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Minimum Bacterial Density for Bacteriophage Replication: Implications for Significance of Bacteriophages in Natural Ecosystems

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Bacteriophage 80α did not increase in number in cultures containing less than about 1.0×10^4 to 1.5×10^4 CFU of *Staphylococcus aureus* per ml, but bacteriophage replication did occur when the number of bacteria exceeded this density, either initially or as a result of host cell multiplication. The minimum density of an asporogenous strain of *Bacillus subtilis* required for an increase in the number of bacteriophage SPβ *c*I was about 3×10^4 CFU/ml. The threshold density of *Escherichia coli* for the multiplication of bacteriophage T4 was about 7×10^3 CFU/ml. In the presence of montmorillonite, bacteriophage T4 did not increase in number until the *E. coli* population exceeded 10^4 CFU/ml. The mineralization of glucose was not affected in *E. coli* cultures inoculated with a low number of bacteriophage T4, but it could not be detected in cultures inoculated with a large number of phage. The numbers of bacteriophage T4 and a bacteriophage that lyses *Pseudomonas putida* declined rapidly after being added to lake water or sewage. We suggest that bacteriophages do not affect the number or activity of bacteria in environments where the density of the host species is below the host cell threshold of about 10^4 CFU/ml.

Since the discovery of bacteriophages, it has often been assumed that they affect the population density or activity of their host bacteria in natural ecosystems. Evidence that bacteriophages may affect the populations of their hosts in natural ecosystems is found in the report of Evans et al. (10), who noted that bacteriophages reduce the population of *Rhizobium trifolii* in the rhizoplane. Barnet (5) found that the presence of bacteriophages increases the proportion of phage-resistant *R. trifolii* in the root zone of clover and increases the proportion of phage-resistant mutants that are not effective in nodulation. However, the studies by Carlucci and Pramer (6), Pretorius (18), and Chambers and Clarke (7) suggest that bacteriophages are not responsible for the decline of coliforms or *Vibrio cholerae* in marine water, sewage, and freshwater. Similarly, Habte and Alexander (12) concluded that bacteriophages are not responsible for the decline of *Xanthomonas campestris* in soil.

A few investigators have tried to determine the conditions in nature that favor bacteriophage replication. Reanney and Marsh (19) suggest that bacteriophages are only found at high titers in soil under conditions suitable for proliferation of their hosts. Anderson (2) reported that a bacteriophage of *Salmonella typhi* does not replicate unless the population density of the host is greater than 3.0×10^4 CFU/ml (i.e., above a threshold level for bacteriophage replication). The occurrence of thresholds for reproduction is common among microbial predators and parasites. For example, protozoa and *Bdellovibrio* spp. do not detectably affect susceptible bacterial species when the densities of those susceptible species are below a threshold value (8, 13). A minimum density of susceptible hosts is also important in the spread of communicable diseases of humans (3).

In view of the paucity of information on the significance of bacteriophages in natural ecosystems, we undertook a study to determine the conditions in nature required for bacteriophages to affect the number and metabolic activity of their hosts.

MATERIALS AND METHODS

Escherichia coli SH305 and bacteriophage T4 were obtained from S. Hilliker (Bucknell University, Lewisburg, Pa.). *Bacillus subtilis* CU3389 and bacteriophage SPβ *c*I were provided by S. A. Zahler (Cornell University, Ithaca, N.Y.). *Staphylococcus aureus* RN450 and bacteriophage 80α were obtained from R. P. Novick (Public Health Research Institute, New York, N.Y.), and *Pseudomonas putida* was supplied by N. C. Dondero (Cornell University). A bacteriophage for *P. putida* was isolated from sewage by the method described by Adams (1) and was designated P10.

The bacteria were grown at 28°C on a rotary shaker operating at 120 rpm. Suspensions containing high densities of bacteriophages were prepared by the method of Adams (1). The bacteria were counted by spreading portions of appropriate dilutions on plates of an agar medium prepared from Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), and bacteriophages were counted by the agar overlay technique (1). The top layer was Trypticase soy broth solidified with 0.6% Bacto-Agar (Difco Laboratories, Detroit, Mich.), and the bottom layer was Trypticase soy broth solidified with 1.1% Bacto-Agar. The mineral salts medium used in studies of glucose mineralization contained 1.5 g of KH_2PO_4, 7.15 g of Na_2HPO_4, 3.0 g of $(NH_4)_2SO_4$, 5 mg of $FeSO_4$, 400 mg of glucose, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.26 g of $CaCl_2$ per liter of distilled water. Dilutions were performed in mineral salts medium with no added glucose.

