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Effects of Suspended Particulates on the Frequency of Transduction among *Pseudomonas aeruginosa* in a Freshwater Environment

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Transduction has been shown to play a significant role in the transfer of plasmid and chromosomal DNA in aquatic ecosystems. Such ecosystems contain a multitude of environmental factors, any one of which may influence the transduction process. It was the purpose of this study to show how one of these factors, particulate matter, affects the frequency of transduction. In situ transduction rates were measured in lake water microcosms containing either high or low concentrations of particulate matter. The microcosms were incubated in a freshwater lake in central Oklahoma. Transduction frequencies were found to be enhanced as much as 100-fold in the presence of particulates. Our results suggest that aggregations of bacteriophages and bacterial cells are stimulated by the presence of these suspended particulates. This aggregation increases the probability of progeny phages and transducing particles finding and infecting new host cells. Consequently, both phage production and transduction frequencies increase in the presence of particulate matter.

Studies conducted during the past 20 years have demonstrated that transduction, the virus-mediated horizontal transfer of genetic material, is a significant gene exchange mechanism among bacteria in aquatic habitats (1, 21–23). We have shown that both plasmid (22) and chromosomal (14, 23) DNA can be transduced in these environments and that naturally occurring bacteriophages are capable of transferring genetic information among members of a natural microbial population obtained from a freshwater lake (18).

Natural environments contain numerous physical factors that may affect the efficiency of transduction (10). Particulate matter, whether present as suspended material or as sediments, is potentially one of the most important of these factors. Several observations which indicate that particulates alter bacteriophage-host interactions and may therefore significantly affect the potential of natural environments to support transduction have been made (6, 8, 9, 19, 24). Because of conflicting interpretations of the effects of particulates on phage-host interactions, we believed that it was important to directly test the potential of suspended particulates to influence transduction frequencies in freshwater ecosystems. We have previously used in situ incubated lake water microcosms to study transduction in freshwater habitats (14, 22, 23) and have used this model system for the studies presented here.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The genetic donor used for these studies was RM2140 (22), a *Pseudomonas aeruginosa* derivative of the prototrophic strain PAO1 (11) which contains the plasmid Rms149 (Cb^r Gm^r Su^r Sm^r). This plasmid is Tra^- Mob^- (4, 6) and is incapable of conjugal transfer, ensuring that only transduction can occur in our system (19, 21, 23). The genetic recipient was *P. aeruginosa* RM296. This strain is an F116 lysogen of PAO515 (17), whose genotype is *met-9011 nalA5 amiE200*. Bacteriophage F116 is a temperate, generalized transducing phage of *P. aeruginosa* (12, 13). Infection by phage F116 occurs via attachment to bacterial flagella (12).

Clay. Mackaloid and montmorillonite clays were made homoionic to Mg^{2+} by the method of Lipson and Alsmadi (9). Dry weights of each clay type were obtained by oven drying to constant weight at 100°C.

Laboratory simulations. For these experiments, the donor (RM2140) and recipient (RM296) strains were inoculated at approximately 10^6 cells per ml into 500 ml of *Pseudomonas* minimal medium (PMM) (11) containing 10^{-5}% yeast extract. Mackaloid clay was added to one flask at a final concentration of 0.01 mg/ml. No clay was added to the second flask. Flasks were incubated at room temperature with gentle shaking (75 rpm), and the frequency of transduction in the two genetically mixed cultures was compared.

In situ incubated microcosms. Microcosms were prepared as described previously (23) with 1-liter Lifecell tissue culture chambers (Fenwal Laboratories, Deerfield, Ill.). These chambers are both gas and UV permeable (16, 23). The chambers were filled with sterilized lake water collected from either the surface or a depth of 6 m from Lake Carl Blackwell, a large freshwater reservoir near Stillwater, Okla. Water was collected with a LaMotte water sampler (LaMotte Chemical, Chestertown, Md.). The lake water-filled chambers were inoculated with the donor and recipient strains, previously washed with sterilized lake water, at approximately 10^6 cells per ml. The chambers were incubated in situ in Lake Carl Blackwell. Concentrations of particulate matter in surface water were determined to be approximately 0.005 mg/ml. Water obtained at the 6-m depth contained particulate-matter concentrations approaching 0.07 mg/ml.

