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Role of Bacteria and Protozoa in the Removal of *Escherichia coli* from Estuarine Waters

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The removal of *Escherichia coli* from estuarine water was investigated. The survival of *E. coli* was dependent on the presence of protozoan predators and not on the presence of lytic bacteria. When indigenous protozoa were removed from estuarine water by filtration, the destruction of coliform populations was negligible. In studies designed to prevent the growth of indigenous bacterial populations without affecting protozoan populations, coliform destruction was significant.

Numerous investigators have reported that marine waters exhibit a marked bactericidal activity toward nonmarine bacteria (2, 6, 11, 13). Various chemical and biological mechanisms such as heavy metal toxicity (8), salinity (2), competition (6), antibiosis (10), and predation (12) have been suggested as playing a role in the bactericidal phenomenon.

Mitchell and Morris (12) and Paoletti (14) demonstrated the existence of microbial predators with their observation that when untreated seawater was added to agar containing dense suspensions of *Escherichia coli*, discrete clear areas (plaques) would develop. Inspection of different plaques revealed a variety of bacteria and protozoa having lytic activity toward *E. coli*.

In recent years there has been considerable speculation on the role of *Bdellovibrio bacteriovorus* in controlling bacterial populations in natural systems. Guelin et al. (7) suggested that bdellovibrios may be important in the autopurification process of polluted rivers based on the observation that salmonella die-off was associated with the presence of bdellovibrios in untreated river water. Mitchell and Morris (12) also reported that seawater, bactericidal to *E. coli*, contained large numbers of bdellovibrios.

Bacteriophage have also been considered a factor in the removal of coliforms from natural environments. Both Guelin et al. (7) and Carlucci and Pramer (1) have detected coliphage in marine waters subject to sewage contamination. However, the mere presence of phage is not an indication of their activity in removing coliforms from marine waters. Investigation into the role of phage in controlling bacterial populations has shown them to be ineffective (1, 15). In order to replicate, phage generally require actively growing hosts. This requirement is usually not met when coliforms are introduced into aquatic or terrestrial environments.

Little is known concerning the role of marine protozoa in controlling bacterial populations. The relationship between bacteria and protozoa in fresh waters was studied by Small (16), who observed an indirect relationship between the numbers of bacteria and the numbers of protozoa. Curds and Fey (3, 4) have conducted the most definitive studies on the effect of protozoa on bacterial populations in sewage treatment processes. They showed that the presence of protozoa dramatically reduced the survival time of *E. coli* in activated sludge and that ciliates were primarily responsible.

This paper reports the results of studies on the role of indigenous microbial populations in removing coliform bacteria from estuarine waters of San Francisco Bay.

MATERIALS AND METHODS

Organisms. The test strain of *E. coli* used throughout this study was isolated from a human fecal stool and characterized as IMVC (++--) and Eijkman positive. Cells from the stock culture were inoculated into 500 ml of nutrient broth (Difco) and incubated on a rotary shaker at 20 C for 48 h. The cells were then washed three times by centrifuging at 8,000 × *g* and resuspending in sterile distilled water containing 2.5% NaCl. After being washed, the suspension was standardized turbidimetrically to obtain a viable count of 5×10^{10} cells/ml in 2.5% NaCl solution. The final suspension was used both as a nutrient source for the enumeration of plaque-forming organisms and, when appropriately diluted, to inoculate *E. coli* into estuarine water samples.

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A double mutant, resistant to both penicillin and streptomycin, was obtained by suspending 10^{10} cells of the parent *E. coli* strain on nutrient agar (Difco) containing 500 mg of penicillin G per liter. After incubation, cells were picked from a colony, transferred to nutrient broth, and incubated for 48 h. The cells were then centrifuged, washed, suspended on nutrient agar containing 500 mg each of penicillin G and streptomycin sulfate per ml, and reincubated. Mutant cells were then picked from one of the resulting colonies and stored on nutrient agar slants until use. This daughter strain was used to determine *E. coli* survival in the absence of indigenous bacterial growth. The latter was controlled by adding penicillin and streptomycin to bay water.

Enumeration of microbial predators and parasites indigenous to estuarine water. Those organisms capable of utilizing *E. coli* as their sole nutrient source were enumerated by counting plaques on dense lawns of *E. coli* in double-layer agar. This technique was a modification of that used by Stolp and Starr (17). A 1.2% agar suspension of Rila marine salts (30 g/liter; Rila Products, Teaneck, N.J.) was added as a base layer to petri dishes. After solidifying, the base layer was overlaid with 4 ml of semisolid (0.6% agar) Rila salt suspension containing 0.5 ml of sample and 0.5 ml of *E. coli* suspension. The plates were incubated at 20 C for 20 days before counting plaques. When necessary, plaques were inspected microscopically to determine whether their origin was bacterial or protozoan.

