To the Graduate Council :

I am submitting herewith a dissertation written by Oladele Abiola Ogunseitan entitled "Molecular Ecology of *Pseudomonas aeruginosa* Bacteriophages in a Freshwater Environment." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Gary S. Sayler, Major Professor.

We have read this dissertation
and recommend its acceptance :

Accepted for the Council :

Vice Provost
and Dean of the Graduate School.

MOLECULAR ECOLOGY OF *Pseudomonas aeruginosa* BACTERIOPHAGES IN A FRESHWATER ENVIRONMENT

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Oladele Abiola Ogunseitan

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ABSTRACT

The distribution, molecular characteristics, and host-specific activities of *Pseudomonas aeruginosa* bacteriophages indigenous to a freshwater habitat were investigated. The occurrence of particulate phage and phage genomic sequences in bacterial populations of environmental samples were evaluated by hybridization reactions. Phage LLPP5, an avirulent *P. aeruginosa*-specific virus characterized by a contractile tail, an octahedral head, and a double stranded DNA genome of approximately 78 kilobases, was isolated from the water column of Lake Loudon. A variety of phage particles specific for *P. aeruginosa* were recovered from the same habitat by enrichment with an indigenous host strain. The interaction of phage LLPP5 and the heterogenous phage population with the indigenous host strain, *P. aeruginosa* LLPA10 was studied in lakewater microcosms over a 45 day incubation period. The transduction of heavy metal resistance, antibiotic resistance and auxotrophic requirement between laboratory strains of *P. aeruginosa* and the lakewater isolate, by means of phage LLPP5 or the heterogenous phage population was investigated.

The distribution of *P. aeruginosa* phage particles in lakewater fluctuate seasonally with the host population. When present, the detectable phage density is about 4.8×10^{-2} per ml of lakewater. DNA sequences that are homologous to phage genomes appear to be common in the bacterial population, but their occurrence may not necessarily indicate a wide distribution of lysogenized bacteria. The development of a phage-carrier state in bacteria hosts seems to be enhanced by competition with the natural microbial community. The stability of phage-host interactions is influenced by prevailing multiplicity of infection. However, phage did not reduce host populations below a threshold density of 10^4 cfu per ml. The transduction of mercury resistance genes was demonstrated for phage LLPP5 at a frequency of 1.67 to 3.89 per 10^7 plaque forming units (pfu). The transduction of chromosomally encoded streptomycin resistance was observed at a frequency of 2.5 per 10^8 pfu. The transduction of plasmid encoded antibiotic resistance (pRms149, 55 kb) was not observed for phage LLPP5. However, the heterogenous phage population from lakewater transduced carbenicillin resistance genes that was carried on this plasmid at a frequency of 1.5 per 10^7 pfu.

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CHAPTER I

LITERATURE REVIEW

INTRODUCTION.

Microbial ecology is defined as the study of the distributions, activities and interactions of microorganisms within their natural habitats. To be true to this definition, an ecological investigation necessitates the application of reliable methodology for the isolation of organisms, their identification, measurement of their activities in both pure and mixed culture, assessment of their interactions with other viable cells, and the determination of their responses to variable environmental factors. Although these ideals are seldom completely fulfilled in practice, they constitute a set of challenges and initiatives for field studies in microbiology. The goal of the present work

concerns the ecological investigation of bacterial viruses that infect *Pseudomonas aeruginosa* with particular reference to their behaviour in freshwater habitats. Emphasis is placed on the molecular aspects of viral interactions in order to facilitate a better understanding of bacteriophage phylogeny, and the basis for host- and environment-dependent properties of phage such as the establishment of cell lysis or lysogeny, bacterial phenotype modification, and genetic transfer.

EARLY VIEWS ON THE NATURE OF BACTERIOPHAGE.

Bacterial viruses were discovered in 1915 when Twort isolated a filterable virus which produces a vitreous transformation of bacterial colonies during their growth on an agar surface. However, D'Herelle (1917) was responsible for coining the name 'bacteriophage' and for detailed experiments which allowed medical bacteriologists to recognize the essential nature of bacterial viruses.