Sampling procedure. Sampling was performed as described by Saye et al. (23). Briefly, 60-ml samples were aseptically removed from the chambers. Nalidixic acid was added to 50 ml of this sample to kill genetic donor cells and inhibit further phage and transducing-particle production. The sample was then filtered and extensively washed to remove all unabsorbed phages and transducing particles. This procedure has previously been shown to be effective in ensuring that transduction does not take place in the sample during workup (22, 23). These filters were placed on Luria agar plates containing 500 μg each of carbenicillin and nalidixic acid per ml to select for transductants which would have a nalidixic acid-resistant (Nal^r), carbenicillin-resistant (Cb^r) phenotype.

The remaining 10 ml of the sample was used for determining total viable, donor, and recipient CFU by plating dilutions prepared in saline on Luria agar, Luria agar containing 500 μg of carbenicillin per ml, and Luria agar containing 500 μg of nalidixic acid per ml, respectively. A portion of this sample was filtered through a 0.45-μm-pore-size syringe filter to remove bacteria, mixed with a phage-sensitive strain of *P. aeruginosa* (PAO1), and plated in a lambda top agar overlay on a Luria agar plate as described by Miller and Ku (11) to determine titers of free phage virions.

Confirmation of transductants by genetic and molecular methods. To confirm that Rms149 was present in transductants, plasmid DNA was isolated by the rapid alkali lysis method of Sambrook et al. (20) and digested with *Eco*RI as specified by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Electrophoresis was then performed on 0.7% agarose gels, and digestion patterns were compared with those of *Eco*RI-digested plasmid DNA from the parental strain, RM2140.

Evaluation of viral interactions with host cells and particulate matter by transmission electron microscopy. *P. aeruginosa* PAO515 and phage F116 at a multiplicity of infection of 1 were mixed with mackaloid clay particles (0.01

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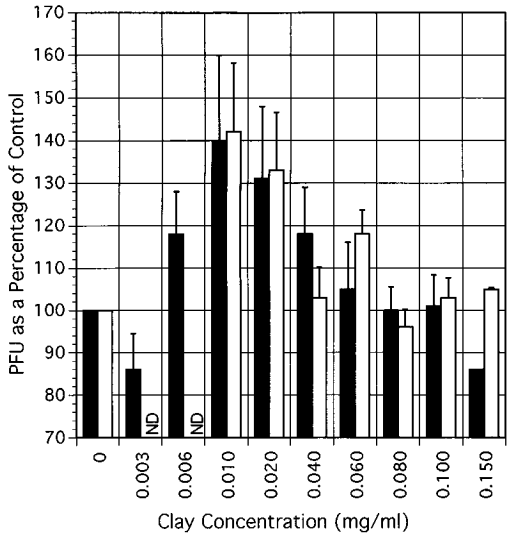

FIG. 1. Effect of clay concentration on phage F116 plaque-forming efficiency. Either mackaloid (■) or montmorillonite (□) clay was mixed with phage F116 and a sensitive indicator strain (PAO1) in a lambda top agar overlay as explained in the text. ND, not done. Error bars indicate standard error of the mean.

mg/ml) and incubated at room temperature in 25 ml of PMM containing 10^{-5}% yeast extract. Samples consisting of single drops (25 to 50 μl) were placed on carbon-coated Formvar grids for 15 min, wicked off, and then stained for 30 s with 2.5% uranyl acetate (2). Air-dried grids were examined in a JEOL Temscan 100CX transmission electron microscope.