Enumeration of *E. coli* in estuarine water. *E. coli* were enumerated by spread plating 0.1 ml of estuarine water on eosin-methylene blue agar. Sample dilutions were made in sterile 2.5% saline solution. All plates were incubated at 37 C for 18 to 24 h before counting.

Seeded and autoclaved estuarine water samples. A 15-liter sample of estuarine water was collected approximately 100 yards (about 91.4 m) offshore during the fall. The water was autoclaved in 500-ml portions and stored in the dark until use. After sterilization, the sample was characterized as having a salinity of 3.1% and a chemical oxygen demand of 14.1 ml/liter. When autoclaved water was required, it was drawn from this sample. At the start of an experiment requiring seeded samples, fresh San Francisco Bay water was collected. Seeded samples consisted of 48 ml of autoclaved bay water, 1.0 ml of an appropriately diluted *E. coli* suspension, and 1.0 ml of freshly collected, untreated bay water, which served as a seed to introduce indigenous microbial populations. The natural decay of coliforms in bay water was determined by adding 1.0 ml of *E. coli* suspension to autoclaved water in the absence of bay water seed. Samples were incubated in 125-ml flasks at 20 C on a rotary shaker (60 rpm).

Filtered estuarine water samples. To selectively monitor the activity of various bacterial populations on the removal of coliforms, freshly collected bay water samples ranging in salinity from 0.6 to 3.1% were passed through sterile membrane filters (Millipore Corp.). The filters ranged in pore size from 0.22 to 3.0 μm. An appropriate number of *E. coli* were added to 49-ml portions of the filtered water. The filtrates were then incubated at 20 C on a rotary shaker.

Estuarine water treated with antibiotics. To prevent growth of indigenous bacteria without affecting protozoa, 500 mg of both streptomycin sulfate and penicillin G (Eli Lilly Co.) per liter were added to 48 ml of autoclaved bay water. The samples were seeded with 1.0 ml of untreated bay water and 1.0 ml of an appropriately diluted suspension of the antibiotic-resistant *E. coli* mutant. To insure that the growth of indigenous bacteria was inhibited, aliquots of the samples were plated at daily intervals on bay water agar, which consisted of 1.5 g of peptone (Difco), 0.5 g of yeast extract, 12 g of agar, 670 ml of 2.5% NaCl solution, and 330 ml of aged bay water.

All experiments were conducted with at least two replicate samples. When enumerating coliforms or plaque-forming microorganisms, triplicate plates were used for each sample.

RESULTS

The relationship between the destruction of *E. coli* and the growth of microbial predators and parasites is presented in Fig. 1. The addition of *E. coli* cells to samples seeded with freshly collected bay water always resulted in a reduction in coliform numbers concurrent with development of predator populations. The number of plaque-forming units (PFU) that were observed in any given estuarine water sample should be considered as a conservative estimate of the number of lytic microorganisms. Lytic bacteria requiring growth factors not supplied by the *E. coli* substrate would probably not develop plaques in the double-layer agar medium used in this study. Furthermore, it was observed that ciliates and some flagellates that were extremely efficient in removing coliforms

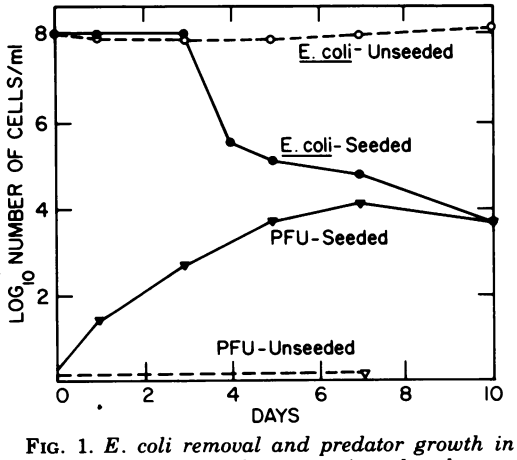

FIG. 1. *E. coli removal and predator growth in estuarine water. Symbols: (——) Autoclaved water seeded with 1.0 ml of freshly collected, untreated bay water; (-----) autoclaved bay water, unseeded.*

were incapable of plaque formation in double-layer agar. Throughout a 2-year period, over 50 samples, representative of all seasons, were seeded with untreated San Francisco Bay water and evaluated for bactericidal activity. In every case, coliform destruction was associated with an increase in the number of PFU. When *E. coli* cells were added to unseeded samples of bay water, there was no significant change in their number (Fig. 1) and, as would be expected, the number of plaque-forming organisms was below the limit of detection.

