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The Importance of the Dynamics of Bacteriophage-Host Interactions to Bacterial Abundance and Genetic Diversity in Aquatic Environments

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Introduction

Using *Pseudomonas aeruginosa* and its bacteriophages as a model system, we have clearly demonstrated a significant potential for viral-mediated gene transfer (transduction) of both plasmid and chromosomal DNA in freshwater microbial populations. These investigations have predicted that the most likely reservoir for environmental bacteriophages capable of transduction is the lysogenized members of the natural microbial population. However, both primary infection of non-lysogens and prophage activation from lysogens can generate significant numbers of transducing particles. These studies have led us to formulate a model for the dispersement of genetic material from an introduced organism to related members of the autochthonous microbial community.

This model requires a unique sequence of events: (a) Phage virions must be produced through spontaneous or stress-stimulated activation of prophages from environmental lysogens. These viral particles must (b) infect, propagate, and (c) lyse the introduced DNA donor. (d) Transducing particles produced during this lytic infection must absorb and transfer DNA to the remaining lysogens where it is established either as a plasmid (e) or, if chromosomal in origin, is recombined into the host chromosome. Hence, environmental lysogens serve as both efficient sources of transducing phages and as viable recipients for transduced DNA.

The potential for this model to lead to genetic diversity in natural bacterial communities has been demonstrated in continuous culture studies designed to determine the evolution of mixed genetic populations as described in the model. We found that at cell densities and generation time (hydraulic residence times) similar to those predicted to occur in the environment, transduction acted to stabilize and even increase the frequency of genotypes which were lost through negative selection in continuous cultures where gene transfer was disallowed. This is an exciting and significant result as transduction has often been dismissed as an important evolutionary process due to its perceived reductive nature.

These studies indicate that our transduction model has the potential of occurring in natural habitats and significantly influencing the genetic makeup and diversity of natural bacterial populations. To determine the true significance of the model in natural ecosystems, it is imperative to understand the dynamics of phage-host interaction and to identify the reservoirs of environmentally observed bacteriophage particles.

Our preliminary studies investigating phage-host dynamics under natural conditions indicated that a primary effector of the outcome of environmental phage-host interaction is nutrient availability: (I) While attachment to starved cells is not impaired, the replication of bacteriophages is significantly altered in starved cells. In primary infection, the latency period is lengthened and the burst size reduced when compared to fed cells. (II) Continuous culture studies have demonstrated that the rate of transduction is directly proportional to the phage-to-bacterium ratio (PER) developed in the microbial consortium. The PER is, in turn, proportional to the extent of starvation of the host organism. (III) The virulence of lytic viruses is reduced in starved hosts potentially allowing alternative outcomes of infection.

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Also, particulate matter, whether present as suspended material or as sediments, is perhaps one of the most important of the physical factors. Several observations have been made which indicate that particulates significantly alter bacteriophage-host interactions and thus have the potential for affecting transduction potentials in natural environments. It has been demonstrated that bacteriophages adhere to clays and that such adhesion protects phages against inactivation. The binding of clay to bacteria to block sites for phage attachment and reduce interaction between the host and parasite has also been hypothesized.

However, homoionic kaolinite and montmorillonite clays were able to enhance ϕX-174 infectivity of *Escherichia coli* threefold at low concentrations of suspended particulates. Optimum infectivity occurred at kaolinite concentrations of 0.14-0.50 mg/ml and at montmorillonite concentrations of 0.01-2 mg/ml. Increased infectivity at higher clay concentrations was not seen, suggesting that the cell may become enveloped by clay particles at these higher concentrations, thus essentially forming a barrier against phage attack.

E. coli is protected from phage infection in the presence of fine clay particulates, but this protection is decreased when the particle size is increased to greater that 0.6 μm. This may be due to the ineffectiveness of large particles in forming a continuous barrier around the cell. Gaps thus allow for phage contact and subsequent cell infection.