RESULTS

Effect of clay concentration on plaque-forming efficiency. We first wished to determine if suspensions of clay particles would stimulate *P. aeruginosa*-F116 interactions as had previously been found for ϕX174 and *Escherichia coli* by Lipson and Alsmadi (9). Two types of clay (mackaloid and montmorillonite) were prepared as described above and added at various concentrations between 0.003 and 0.2 mg/ml to a mixture of phage F116 at 10 PFU/ml and PAO515 grown to mid-exponential phase (50 Klett units at 660 nm). Then 3 ml of lambda top agar was added, and the mixture was poured onto a Luria agar plate and analyzed by the method of Miller and Ku (11). The efficiency of successful phage-host interactions was measured by enumerating the resulting PFU (Fig. 1). At low clay concentrations (0.006 to 0.06 mg/ml), plaque-forming efficiency was greatly enhanced over results obtained in the absence of particulate matter. As clay concentrations increased, the frequency of phage infection plateaued and then steadily declined.

Direct examination of bacteriophage-cell-particulate aggregates by transmission electron microscopy. Bacteria, phages, and mackaloid clay were mixed in PMM and observed by transmission electron microscopy as described above. Electron micrographs revealed that aggregates of phages, host cells, and clay particles are formed in the liquid medium (Fig. 2).

Laboratory simulations of environmental transduction in the presence of particulates. Before experiments were actually performed in situ, trials were carried out in the laboratory under more controlled conditions. A donor of genetic material (RM2140) and a recipient strain (RM296) were inoculated into flasks either containing mackaloid clay (0.01 mg/ml) or not containing clay, and the frequency of transduction in the two genetically mixed cultures was assessed over a 44-day period (Fig. 3). Culturable cell concentrations were unaffected by the presence of clay and remained essentially the same in the two chambers (Fig. 4). Donor and recipient ratios likewise remained equal. Although there was significant fluctuation in the amount of transductants recovered over the duration of the experiment, as much as a 10-fold increase in the number of transductants was observed in some of the samples when clay was present in the culture (Fig. 3). No transductants were seen at the zero time point. Of the 42 presumptive transductants obtained, all were confirmed as containing Rms149 by restriction endonuclease digestion patterns.

As might be predicted from the previous experiments, there was also an increase in the ratio of phage particles per bacterial cell (PBR) (15). This increase approached 10-fold compared with the ratio in cultures which did not contain added particulates (Fig. 5). These simulations of aquatic milieus containing low and high concentrations of suspended particulates suggested that increases in phage production due to the presence of suspended clay particles could lead to an increased frequency of transduction.

Transduction in in situ incubated lake water microcosms. We next asked if water collected from near the bottom of a freshwater lake, which was rich in suspended particulates (0.07 mg/ml), would support higher levels of transduction than surface water, which was relatively depleted of suspended particulates (0.005 mg/ml). Environmental test chambers (23) were prepared as described above, and these microcosms were incubated in situ at our freshwater-lake field site. Incubation in the lake continued for 3 weeks. A 100-fold increase in the number of transductants recovered was observed in the bottom water microcosms compared with the surface water microcosms (Fig. 6). All transductants were confirmed as containing Rms149 by restriction endonuclease digestion patterns.

The relative number of phages sustained in these microcosms was measured by determining the PBR which developed during incubation in situ. PBRs were higher in the bottom water microcosms than in the surface water microcosms (Fig. 7). In addition, as had been previously observed by other investigators (3, 5, 27), the microbial community sustained by the habitat, measured as the concentration of total viable cells, was found to be greater in the bottom water, which contained higher concentrations of particulates (Fig. 8).

DISCUSSION

The experiments described here indicate that suspended particles have the potential to stimulate phage-host interaction by allowing the formation of compact microenvironments consisting of aggregates of bacterial cells, phage virions, and particulates. This close contact among host cells and virions increases the probability that progeny phages (and transducing particles) released from one infected cell will encounter and infect a neighboring host cell. Consequently, we observed greater numbers of phages and transductants when particulates were present in the aquatic microcosms. Photomicrographs (Fig. 2) directly demonstrated the formation of these aggregates.