Random microscopic examination of plaques showed they were formed by a wide variety of microorganisms. The most common were small limax amoebae, microflagellates, bdellovibrios, nonfruiting myxobacteria, and numerous types of lytic gram-negative bacteria.

To evaluate the relative importance of bacteria and protozoa in the destruction of *E. coli*, two different methods were used to separate their activity in estuarine water samples: the addition of prokaryote-specific antibiotics and the use of differential membrane filtration, which allowed the passage of representative species of bacteria while entrapping protozoa.

The addition of penicillin and streptomycin to bay water allowed measurement of coliform survival in the absence of indigenous bacteria that were potentially antagonistic, competitive, or lytic towards *E. coli*. When a suspension of the antibiotic-resistant mutants was added to autoclaved bay water containing penicillin and streptomycin and then seeded with untreated bay water, there was a significant reduction in the number of *E. coli* (Fig. 2). By comparison, there was only a gradual decline in the number of mutants in the unseeded samples.

It was noted that the presence of antibiotics did not always inhibit indigenous bacterial growth. For this reason, each trial was started with ten replicate samples to insure that at least two samples maintained suppression of indigenous bacteria until the trial was terminated. Contaminated samples and the respective survival data were discarded when bacterial growth became apparent.

The survival curves in Fig. 2 show that significant bactericidal activity existed in samples seeded with freshly collected bay water, indicating that the coliform removal was not necessarily dependent on the presence of lytic or antagonistic bacterial populations. It was observed that the destruction of *E. coli* was most severe between days 2 and 4. During this time there was also a 10-fold increase in the number of protozoan predators as indicated by the increase in the number of PFU. In this particular

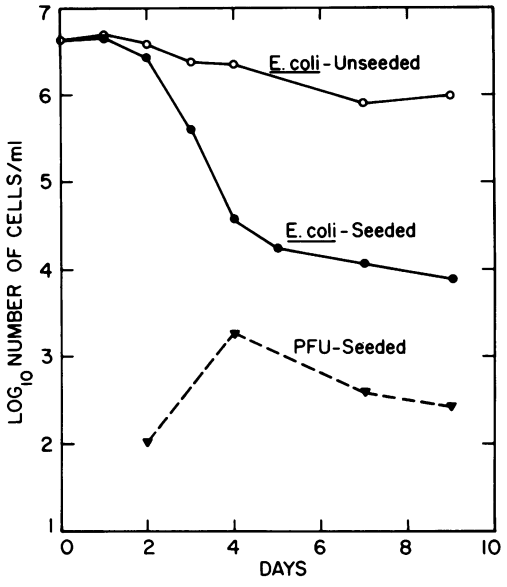

FIG. 2. *E. coli removal and predator growth in samples containing penicillin and streptomycin (500 mg of each per liter). Symbols: (○——○) E. coli in unseeded autoclaved bay water; (●——●) E. coli survival in autoclaved water seeded with 1.0 ml of freshly collected untreated bay water; (▼- - -▼) predator growth in autoclaved water seeded with 1.0 ml of freshly collected, untreated bay water.*

trial the samples contained a mixed population of amoebae and microflagellates which, when subcultured, were shown to have obligate growth requirements for viable host cells. Both organisms were maintained for over 6 months by subculturing in 3.0% Rila salt solution. *E. coli* cells were added to the Rila salt solution as the sole nutrient source (10^9 cells/ml). After subculturing, turbid suspensions of *E. coli* were cleared by both organisms in a matter of days.

To evaluate the effects of indigenous bacterial activity on the survival of *E. coli*, attempts were made to inhibit protozoan growth by the use of polyene antibiotics. Combinations of nystatin and amphotericin B were added to estuarine water without success. Ciliate growth consistently occurred in the presence of these antibiotics. Membrane filtration was finally used to separate the two populations by entrapment of protozoa on the filters while allowing representative species of bacteria to pass into the filtrates. In all cases, the protozoa in freshly collected bay water were quantitatively trapped by membranes 1.2 μm and smaller, and conversely would generally pass through membranes having pore sizes of 3.0 μm or greater.