The interpretation of phage-induced lysis of infected bacterial cultures was one of the many controversies during the early phase of bacteriophage research. While D'Herelle (1917) correctly thought that intracellular phage growth leads to lysis of the host cell and liberation of the

phage progeny, Bordet and Ciuca (1920 a & b) maintained that phage-induced dissolution of bacterial cultures is merely a consequence of the stimulation of lytic enzymes endogenous to the bacteria. Other workers such as Bronfenbrenner (1928) and Krueger and Northrop (1931) thought that lysis of the bacteria was only a secondary phenomenon, which may or may not follow the growth of the phage, and they hypothesized that bacteriophage can pass freely in and out of the bacterial cells. Delbruck (1940) finally showed that these arguments were confused by the observation that there are two completely different processes by which bacteriophages can lyse susceptible bacterial cells. One of these, lysis-from-without, represents an immediate dissolution of bacteria often encountered when the multiplicity of infection is much greater than one phage per bacterium (Mueli, 1923 ; see Figure 1.1). Loss of the input phages, rather than their multiplication, ensues from this form of lysis. Only the second of the lytic processes, lysis-from-within, is really the form of lysis connected with intracellular phage replication, and its onset usually signals the end of a latent period. The adoption of bacteriophage as a model system for investigating genetic phenomena, and their studies by Delbruck and his collaborators, may have been the foundation of modern molecular biology. Within a few years of the discovery of bacteriophage, lysogenic bacteria strains were found which appear to carry bacteriophages in the sense that phage particles are always



Figure 1.1. Lysis-from-without. A lakewater strain of *Pseudomonas aeruginosa* infected with phage from the same habitat at a multiplicity of infection of approximately 1000.

present in the culture fluid of such strains (Bordet and Ciuca, 1921). Lysogeny is therefore defined as the hereditary property of producing bacteriophage without infection with external particles. A lysogenic bacterium possesses and transmits to its progeny the capacity to produce infective phage. It was recognized by many workers (e.g. Gildemeister, 1921) that filtrates of bacterial cultures isolated from nature often contain phages that lyse other 'indicator' strains of the same species, or of related species. Two types may be distinguished among such phage-producing strains. In the so called carrier strains, also called pseudolysogens, phage production can be ultimately ascribed to a population equilibrium between resistant and sensitive cells which are constantly being infected by free phage particles. These cultures can easily be freed from phage by successive colony re-isolation or by exposure to antiphage serum. On the contrary, in truly lysogenic bacteria, each cell can potentially produce phage. Even after many successive re-isolations (Bordet, 1925) or after prolonged growth in antiphage serum (McKinley, 1925), every bacterium gives rise to cultures containing bacteriophage. It was realized that this association between phage and bacteria can not be superficial since it is impossible to permanently free lysogenic strains from the phage they carry by methods which ought to kill or remove the virus particles (Bordet, 1925 ; McKinley, 1925). Some lysogenic cultures are known to have been maintained for over 77 years without loss of

phage (Burnet and McKie (1929), as reported by Barksdale and Arden (1974)).

The nature and significance of lysogeny remained a controversial subject for about thirty years some workers denying the existence of true lysogeny and others claiming that lysogeny disproves the whole notion that bacterial lysis involves an infection of bacteria by virus particles. Nevertheless, Burnet and McKie (1929) and Wollman (1928) maintained that lysogeny represents an innate nature of bacteria for phage production. In order to establish firmly some of the basic but controversial facts of lysogeny, Lwoff and Gutman (1950) demonstrated unequivocally that each bacterium of lysogenic strain harbors and maintains a non-infective structure, the prophage, which endows the cell with the ability to give rise to infective phage without further intervention of exogenous phage particles. Lwoff and Gutman (1950) also inferred from their experiments that the induction of phage development in a lysogenic cell is under control of external factors, and their subsequent collaborations with Siminovitch and Kjeldgaard (1950) showed that treatment with various reagents, in particular irradiation with ultraviolet light, will induce phage production and ultimately lysis of almost every cell in a culture of lysogenic bacteria. The presence of the prophage not only endows the bacterium with the capacity to produce phage, but also confers upon the cell an immunity to infection by a homologous phage (McKinley, 1925). Hayes

(1968) suggested that the existence of lysogeny should be judged by a rather rigorous criterium of stability since interaction of some virulent phages with their hosts may superficially simulate the condition.