The enhanced interaction which we observed between bacteria and viruses in the presence of particulate matter resulted in conspicuous increases in transduction frequencies in both the laboratory simulations and in situ experiments. Although we believe that this enhancement is due primarily to phage-host aggregation in the presence of particulate matter, other factors may also contribute to increased transduction potential.

Greater host metabolic activity has been observed in particle-associated organisms because of the increased availability of nutrients (21, 27). Since both the length of the latency

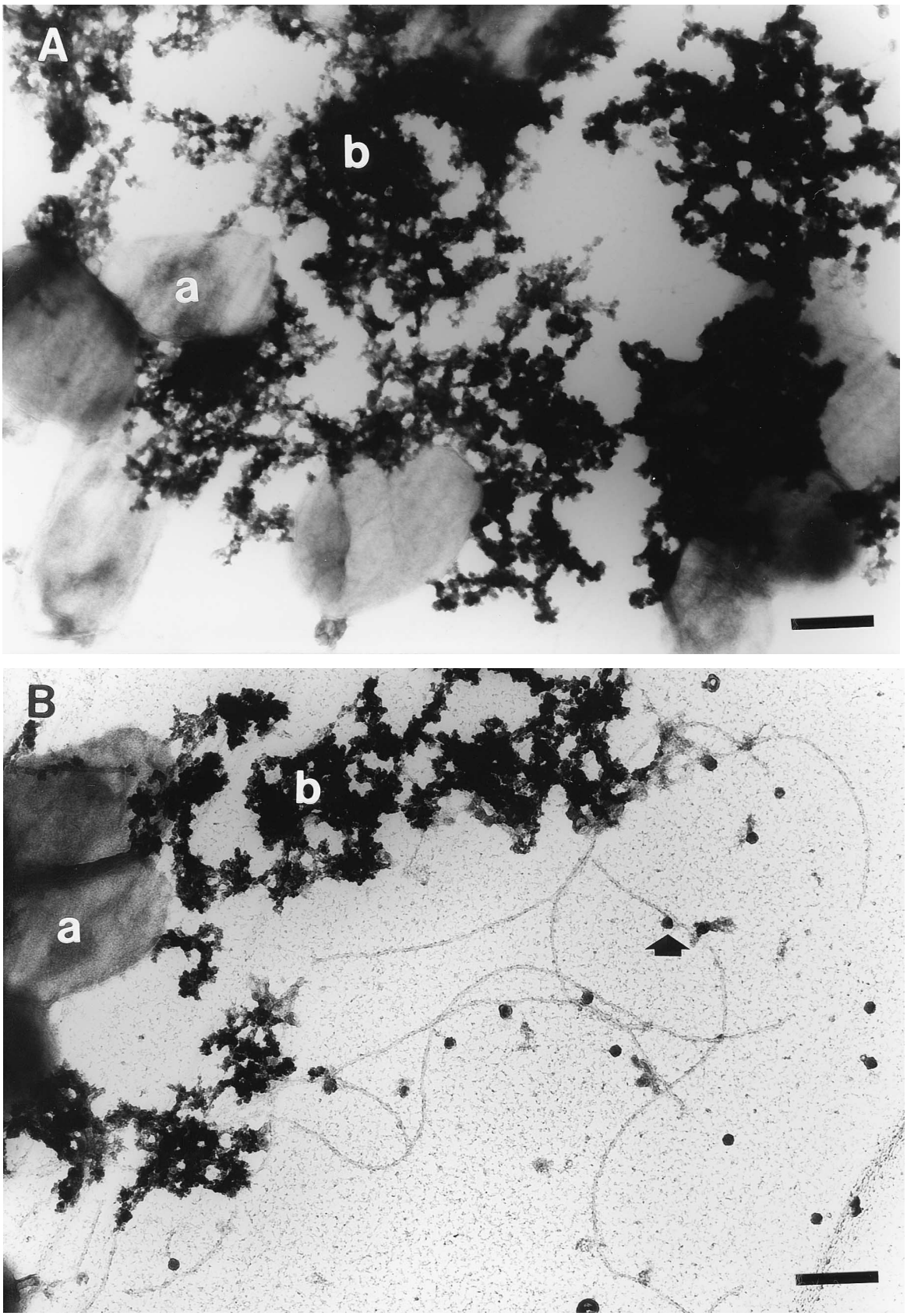

FIG. 2. Electron micrographs depicting aggregate formation. Magnification, ×17,600; bars, 400 nm. (A) Aggregates of clay particles (b) and bacterial cells (a) as seen on grids stained with uranyl acetate. (B) A group of cells at the edge of an aggregation. The flagella to which phage F116 are attached are kept in relatively close contact because of binding by clay particles, allowing for rapid cell-to-cell infection. Arrow, bacteriophage attached to flagellum.