Coliform reduction in the filtrates obtained from the 0.45-, 0.80-, and 1.2-μm membranes

(Fig. 3) was approximately the same after 10 days of incubation. Microscopic inspection of the filtrates (concentrated by centrifugation) and of plaques derived from the filtrates showed that they were free of protozoa. Maximum destruction of coliforms occurred in the 3.0-μm filtrates and in untreated bay water samples, all of which were shown by microscopic examination to contain numerous microflagellates and microciliates.

By filtering bay water through membranes of various pore sizes, it was possible to control the microbial diversity in the filtrates. Filtrates obtained from 0.22-μm membranes were usually free of indigenous microorganisms capable of forming plaques (Fig. 3). The number of *E. coli* remained constant throughout the trial. We consider it significant that the removal of all plaque-forming organisms resulted in the complete elimination of the bactericidal activity towards *E. coli*. Bdellovibrios were the only plaque-forming organisms present in the 0.45-μm filtrates. Although there was a tremendous increase in their numbers (Fig. 3), there was little effect on *E. coli* survival, which indicates that bdellovibrios were probably not a major factor participating in the bactericidal activity of estuarine water. The numbers of PFU in the 0.45-μm filtrates were always significantly greater than in any of the other samples, suggesting that bdellovibrio growth was suppressed by the presence of other indigenous microorganisms that pass through filters larger than 0.45 μm.

Filtrates from 0.8-μm membranes generally contained bdellovibrios and one to two additional species of lytic bacteria. The diversity of bacterial plaque-forming organisms in the 1.2-μm filtrates was comparable to that in 3.0-μm filtrates. Regardless of bacterial diversity, maximum coliform destruction was correlated with the presence of protozoa. The effect of indigenous protozoa on coliform survival is most dramatically illustrated in Fig. 4. When coliform survival was monitored simultaneously in a series of five different 3.0-μm filtrates, coliform destruction in four of the samples was comparable to that in unfiltered bay water. When these samples were examined microscopically, protozoa were observed to be present. The number of coliforms remained constant in the fifth sample, and microscopic inspection of plaques and filtrate showed that protozoa were absent.

Although the number of lytic bacteria increased in this sample, there was little effect on the survival of *E. coli*. This observation supports the contention that lytic bacteria, including bdellovibrios, are much less efficient in removing coliforms than are the protozoa.

DISCUSSION

The results obtained in this study indicate that bacterial competition, antagonism, and even bacterial predation were relatively unimportant in removing coliforms from estuarine waters. When indigenous bacterial activity was suppressed by addition of antibiotics, there remained a marked bactericidal activity towards

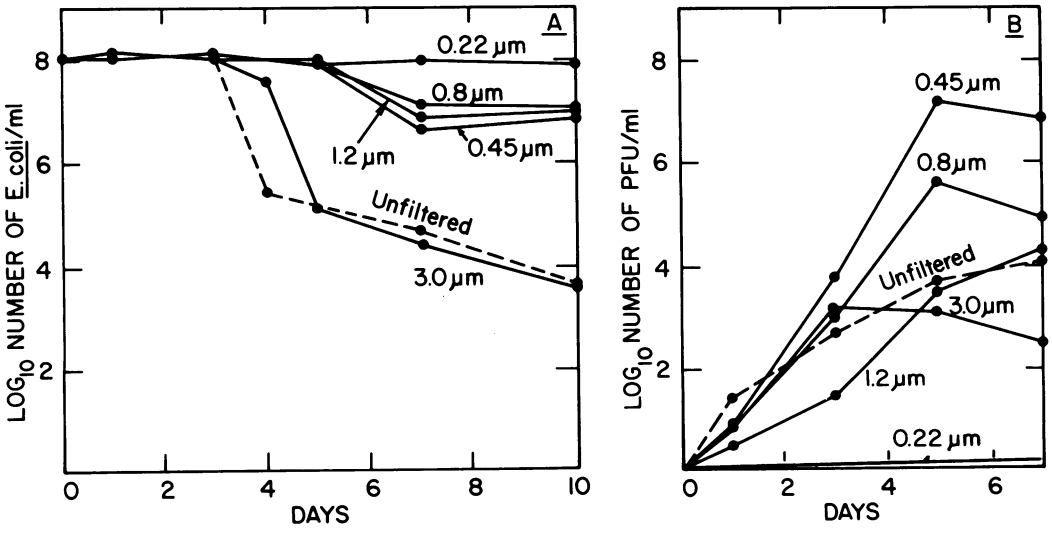

FIG. 3. *E. coli survival (A) and predator growth (B) in bay water filtered through membranes having different pore sizes.*