To determine the minimum host density required for bacteriophage replication, bacteriophages and exponentially growing cells were added to 250-ml Erlenmeyer flasks containing 50 ml of Trypticase soy broth. The cultures were incubated at 28°C on a rotary shaker operating at 120 rpm, and at regular intervals, samples were taken, diluted, and plated to determine the numbers of bacteria and bacteriophages. The plates were incubated at 28°C.

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To determine the effect of bacteriophages on the metabolism of their hosts, uniformly labeled [^{14}C]glucose (specific activity, 318 mCi/mmol; New England Nuclear Corp., Boston, Mass.) was added to 500-ml Erlenmeyer flasks containing 50 ml of mineral salts medium. Radioactivity in the culture medium was approximately 1,000 to 1,500 dpm/ml. The total glucose concentration in the medium was 400 μg/ml, of which 3.6 to 5.4 ng/ml was derived from the radioactive glucose. The medium was inoculated with bacteriophage T4 and *E. coli* derived from a culture in the exponential phase of growth, and the flasks were then incubated on a rotary shaker at 28°C. At various times, the amount of radioactivity remaining in the culture was determined by the method of Subba-Rao et al. (23).

To determine the potential for bacteriophage survival in natural ecosystems, phages T4 and P10 were added to 250-ml Erlenmeyer flasks containing 50 ml of autoclaved or nonsterile lake water or sewage. The lake water was obtained from Cayuga Lake (Ithaca, N.Y.) and filtered through a no. 66085 glass fiber filter (Gelman Sciences, Inc., Ann Arbor, Mich.). Fresh, untreated sewage was obtained from the primary settling tank of the Ithaca sewage treatment plant and filtered through a Whatman no. 41 filter. The lake water and sewage were sterilized by autoclaving for 20 min.

To determine the effect of clay on bacteriophage replication, 5 mg of montmorillonite was added per ml of Trypticase soy broth. The clay was prepared by suspending crude bentonite (Wards Scientific, Rochester, N.Y.) in distilled water, decanting the liquid to remove heavy impurities, and centrifuging the decanted suspension at 3,020 × *g* for 10 min. The broth containing the clay was sterilized by autoclaving for 20 min and then inoculated with T4 and *E. coli* derived from a culture in the exponential phase of growth. Bacterial and bacteriophage counts are the means of values from duplicate plates unless stated otherwise.

RESULTS

Bacteriophage 80α was incubated with *S. aureus* at three different initial densities of the host bacterium. When the initial number of *S. aureus* was 1,600 cells per ml, the density of the virus remained essentially constant for 3 h (Fig. 1A). The number of bacteriophages increased at 4 h, when the density of *S. aureus* reached approximately 3 × 10^4 CFU/ml. Taking into account a latent period of 40 min for virus 80α (20), the density of *S. aureus* at which bacteriophage replication actually began was approximately 1.5 × 10^4 cells per ml. This density of cells present 40 min before the increase in the number of viruses was considered the threshold value.

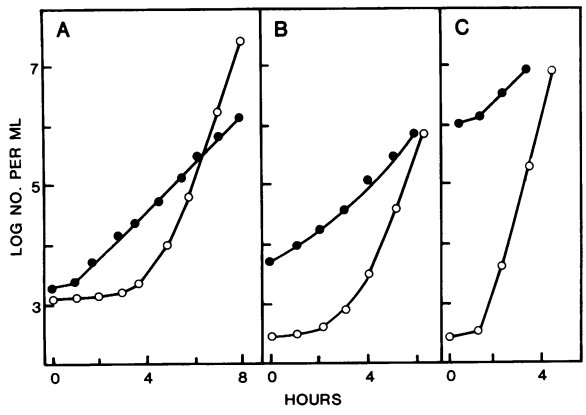

FIG. 1. Growth of bacteriophage 80α (○) on *S. aureus* (●) provided at three initial densities.