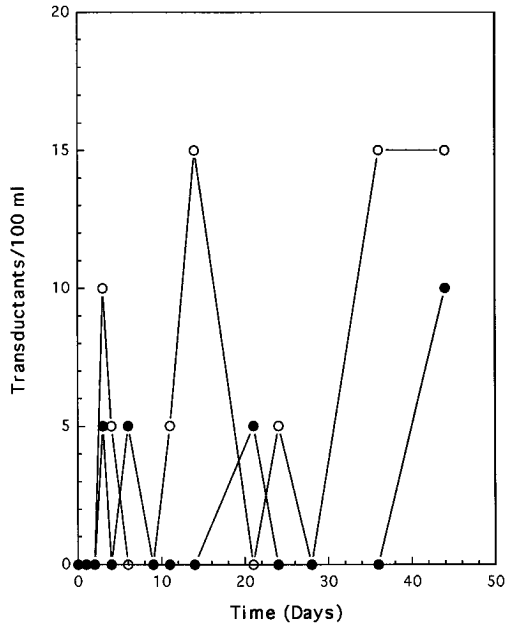

FIG. 3. Transductant concentrations in laboratory simulations with donor (RM2140) and recipient (RM296) cells grown in PMM in the presence (○) or absence (●) of mackaloid clay particles (0.01 mg/ml).

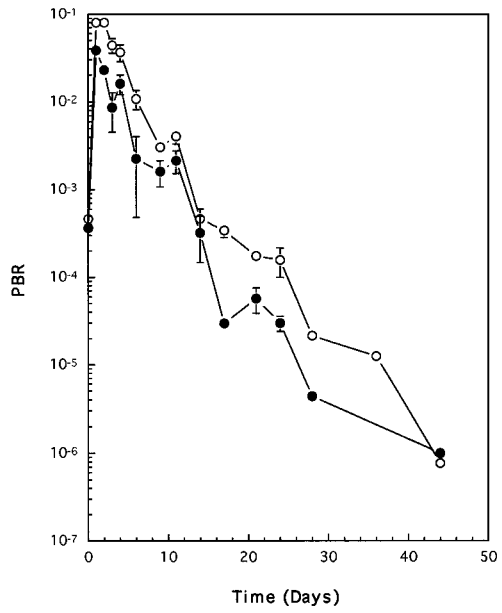

FIG. 5. PBRs in laboratory simulations. Phage production was found to increase in the presence of mackaloid clay particles at 0.01 mg/ml (○) in comparison with microcosms in which clay particles were absent (●).

period and the size of the burst of progeny virus particles are dependent on the metabolic rate of the host (8), it is likely that attached cells produce more phage particles than unattached cells do, thus increasing the PBR. Increased PBRs have been shown by Replicon and Miller (17a) to amplify transduction frequencies in continuous-culture experiments. Consistent with this hypothesis, elevated PBRs were observed in in situ incubated microcosms containing water rich in suspended particulates (Fig. 7).