Alternatively, suspended particulates may act to alter phage-host interactions by providing a surface which facilitates contact between the virus and its prey. The most effective source of virus particles in the environment is lysogenized bacteria present in the microbial community. Therefore, mixed microcolony formation on suspended particle surfaces may act to place the source of phages (i.e., lysogens) in close proximity to the prospective hosts, thus forming centers of phage activity within the aquatic environment. In this light, results which indicate that the stimulation of clays on phage infection is dependent on both the concentrations and average particle size, can be interpreted as resulting from altered phage-host interactions by altering the total surface area on which interaction can take place. As the concentration of clay increases or the average diameter of the clay particle decreases, the total surface area increases. In turn, increased surface area will reduce the probability that hosts and viruses will be in close enough proximity to interact.

Several studies have found that attached microorganisms are more metabolically active than unattached cells. Since both the length of the latency period and the size of the burst of progeny virus particles have been shown to be dependent on the metabolic rate of the host, it is likely that attached cells produce more phage than unattached microorganisms. This, too, would increase phage-host activity in environments containing suspended particulate surfaces as compared to purely liquid phase habitats.

These observations encouraged us to attempt to determine the effects of suspended particulates on transduction rates in freshwater lake microbial communities. We have previously used *in situ* incubated lake water microcosms to study transduction in freshwater habitats and we employed this model system for these studies as well. Data concerning the effects of environmental factors, such as particulate matter, on these microcosms significantly enhanced our understanding of host-phage interactions and, hence, phage-mediated horizontal gene transfer in natural aquatic ecosystems. Since half-lives of phage particles in these environments are only 12-24 h, the mechanisms by which the observed levels of phage particles are maintained in natural aquatic ecosystems are currently undefined. We initiated studies to investigate the natural reservoirs of bacteriophages and the dynamics of phage-host interactions in the aquatic environment. This report summarizes our observations.

Methods

Bacterial Strains and Bacteriophages

The bacterial strains used in this study are all derivatives of *P. aeruginosa* (PAO) or *P. aeruginosa* (PAT). PAO1 is the prototrophic parental strain of all PAO derivatives. PAT2 is a prototrophic PAT derivative which carries the *P. aeruginosa* fertility factor FP2. RM242 is a non-lysogenic PAO strain with a nal-933 met-1011 genotype, and RM132 is a prototrophic PAO that is lysogenic for ϕF116. PA0303 is an argB2I non-lysogen. ϕF116 is a generalized transducing phage and ϕD3 is a specialized transducing phage. ϕUTI is a generalized transducing phage isolated from Fort Loudoun Lake near Knoxville, TN.

In the experiments on suspended particulates, the genetic donor used for these studies was RM2140, a *P. aeruginosa* derivative of the prototrophic strain PAO1 which contains the plasmid Rms 149 (Cb^1, Gm^1, Su^1, Sm^1). This plasmid is Tra-Mob-, which renders it incapable of being transferred by conjugation, thus ensuring that only transduction can occur in our system. The genetic recipient was RM296. It is an ϕFI16 lysogen of PA05-15 whose genotype is met-9011 nalAS amiE200. Bacteriophage ϕF116 is a temperate, generalized transducing phage of *P. aeruginosa*.

Environmental Sampling and Analysis of Bacteriophage, Bacterial, and Lysogenic Populations

Samples were collected from Fisher Pond in the Morton Arboretum, Downers Grove, IL. *P. aeruginosa* strains PAO1 and PAT2 were used as indicators for the enumeration of phage titers.

Identification of Lysogens in Laboratory and Microcosm Studies

Lysogeny was identified by demonstrating super-infection immunity and spontaneous release of bacteriophages. *P. aeruginosa* PAO1 was used as the indicator strain for enumerating release of bacteriophages from lysogens.

Clay

Mackaloid, montmorillonite, and barite clays were made homoionic. Dry weights of each clay type were obtained by oven drying at 50°C until three similar consecutive weights were acquired.

In Situ *incubated Microcosms*

Microcosms were prepared using one-liter Lifecell tissue culture chambers (Fenwal Laboratories, Deerfield, IL). These chambers are both gas and ultraviolet (UV) light permeable. Chambers were filled with sterilized lake water collected from either the surface or at a depth of 6 m from Lake Carl Blackwell, a large freshwater reservoir in Stillwater, OK, and inoculated with the donor and recipient strains, previously washed with sterilized lakewater, at approximately 1 x 106 cells/mi. The chambers were then suspended 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 approaching 0.07 mg/ml.