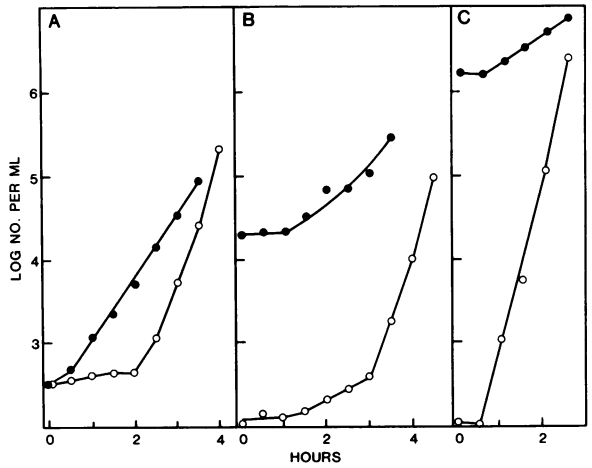

FIG. 2. Replication of bacteriophage T4 (○) on *E. coli* (●) provided at three initial densities. Data are the averages of triplicate counts.

In flasks containing *S. aureus* at an initial density of 6,700 CFU/ml, the number of bacteriophage 80α particles began to increase after 2 h, when the host population reached a density of approximately 2 × 10^4 CFU/ml (Fig. 1B). After subtraction of the time for the latent period, the *S. aureus* density present when virus 80α started to replicate was approximately 10^4 CFU/ml. With an initial density of 1.1 × 10^6 CFU/ml, the number of bacteriophage 80α increased soon after 90 min and then began to increase rapidly (Fig. 1C).

Bacteriophage T4 was added to a culture of *E. coli* containing an initial density of 310 CFU/ml. The number of bacteriophage particles did not begin to increase appreciably until after 2 h (Fig. 2A). When T4 showed the first marked increase, the density of the host bacteria was approximately 1.5 × 10^4 CFU/ml. The latent period for T4 is approximately 25 min (9). Because the bacterial count of *E. coli* 25 min before the increase in phage numbers was approximately 7 × 10^3 CFU/ml, this value represents the minimum density of host cells required for increases in virus numbers in culture. When the initial bacterial density of *E. coli* was 2.1 × 10^4 CFU/ml, the number of host cells did not increase for 1 h (Fig. 2B). Because the intracellular development of bacteriophages is dependent on actively growing hosts (22), the phage did not increase in number rapidly during this time. However, when the host *E. coli* cells began to grow after 1 h, the T4 density also began to increase. The density of T4 began to increase after 30 min (the length of the latent period) when added to a culture initially containing 2.0 × 10^6 CFU of *E. coli* per ml (Fig. 2C).

A similar experiment was performed with an asporogenous mutant of *B. subtilis* and bacteriophage SPβ *c*I (a lysogenic phage with a mutation in its repressor gene). In cultures with 270 *B. subtilis* CFU/ml present initially, an appreciable increase in the number of bacteriophages was only evident at 5 h, when the host population density was approximately 8 × 10^4 CFU/ml (Fig. 3A). After correcting for a 45-min latent period (24), the threshold density required for bacteriophage SPβ replication was approximately 3 × 10^4 CFU of *B. subtilis* per ml. When the initial number of *B. subtilis* cells was 1.0 × 10^4 CFU/ml, the phage density did

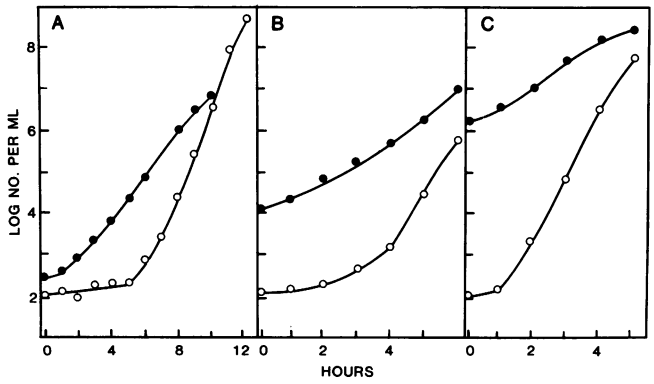

FIG. 3. Growth of bacteriophage SPβ *cI* (○) on *B. subtilis* (●) provided at three initial densities.

not change for some time. However, as the bacteria multiplied and their density exceeded the apparent threshold value, the phage density started to increase (Fig. 3B). When 1.3×10^6 CFU of *B. subtilis* per ml were present initially, the phage density began to increase slowly for 60 min and then increased rapidly (Fig. 3C).