Our initial observations on the effects of clay particles on phage F116 plaque-forming efficiency (Fig. 1) are consistent with those made by Lipson and Alsmadi (9). These investigators determined that the effects of clay on the interaction between ϕX174 and its host *E. coli* were concentration dependent. They showed that phage-host interactions were first in-

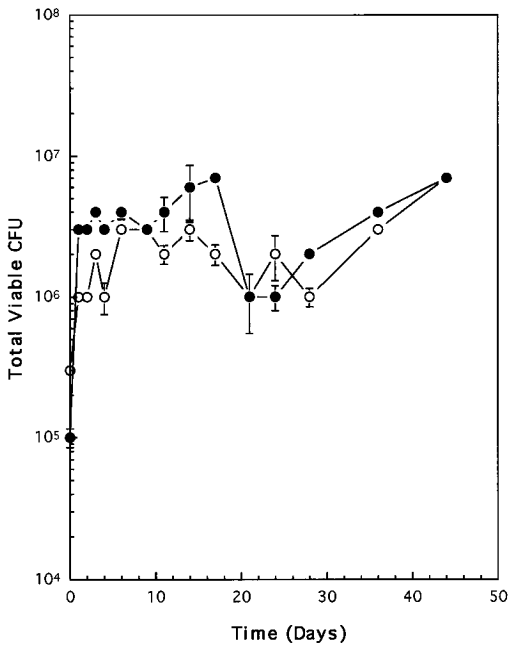

FIG. 4. Total viable-cell concentrations in laboratory simulations in the presence (○) or absence (●) of mackaloid clay particles at 0.01 mg/ml.

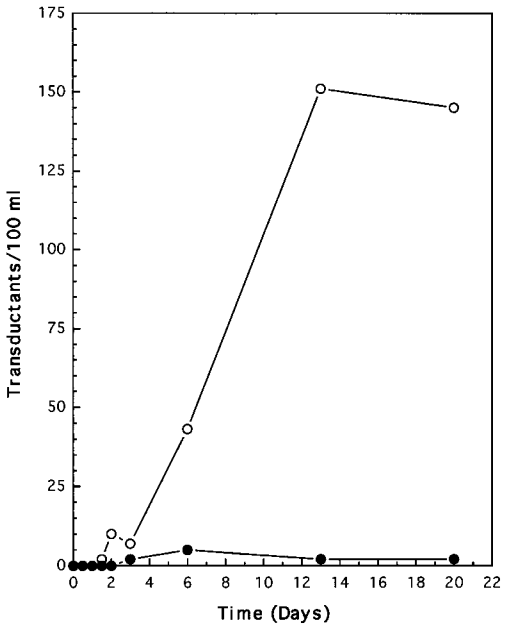

FIG. 6. Concentration of transductants in in situ incubated microcosms. Environmental test chambers were filled with water obtained from the surface (●) (0.005 mg of particulates per ml) or from a depth of 6 m (○) (0.07 mg of particulates per ml) from a freshwater lake.

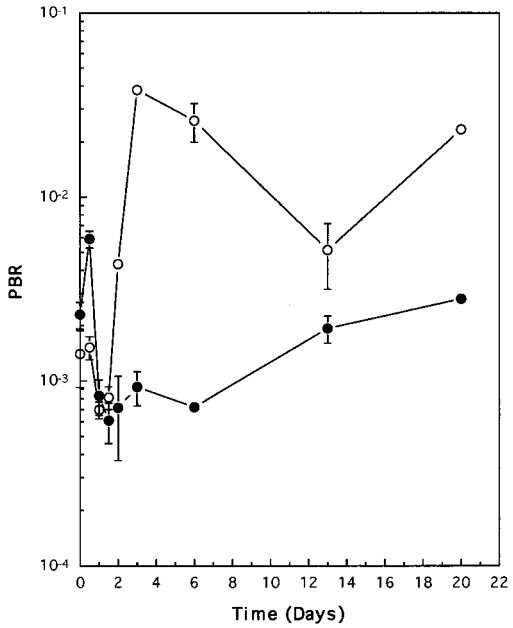

FIG. 7. PBRs in in situ incubated bottom water microcosms (high suspended particulate concentrations) (○) and surface water microcosms (low suspended particulate concentrations) (●).