Lysogeny appears to be wide spread in nature. When systematic investigation have been conducted, it has been detected in many strains of various bacteria species and genera, including *Pseudomonas* . The recognition of the existence and nature of the proviral stage ultimately became the bridge leading from the study of the genetics of viruses to that of the host bacteria and engendered new ideas concerning the origin, evolution and biological function of viruses (Lwoff, 1953 ; Bertani, 1958 ; Jacob, 1960).

MEDIATION OF BACTERIAL GENETIC EXCHANGE BY BACTERIOPHAGE.

The mechanism allowing the transfer of genes from one bacterial strain to another by means of viral particles was first observed in *Salmonella* by Zinder and Lederberg (1952) who subsequently used the term transduction to describe the phenomenon. By transduction, recombinant bacteria can arise naturally, which have derived a limited part of their genome from a donor cell. Transduction was immediately recognized as a powerful tool for genetic analysis of lysogeny as an interaction between phage and host (Jacob,

1955). One of the important pieces of information about transduction reported by Jacob (1955) was that a transducing virus can carry the prophage of an entirely different virus particle from a lysogenic donor to a nonlysogenic recipient cell, usually in association with a contiguous region of the donor chromosome, demonstrating the simultaneous exchange of both foreign and indigenous genetic material. The temperate and the transducing phages became one of the most effective tools in the study of the structure and function of bacterial genes (Stent, 1965). The classification of the transducing virus as either specialized or generalized is based on the relative frequency of transfer of specific gene loci.

Limited data are available concerning the ecological impacts of genetic transfer processes in nature. However, certain observations, including the rapid proliferation of traits such as multiple antibiotic resistance and the metabolism of exotic compounds, constitute *a priori* evidence for the efficiency of genetic exchange mechanisms in nature. It is axiomatic among bacteriophage microbiologists that there is somewhere in nature an infecting phage for each species of bacterium. The ecological and evolutionary implications of such phage distributions and contributions to environmental gene flow remain to a large extent uninvestigated. The current and future applications of genetically engineered microorganisms (GEM) in waste treatment, agronomy, pest control, and energy product recovery will probably

require release of the altered bacteria in large quantities to the environment in order to be effective. There has been a number of concerns raised over the feasibility and the potential perturbations of the ecosystem from the release of GEM (Curtis, 1976 ; Liang *et al*., 1982 ; Rissler, 1984, and Brill , 1985). Assessment of the risks involved in the release of GEM requires charting the environmental fate of the organisms to determine their survival, growth, dissemination and persistence of the recombinant genes. The occurrence of bacteriophages in environments where deliberate release of GEM are targeted may influence the assessment process in a number of ways. First, obligately virulent infection may predominate due to an initial low multiplicity of infection, resulting in a drastic reduction of the population of introduced organisms. Second, nonvirulent infections may occur which could lead to a phenotype conversion in the lysogenized cells, leading to a confoundment of tracking protocols. Thirdly, lysogenic infections may enhance transduction of genes from introduced GEM to indigenous populations of related strains, causing multiple dissemination problems.

It is generally recognized that the various microbial habitats, including soils, sediments, water, sewage, plants and animals surfaces, vary widely in their species composition, and in the physical-chemical parameters that define their components. All of these ecosystems are possible targets for GEM applications. There is an ongoing debate concerning the modelling of

the response of each system to invasion by GEM. Although tests performed in one habitat may not be used strictly as guidelines for assessing the response of other systems, aquatic environments represents a unique habitat because they are form a continous link with other environments. The aquatic microbial community therefore represent a primary system in which interactions that may affect the ultimate fate of GEM ought to be adequately understood.

IMPLICATIONS OF *Pseudomonas* BACTERIOPHAGE IN FRESHWATER.

Bacteria in freshwater systems comprise at least four distinct communities. The planktonic population floats in the water column with the particle associated species. The epilithic and the epiphytic populations are attached to submerged surfaces. The sediment population occupies the water-filled spaces of the soft sediment layer. Potentially, genetic interactions may occur within all of these populations. In spite of the great variety of species found in the aquatic community, pseudomonads are essentially ubiquitous in the water profile. The metabolic versatility and potential pathogenicity of certain pseudomonads have made members of this species prime targets for genetic engineering manipulations. It has been suggested