Microcosm Sampling Procedure

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 formation. This sample was then filtered (0.45μ) 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. These filters were placed on Luria agar plates containing 500 μg/ml each of carbenicillin and nalidixic acid to select for transductants.

The remaining 10 ml of the sample were used for determining total viable, donor, and recipient CFU concentrations by plating dilutions prepared in saline on Luria agar, Luria agar containing 500 μg/ml carbenicillin, and Luria agar containing 500 μg/ml nalidixic acid, respectively. A portion of this sample was filtered through a 0.45 μ syringe filter to remove bacteria, mixed with a phage sensitive strain (PAO1), and plated in a lambda top agar overlay on a Lucia agar plate to determine free phage titers.

Confirmation of Transductants by Genetic and Molecular Methods

To confirm that Rms 149 was present in the transductants, plasmid DNA was isolated by rapid alkaline lysis, and digested with EcoRI according to the manufacturer's directions (Boehringer Mannheim Biochemicals, Indianapolis, IN). Electrophoresis was then performed on 0.7% agarose gels and digestion patterns were compared to those of EcoRI digested plasmid DNA from the parental strain, RM2140.

Results and Discussion

Abundance of Phages and Bacteria in a Freshwater Lake

To begin our investigations, we monitored the occurrence of bacteriophages, potential host bacteria, and lysogens at a freshwater lake over a nine month period (Table 1). This study indicated that not only are phage-like particles observed at significant concentrations by transmission electron microscopy in aquatic environments but that phages capable of producing plaques on an environmentally prominent bacterial host are present in significant titers. When lysogeny was evaluated, between I% and 7% of the isolates tested positive by the criterion of release of virions infectious on *P. aeruginosa.* Colony hybridization studies using various DNA probes specific for *P. aeruginosa* phages of environmental origin revealed that a much larger fraction (70%) of Pseudomonas isolates contained at least one phage-specific DNA sequence (Table 2).

Table 1. Abundance of bacteria and *P. aeruginosa*-specific bacteriophages in a freshwater lake during a nine-month period.

Month Sampled	Total Recoverable CFU (X 10^4 CFU ml^{-1})	Pseudomonas spp. Specific (X 10^3 CFU ml^{-1})	P. aeruginosa-Phages X 10^3 PFU ml^{-1})
October	20	10	10
November	20	0.6	10
December	9	0.5	1
January	20	2	0.5
March	10	10	0.003
April	20	20	1
May	40	10	1
June	20	9	0.9

Table 2. Relative abundance of sequences related to Pseudomonas phage DNAs in bacterial colonies recovered from a freshwater habitat.

In Situ *Incubated Lakewater Microcosms*

We next asked if water collected from near the bottom of a freshwater lake which was enriched for suspended particulates would support higher levels of transduction than surface water that was relatively depleted of suspended particulates. These experiments were performed in microcosm chambers incubated *in situ* at our freshwater-lake field site. The chambers were filled with sterilized lakewater obtained either from the extreme surface of the lake or from a depth of 6 m using a LaMotte water sampler (LaMotte Chemical, Chestertown, MD). These chambers were inoculated with the genetic donor and recipient strains at approximately 1 x 10^6 cells/ml and incubated in the lake over a period of three weeks (Figure 4). A 10-fold increase in transduction frequency was observed in the bottom water microcosms as compared to the surface water microcosms. As had been observed in the laboratory simulations, the PER was also significantly higher in the bottom water than in the surface water microcosms.

The experiments described here indicate that suspended particles significantly influence cell-phage interactions in aquatic milieus. Enhanced infection probably occurs because aggregates consisting of cells, phage, and particulates form, thereby allowing for progeny phage released from one infected cell to readily interact with a neighboring host cell. Consequently, greater numbers of transductants are seen when particulates are present in aqueous environments. In the case of the laboratory simulations, an almost 100-fold increase was seen in the numbers of transductants when clay was added. Similarly, *in situ* incubated locator microcosms exhibited a higher number of transductants in bottom water (Figure 4).

At high clay concentrations, phage-host interactions were reduced (Figure 3), this may be due to the production of a continuous clay envelope surrounding the cell and acting as a barrier to phage infection or simply to a dilution effect caused by the increased particle surface area to which bacteria and bacteriophage may become attached. Factors other than simple interaction with the particle may influence phage-host interaction. These may include the increased metabolism and, hence, phage production, of attached organisms due to increased availability of nutrients. In addition, association with particles may protect phages by inactivating environmental stresses such as UV radiation or changes in pH.