creased and then reduced as the concentration of clay particles in a liquid milieu increased. These findings suggested to Lipson and Alsmadi that the host cell became enveloped by clay particles at these higher concentrations, forming a barrier to phage attack. An alternative interpretation is that clay particle concentrations simply reduce the probability that phages will encounter host bacteria because of the increased particle surface area to which bacteria and bacteriophages can become attached.

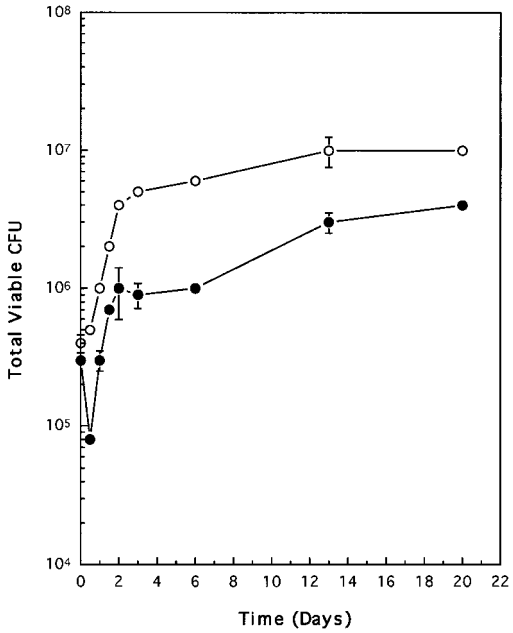

FIG. 8. Total cellular concentrations in bottom water microcosms (○), which were rich in particulate matter, and in surface water microcosms (●), which were relatively depleted of particulate matter.

In complementary studies, Roper and Marshall (19) found infection of *E. coli* by its phages to be influenced by particle size. While large-diameter (>0.6-μm) particles stimulated phage-host interaction, small-diameter particles appeared to protect *E. coli* from infection. These results may be interpreted to indicate that large particles are ineffective in forming a continuous barrier around the cell and that gaps between the large particles allow phage contact with the cell and subsequent cell infection. Alternatively, the effect may be because small-diameter clay particles have a much larger surface-to-volume ratio than large-diameter ones do. The apparent protection provided by small particles may therefore be due to reduced phage-host interaction caused by spatial separation on the increased surface area provided by these particles.

The effects of particulates on all of the measured parameters were amplified during in situ incubation compared with the laboratory simulations. This observation suggests that association with suspended particulates may be exacerbated by other environmental factors affecting phage-host interaction in the aquatic environment. In addition to stimulating bacterial metabolism and biosynthesis of phages, association with particles may protect phages and transducing particles, as well as their host bacteria, from inactivating environmental stresses such as UV irradiation or changes in pH (25, 26). This protection may increase the time frame in which effective interaction between virions or transducing particles and their host organisms can take place—a phenomenon observable as increased bacteriophage activity.

In our previous work (7, 14, 15, 18, 21), we have shown that transduction is a viable means of gene exchange in freshwater ecosystems. It is evident from the data presented here that physical factors such as particulate matter, which can vary greatly among ecosystems, can substantially alter transduction potentials in aquatic habitats. Information concerning the effects of environmental factors such as particulate matter on in situ phage-host interactions significantly enhances our understanding of the parameters regulating phage-mediated horizontal gene transfer in natural aquatic ecosystems and increases our ability to evaluate its effect on genetic diversity and evolution in aquatic microbial populations.

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