that pseudomonads be accepted as model organisms for studying the deliberate release of GEM (Cuskey *et al* , 1986). The abundance of information available concerning the genetics and physiology of *P. aeruginosa* warrants its use in the present studies. Other factors provide rationale for studying molecular ecology of bacteriophage in *P. aeruginosa* . In addition to being able to produce phage spontaneously in culture and to be immune to superinfection by related bacteriophage, lysogenic bacteria usually acquire other phenotypic traits (Barksdale and Arden, 1974). This phenomenon of lysogenic conversion was first observed as antigenic variability resulting from phage infection of *Salmonella anatum* [3, 10] and *S. newington* [3, 15] by Bruner and Edwards (1948). In *P. aeruginosa* lysogenization with phage D3 confers a surface antigenic phenotype change (Holloway and Cooper, 1962). Liu (1969) has shown that phage conversion occurs with a large number of serotypically distinct strains of *P. aeruginosa*. These changes affect surface components, synthesis of aeruginocins, melanogenic pigments, fluoresceins, and proteases. Some *Pseudomonas* phages also encode the ability of the host strain to produce various classes of slime polysaccharide depolymerases (Bartell and Orr, 1969b). For one phage there was conclusive evidence that the phage genome encodes upon infection the *de novo* synthesis of a unique depolymerase, not by derepression or activation of a host encoded gene (Bartell and Orr, 1969a).

The production of slime polysaccharide depolymerases are crucial for pathological and ecological reasons pertaining to the invasion of surfaces. The distribution and activites of phages able to confer such phenotypes need to be characterized. The major phage property usually stressed in these conversions is the ability to establish a stable relationship between the phage genome and the host replication system. The potential for transduction may fit into such schemes of host modification activities by phage.

The phenomenon of Host-Controlled-Modification (HCM) is another rationale for this study of phage molecular ecology. First recognized through aberrations in the plating efficiencies of phage on different host strains, HCM has been of considerable importance in establishing the nature of genetic transfer mechanisms in bacteria. HCM is detected when phage grown on one host plates at low efficiency on a second host. Plaques isolated from the second host would show normal efficiency when plated back on the original host, indicating that the effect is attributable to the hosts. There are two mechanisms involved in HCM. Each bacterial strain imposes its own specificity on DNA replicating within that strain by means of methylases which produce specific patterns of methylated adenine or cytosine residues. This phenomenon is known as DNA modification. Secondly, each strain has a recognition system by which it can differentiate between different patterns of DNA methylation, and thus between 'self' and 'foreign' DNA. An

endonuclease acts on foreign DNA to inactivate its biological function. This process is known as restriction.

Host-Controlled-Modification was first detected in *P. aeruginosa* by means of phage B3 (Holloway and Rolfe, 1964). Subsequently, a variety of other phages were found to be subject to this phenomenon. The transducing phage F116 is not susceptible to restriction or modification nor are the bacterial DNA fragments which it may transduce (Dunn and Holloway, 1971). Phage F126 which is related to F116 in its serological and immunological properties also shows no susceptibility to HCM. However, the bacterial DNA transduced by phage F126 is not protected as the phage genome is. The differential nature of the restriction response to different kinds of DNA indicated the existence of various restriction enzymes in *P. aeruginosa* PAO strains (Krishnapillai, 1971).

The genetic basis of HCM appears to be varied in nature. *P. aeruginosa* mutants that are deficient in restriction abilities (Res^-) may be isolated directly from nature, and some may have altered modification phenotypes (Mod^-) (Rolfe and Holloway, 1968). The temperature of cultivation, mutagens and components of bacterial genomes have been shown to influence the outcome of HCM in *P. aeruginosa* (Holloway, 1965; Olsen *et al.*, 1968, and Dunn and Holloway, 1971).

Investigation of the environmental distribution of *P. aeruginosa* bacteriophages is scanty in spite of an early start (Holloway *et al.,* 1960). Most of the reported studies have been conducted on clinical isolates (e.g. Akhverdyan *et al.*, 1985). However, a variety of temperate and virulent phages of this species are available for study. Up to 2.0 x 10^3 particles per ml of sewage have been reported (Primrose and Day, 1977). Many, if not all, strains of *P. aeruginosa* are lysogenic and this associative state may be more common in environmental isolates (Holloway *et al.*, 1960). Methods previously available for phage and prophage detection were developed independently and did not allow for a comprehensive study of phage ecology in the environment. The frequency of lysogenic strains in *P. aeruginosa* strains isolated from clinical samples is probably close to 100% (Hammon *et al.*, 1961; Patterson, 1965), in contrast to *Escherichia coli* where only about 10% of strains are lysogenic. Polylysogeny also appears to be common in *P. aeruginosa*, including an example of a strain harboring up to 10 different prophages (Holloway, 1960 ; Zierdt and Schmidt, 1964, and Shionoya, 1967). Lysogeny in other species of *Pseudomonas* appear to be rare or undetectable (Hammon *et al.*, 1961; Patterson, 1965).