A study was performed to determine the effect of bacteriophages on the metabolic activities of the host population. The initial host density was held constant while the initial phage densities were varied. When added to a culture inoculated with 110 CFU of *E. coli* per ml, bacteriophage T4 inoculated at a density of 50 PFU/ml had no effect on glucose mineralization (Fig. 4A). In contrast, in cultures with an initial density of 1,100 PFU of bacteriophage T4 per ml, the rate of mineralization by an *E. coli* culture initially containing 130 CFU/ml was decreased, and cultures initially receiving 1.0×10^4 PFU of T4 per ml had an even lower mineralization rate (Fig. 4B). The long delay between the addition of the cells and the onset of mineralization in these experiments resulted from the long lag phase in the growth from the small inoculum of *E. coli* in mineral salts medium.

In another experiment, in which the initial population of *E. coli* in one-third strength Trypticase soy broth was 1,300 CFU/ml, 19% of the glucose carbon was mineralized after 20 h when no phages were present. If the culture initially received 10 or 1,000 PFU of phage per ml, 17 and 12%, respectively, of the glucose carbon was mineralized. At initial phage densities of 1.0×10^5 and 1.0×10^7 PFU/ml, no mineralization of glucose was detected in the test period.

When bacteriophages T4 and P10 were added to sterile lake water, their numbers did not decline in 30 days (Fig. 5A). In nonsterile lake water, however, the density of both phages decreased rapidly. At 30 days, less than 10 PFU of phage per ml remained. In autoclaved sewage, neither of these two bacteriophages declined appreciably in number (Fig. 5B). When the bacteriophages were added to nonsterile sewage, the density of both viruses decreased, but the decline was slower than in lake water; fewer than 1,000 PFU/ml remained at 30 days.

When 100 *E. coli* cells and 100 bacteriophage T4 particles were added per ml of Trypticase soy broth containing montmorillonite (5 mg/ml), the rate of increase in CFU of *E. coli* was slower than was previously noted when no clay was present. Microscopic examination showed that the surfaces of the clay particles were covered with many cells. The density of bacteriophage T4 remained constant for at least 4 h and then increased (Fig. 6). The first detectable increase in the number of bacteriophage occurred at an *E. coli* density of approximately 2×10^4 CFU/ml. Taking into account the 25-min latent period, the apparent threshold host density for T4 replication in the presence of clay was approximately 10^4 CFU/ml.

DISCUSSION

The behaviors of each of the three bacteriophages were similar. Thus, at low host densities, a delay was evident prior to the increase in virus particles. This delay lasted until the host had proliferated to achieve a threshold density. Thereafter, the number of viruses increased rapidly. When added to host cells at an initial density above this threshold, the number of phages remained constant or increased slightly and then increased rapidly. The length of time between the start of the experiment and the first measurement was greater than the latent period for bacteriophages 80α and