In our previous work, we have shown that transduction is a viable means of gene exchange in freshwater ecosystems. It is evident from the study reported here that physical factors such as particulate matter that can vary significantly among aquatic habitats, can significantly alter transduction potentials in these ecosystems.

Conclusion

The observations reported here suggest that the evaluation of phage-host relationships will provide important and sometimes unsuspected insights into microbial population ecology. They suggest that pseudolysogeny may be an important host-interactive state for bacteriophages in aquatic environments. Data from this and other studies suggest that both temperate and virulent DNA phages and RNA phages establish pseudolysogenic relationships with their hosts under environmental conditions. If pseudolysogeny does indeed occur in aquatic environments, it would allow phage genomes to coexist

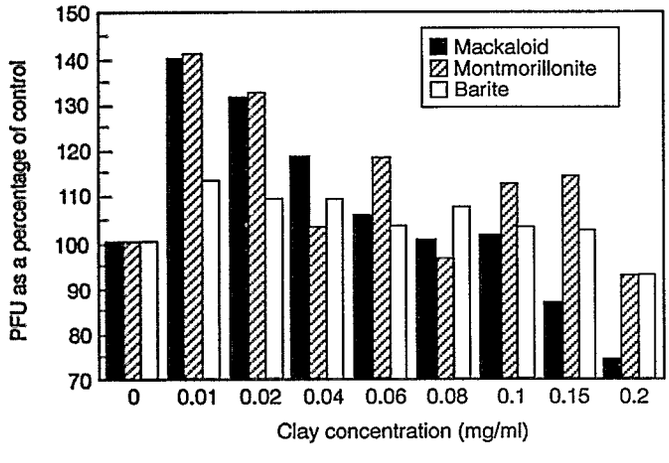

Figure 3. Effect of clay concentration on ϕF116 plaquing efficiency.

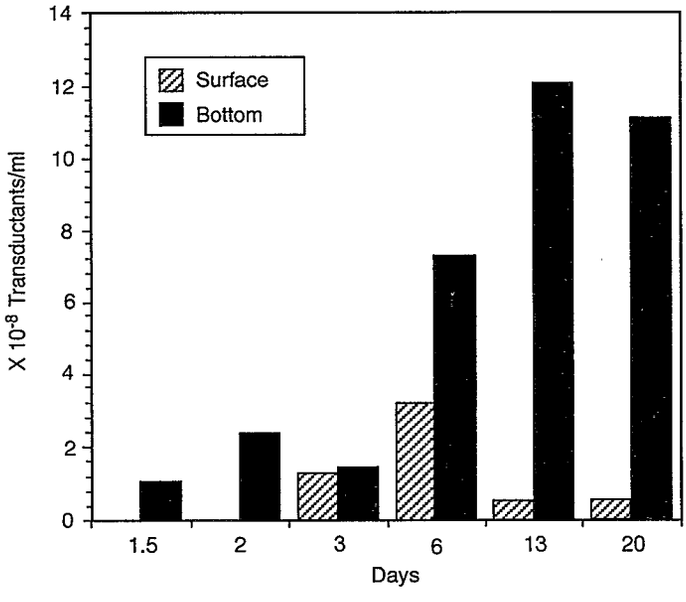

Figure 4. Comparison of transductant concentrations *in situ* incubated lakewater microcosms.

with their starved host bacteria in a relatively protected state during prolonged periods of starvation. When nutrients become available, pseudolysogens may be transformed into true lysogens, or the lytic response may be induced. Although the physical and molecular manifestations of the pseudolysogenic phage genome are not currently understood, it may be sequestered in the host cytoplasm in a non-replicative form or achieved in the host chromosome as described for ϕP22.

Disclaimer

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All research projects making conclusions or recommendations based on environmentally related measurements and funded by the Environmental Protection Agency are required to participate in the Agency Quality Assurance Program. This project was conducted under an approved Quality Assurance Program Plan. The procedures specified in the plan were used without exception. Information on the plan and documentation of the quality assurance activities and results are available from the Principal Investigator.

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