toxic effects of various agents on microbial populations.

The use of autoclaved water for studies of an environmental nature is also open to criticism. Taylor (30) has stated that “raising water to temperatures 100 C and higher destroys living antagonistic organisms, modifies bacteriocidal and toxic agents, and alters the chemical composition of organic matter so it becomes more assimilable to E. coli to produce sufficient effect for these organisms to multiply.” Water passed through a washed bacteriological filter is not a satisfactory alternative in itself, but no chemical or biological effect was noted in this study that could be attributed to the sterilization process.

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