GENERAL PROPERTIES OF *Pseudomonas* BACTERIOPHAGES.

The structural range of pseudomonad phages is as diverse as those found for other groups of bacteria. Some phages have the typical icosahedral head, tail with or without sheath, tail plate with attached fibers like those of the T-even coliphages. In addition to this type, phages similar to Lambda, filamentous forms and spherical phages have been found (Slayter *et al.*, 1964; Takeya and Amako, 1966; Bradley, 1967; Bradley and Robertson, 1968 and Lapchine *et al.*, 1969). The genomes of pseudomonad phages are usually of the double stranded DNA form, ranging in size from 9 to about 180 kilobases. Several RNA phages have also been found (Feary *et al.*, 1963a; Bradley, 1966 and Olsen and Shipley, 1973). Unlike the RNA phages of *E. coli*, only one pseudomonad RNA phage shows any degree of host specificity,this being PRR1 which only plates on bacteria strains containing the RP plasmid (Olsen and Shipley, 1973). *P. aeruginosa* phages usually contain double stranded DNA of mole % (G + C ratio) in the range of 46 - 55% (Chow and Yamamoto,1968) which is lower than the ratio found for the hosts (67%) (Duodoroff and Palleroni, 1974). Bacteriophage may be classified according to their initial host recognition mechanisms. Appendage bacteriophage recognize flagella or pili (e.g. phage F116). Capsule phage attach to outer layer polysaccharides while somatic phages recognize the cell wall directly.

All of these types have been reported for pseudomonads (Holloway and Krishnapillai, 1975). The period of latent infection for most pseudomonad phages have been found to be about 35 to 65 minutes with a burst size ranging between 10 and 200 particles per host cell (O'Callaghan *et al.*, 1969; Chow and Yamamoto, 1969).

Physical survival of *P. aeruginosa* bacteriophages in the environment has not been investigated beyond the observation that they are consistently recoverable from high bacterial density samples. However, *in vitro* experiments have shown these phages to be resistant to temperatures up to 60 ^{o}C but are inactivated at 65 ^{o}C. They are usually stable at pH 6.5 - 10.0, but not below pH 6.5 (Dickinson, 1948). These phages usually do not contain phospholipids or glycoproteins (as do phages PM2 of a marine pseudomonad and phi6 of *P. phaseolicola*).

Traditionally, relationships between viruses have been measured by cross-reactivity in serotyping reactions. Recently, molecular relationships in terms of genomic sequence similarities have become popular due to the fundamental nature of information generated by such investigations. In the present study the distribution pattern of free and engaged phage is investigated in an aqueous environment. Molecular relationships and the determinants of the interaction of these phages and their usual hosts in freshwater were also studied. In recognition of the urgent need for more

competent research techniques in phage ecology (Goyal, 1987), the present effort applies currently available molecular hybridization methods for phage ecology studies. In the following chapter the distribution and integrity of *P. aeruginosa*-specific bacteriophages, and their hosts in the freshwater habitat studied (Fort Loudon Reservoir, Knoxville, Tennessee) are evaluated. The distribution pattern of these organisms in the environment is considered necessary background information for the proper interpretation of results gathered by studying phage-host interactions under laboratory conditions.

The characterization of an isolated bacteriophage and a susceptible lakewater bacterium constitute the subject of Chapter III. The autecological analysis of the phage-host couple was conducted to gain insight into the possible molecular determinants of their association.

The regulation of natural populations of bacteria is one of the ecological functions traditionally attributed to phage occurrence. Chapter IV covers the dynamic nature of the interaction of bacteriophage with host cells in the freshwater environment.

In the final Chapter, the most prominent consequence of bacterial infection by phage, that is genetic transfer by transduction is investigated. Here the confluence of basic phage genetics research and emerging ecological investigations should allows new insights into the structure and functions of the evolutionary machinery in microbial systems.