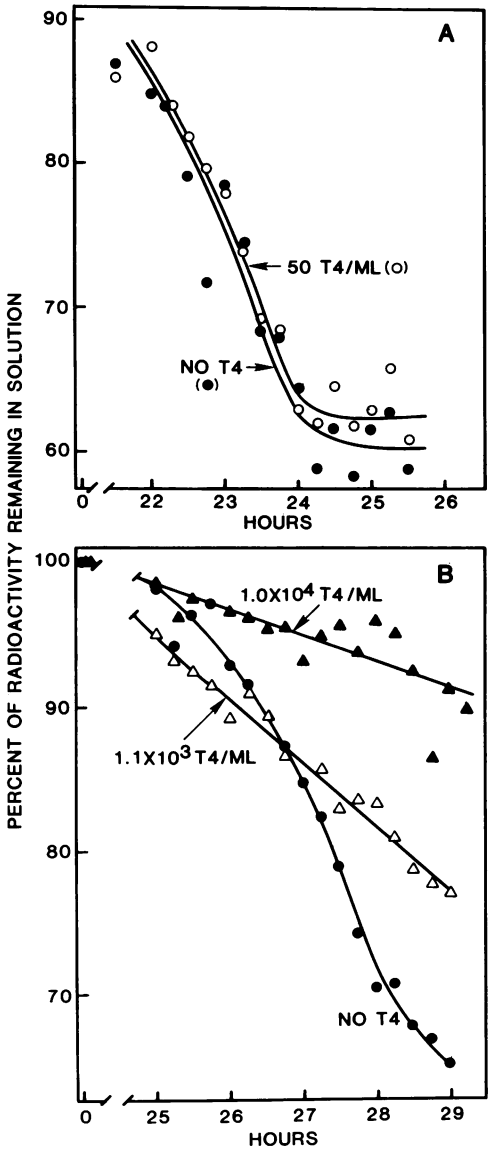

FIG. 4. Effect of initial density of bacteriophage T4 on the mineralization of [^{14}C]glucose by *E. coli*. A and B represent different experiments.

SPβ and was equal to the latent period for bacteriophage T4. The failure of a virus to multiply after the latent period could be the result of variations in latent period duration, which depends on the growth rate of each host cell (22). Many of the infected cells may have not yet burst when the early counts were made. When enough time had elapsed for all of the initially infected cells to burst, the number of viruses increased rapidly. That the threshold host densities were similar at about 10^4 CFU/ml for gram-negative and gram-positive rods, a gram-positive coccus, and both lytic and lysogenic phages suggests that the threshold for replication is probably similar for most if not all phage-host combinations.

If thresholds are universal, it is likely that bacteriophages would only affect the number of their hosts in environments where bacterial hosts are present at population densities above this threshold. Because these experiments were performed in the laboratory, the conditions were probably

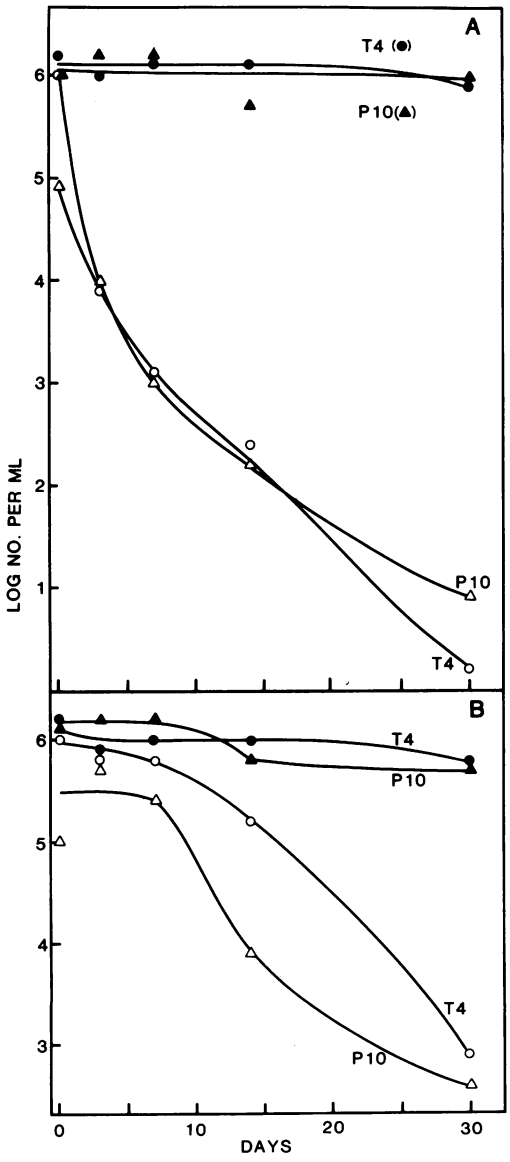

FIG. 5. Survival of bacteriophages in Cayuga Lake water (A) and sewage (B). Closed symbols indicate autoclaved lake water or sewage, and open symbols indicate nonsterile lake water or sewage.