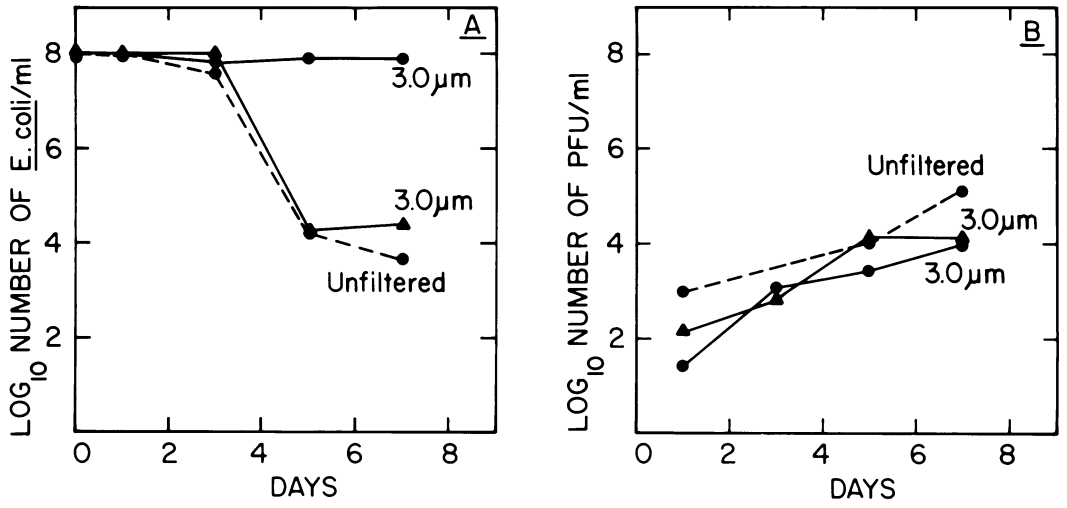

FIG. 4. *E. coli survival (A) and predator growth (B) in 3.0-μm filtrates. Symbols: (●——●) 3.0-μm filtrate with protozoa absent; (▲——▲) 3.0-μm filtrate with protozoa present; (●-----●) autoclaved water seeded with freshly collected, untreated bay water (protozoa present).*

the antibiotic-resistant strain of *E. coli*. The decline in *E. coli* number was associated with an increase in the number of protozoa. The association between the presence of protozoa and coliform destruction was further supported when bay water was filtered through membranes of different pore sizes. In the absence of microciliates or flagellates in the filtrates, *E. coli* survival was not significantly affected regardless of the types or number of bacterial predators present. In those filtrates containing protozoa, there was a 2- to 4-day lag period in the survival of *E. coli* followed by logarithmic death. The lag can be explained by the time required for protozoa to reach sufficient density to effect a detectable removal of *E. coli*. It was observed that the lag time in survival could be reduced by adding a less concentrated suspension of *E. coli* at the start of an experiment.

Recently, Habte and Alexander (8) reported that protozoan predation was responsible for the decline in number of *Xanthomonas campestris* in natural soil. It appears that protozoan predation may exert pressure on bacterial populations in a variety of environments, both aqueous and terrestrial.

An important question remains which concerns the bactericidal activity of marine waters. Why is the destruction of foreign bacteria apparently more pronounced in marine waters than in fresh waters? It seems unlikely that marine protozoa have evolved into forms that are more active against enteric bacteria than are those protozoa indigenous to fresh waters. The adverse chemical and physical environment of bay water would be suspect, except that the results of this study demonstrated that the presence of protozoa was required for significant coliform destruction.

It is proposed that the chemical and physical properties of marine waters indirectly influence the survival of coliform bacteria. When coliforms are introduced into such an adverse environment, their death is not affected by these properties per se, but their ability to compete for nutrients with indigenous marine microorganisms may be affected. Bearing this in mind, it is proposed that micropredators exert a destructive pressure on what would be in their absence a static population of coliforms subject to slow natural decay. Although confirmatory experimental data are not known to exist, it is proposed that coliforms may be better equipped to compete with indigenous bacteria in freshwater systems. Possibly, the protozoan activity in fresh waters is comparable to that in marine waters, but is counteracted in the former by the ability of *E. coli* to compete more successfully.

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