CHAPTER II

DISTRIBUTION OF *Pseudomonas aeruginosa* BACTERIOPHAGES AND THEIR HOSTS IN THE ENVIRONMENT

INTRODUCTION.

There is an abundance of highly diverse species of microorganisms in natural aquatic environments. Sources of freshwater that are lentic probably support more microbial life than comparable lotic surface or groundwater environments. In freshwater different microorganisms may interact in many ways, including symbiotic, commensal and parasitic relationships. To facilitate the investigation of such interactions, there is a need for methods capable of detecting and isolating individual members of the freshwater microbial community. The specific isolation of bacterial species

and strains from freshwater has been the subject of numerous studies (Gibson *et al.*, 1977). For the fluorescent pseudomonad group in particular, there has been several approaches to their selective detection and cultivation. These approaches include the use of selective growth media (Gould *et al.*, 1985), DNA probes (Festl *et al.*, 1986), and immunofluorescent tagging (Mills and Bell, 1986). Due to the pathological significance of *Pseudomonas aeruginosa*, special attention has been attributed to its detection and isolation from clinical and environmental specimens (Lowbury, 1975). Techniques for studying the distribution of bacteriophages in environmental samples have developed only slowly due to the limitations posed by the high dilution (Seely and Primrose, 1982). The majority of studies conducted on the ecology of bacteriophages in the environment have focused on their distribution in wastewater treatment plants and their behavior after release into receiving waters (e.g. Safferman and Morris, 1976; Havelaar and Hogeboom, 1983, and Shields *et al.*, 1986). Consequently, many of the methods currently available are adapted from those developed for detecting human enteric viruses. Since nutrient resources and therefore populations of host bacteria in sewage are invariably higher than those found in oligotrophic freshwater habitats, those techniques can not be applied to study aquatic phage ecology without substantial loss of sensitivity. The traditional method of phage detection has been to incubate active cultures of host bacteria with a volume of test sample that is expected to

contain phage. After an appropriate incubation period, the culture fluid is rid of cells and the supernatant assayed for plaque forming phage particles (Delisle and Levin, 1969, for pseudomonads). The application of these enrichment procedures is limited by lack of quantitation. The small amount of inoculum that is often used may preclude the detection of sparsely distributed particles. Another limitation is the tendency to select for the most vigorously virulent strains in a heterogenous phage population. However, the detection of less productive particles may be masked. An alternative phage detection and recovery procedure is to concentrate large volumes of water, sometimes up to 200 l (Seely and Primrose, 1982), prior to incubation and titering. Other techniques that have been used include direct electron microscopy of concentrated environmental samples (Hackerman and Nguyen, 1983). This technique is useful only for samples containing high phage densities. A review of currently available methodology for phage ecology studies has been published (Goyal, 1987). All of the techniques discussed above lack the ability to combine the study of phage distribution patterns with the fate of host bacteria present in the same environment. The preponderance of temperate and non-obligately virulent phage in natural environments suggest that a substantial proportion of phage populations remain undetected by protocols that depend largely on plaque assays of concentrated samples. In the present study the traditional techniques of concentration and enrichment are used in

addition to a novel approach of detecting prophages in bacterial fractions of samples being investigated. Bacterial colonies that harbor a prophage may be detected by hybridization to phage genomic probes. This technique allows the simultaneous evaluation of specific phage interaction with their hosts in their natural state. Phylogenetic studies of phage populations are also enhanced since different phage from the same habitat may be compared according to the level of homology they share.

Understanding the role of bacteriophage in the ecology of freshwater requires a knowledge of the numbers of individual bacteria and their phage. Previous studies concerning the distribution of phage in freshwater have concentrated on *Escherichia coli*, which reflects the current interest in the possibility of using coliphages as indicators of fecal pollution (Furuse, 1987). This chapter deals with work done to initiate investigation into the distribution and interaction of *P. aeruginosa* - infecting phages indigenous to a freshwater environment.

MATERIALS AND METHODS.

Environmental samples.