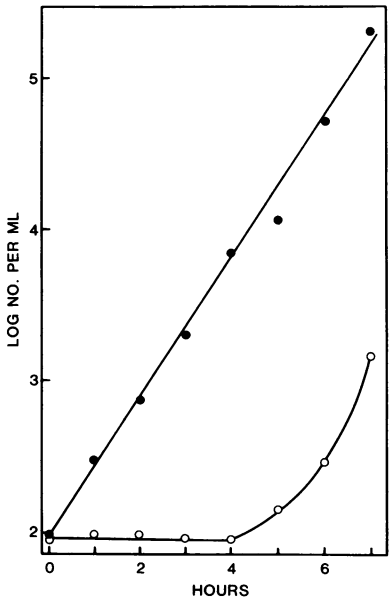

FIG. 6. Replication of *E. coli* (●) and bacteriophage T4 (○) in a medium containing montmorillonite.

nearly optimal for the growth and replication of the bacteria and their phages. In nature, host cells may not grow or metabolize at their maximum rates. There could also be a higher proportion of nonviable cells, which may adsorb phage particles but not allow them to replicate. Thus, the bacterial population density required for bacteriophage replication in nature may be somewhat different from the value of 10^4 CFU/ml reported here. It is not certain how many natural ecosystems contain populations of individual species or strains greater than this threshold. If the cell density of individual bacterial species in marine environments and in soils is low, then the existence of a threshold may account for the low densities of bacteriophages in these environments (2, 4, 19, 21). Therefore, bacteriophages probably do not account for the frequent disappearance of species introduced into such environments. Conversely, in environments in which high densities of hosts exist, bacteriophages would flourish and could affect the number and activity of the bacteria present. Such high bacteriophage densities are found in human feces (2), in sewage and sewage sludge (11), in the rumen (17), and in the senescent nodules of legumes (15).

The threshold host density for bacteriophages could be a consequence of the time needed for the virus particles to contact host cells. The time required for one bacteriophage to contact a host can be calculated from a first-order equation describing the adsorption of bacteriophages to host cells (22). With measured constants for bacteriophage T4 (22) and an *E. coli* density of 100 CFU/ml, which was below the measured threshold, it could be calculated that an average of 4,000 min would be required for one virus particle in an initial density of 1,000 PFU/ml to contact a host. Under such circumstances, the initiation of replication would be delayed. At a host density of 10^5 CFU/ml, which is above the threshold, it would take 4 min.

The lysis by bacteriophages of large bacterial populations in culture has a dramatic impact on the metabolism of the host species. The present data show that the low densities of bacteriophages characteristic of many natural ecosystems did not affect the mineralization of glucose by *E. coli*. Thus,

in an ecosystem in which the population of no single species greatly exceeded the threshold density, the impact of these viruses on the metabolism of the community would be negligible.

Although bacteriophages may have no significant impact on bacterial populations and activity in marine and fresh water and in soils in which the density of their hosts is low, one must explain the occurrence of the viruses there. One possibility is a constant inflow of new bacteriophages from environments with high host densities, as from sewage discharged into surface waters. Another possibility is a long survival time of the virus in the absence of its host (16), which would allow the bacteriophage a longer time to find a host present at a low density. However, although bacteriophages T4 and P10 survived in large numbers in sterile lake water and sewage, their numbers fell rapidly in nonsterile lake water and sewage, presumably because of some biological factor.

The presence of localized high cell densities on clay surfaces or clumps of detritus would also allow viruses to replicate in environments where the total host density is low. However, although the methods we used would not detect an increase in the density of viruses that remained adsorbed to clay surfaces, the finding that the threshold densities for host cells were similar in the presence and absence of montmorillonite made it less likely that the bacteriophage has a major impact on the host population in the presence of clay. In fact, surfaces may inhibit the replication of bacteriophages (14).

Thus, it is plausible that bacteriophages have little or no impact on the growth, activity, and survival of bacteria in natural environments where the densities of individual bacterial populations are low. If so, then the biological agents causing the decline of small populations of alien species and the biological interactions affecting the metabolism of natural communities must be attributed to other types of organisms.

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