Freshwater samples were collected from Fort Loudon Reservoir, Knoxville, Tennessee. Pertinent characteristics of this study site have been described (Morrison *et al.*, 1978) and are summarized in Table 2.1. For bacterial population analyses and for enrichment assays, 2.5 l of lakewater were collected into sterile Niskin bags samplers from a depth of 0.3 m. When larger volumes of water were required for phage concentration, 25 liters of water were pumped into sterile carboys. Water samples were kept at 4 ^{o}C and processed within 24 hours of collection.

Soil samples were obtained from the embankment area of Fort Loudon reservoir. Agricultural soil samples were collected from a cultivated plot of the Plant Science Field Laboratory of the University of Tennessee, Knoxville. Soils were generally taken from within 5 and 15 cm of the surface layer, sieved to remove stones and twigs, and stored at 4 ^{o}C in plastic containers. Sediment and soil dry weights were determined by oven drying to constant weight at 105 ^{o}C. Sewage samples were collected from the port of entry into the Municipal wastewater treatment plant of Knoxville, Tennessee. Raw sewage was collected from homogenized wells and placed in 2.5 l plastic containers and stored at 4 ^{o}C until processed.

Table 2.1. Salient Physical and Chemical Parameters of Ft. Loudon Lakewater.[a]

Parameter	Range
Temperature (^{o}C)	8.0 - 28.5
pH	6.84 - 8.33
Dissolved Oxygen (mg L^{-1})	2.5 - 10.7
Conductivity (υmho)	150.0 - 260.0
Turbidity (JTU)[b]	100 - 200
Depth (m)	4.0 - 7.8

[a]Range for individual sampling periods during the course of a two year investigation.
[b]Jackson Turbidity Units.

Recovery and Enumeration of Bacteria.

Serial dilution of environmental samples were made in Phosphate Buffered Saline (PBS). Aliquots of each dilution were spread plated onto full strength and 10% Yeast Extract Peptone Glucose (YEPG) agar (Sayler *et al.*, 1979), Luria-Bertani (LB) agar (Difco) and *Pseudomonas* Isolation Agar (PIA) (Difco). YEPG has been shown to be adequately selective for heterotrophic bacteria in oligotrophic environments (Mallory and Sayler, 1983). LB-agar was used to select for enterobacterial groups that may be present in fecally polluted samples. YEPG plates were incubated at 22 ^{o}C for 10 days while LB and PIA plates were incubated at 37 ^{o}C (or 42 ^{o}C for selecting *P. aeruginosa*) for 24 hours. Bacterial colonies recovered on LB and PIA media were lifted onto Nylon membranes (Biotrans) and probed with a fluorescent pseudomonad specific DNA sequence (pHF360) derived by Festl *et al.*, (1986). Taxonomic tests for isolated pseudomonads growing at 41 to 42 ^{o}C were conducted according to Bergeys Manual specifications (Duodoroff and Palleroni, 1974).

Direct Recovery of Bacteriophage.

The water-concentration protocol of Primrose and Day (1977) was employed to detect the presence of *P. aeruginosa* specific plaque forming

units in freshwater. 25 l samples of freshwater were filtered onto a 20 ml hydroxyapatite (Bio-Rad) slurry. The phage particles were eluted with 10 ml of 0.8 M sodium phosphate buffer (pH 7.2) and titered on *P. aeruginosa* RM 273 or the lakewater strain LLPA10 (Table 2.2) using the soft agar overlay method as described by Silhavy *et al.*, (1984). All plaques obtained were purified by transfer onto fresh bacterial lawns. High titers were obtained from purified plaques by lysing 2.5 ml broth culture of the host strain (Silhavy *et al.*, 1984).

Enrichment of lakewater for bacteriophage production was conducted essentially as described by Grimont *et al.*, (1978). *P. aeruginosa* LLPA10 was used as the susceptible host in the enrichment procedures. All phage lysates were clarified by centrifugation and filtration through 0.45 um pore size polycarbonate membranes (Nucleopore Corp., Pleasanton, CA). Further purification was achieved by ultracentrifugation at 35,000 rpm for 2 hours at 4°C in a Beckman TL 100 rotor. Phage pellets were resuspended in 1% ammonium acetate for electron microscopic studies or in SM buffer (Silhavy *et al*, 1984) for other characterization studies. The technique of Lanning and Williams (1982) was attempted for the direct detection of free *P. aeruginosa* phage in sediment and soil samples, but without success. Particulate phage were isolated from sewage by direct titration of low speed centrifugation clarified samples on *P. aeruginosa* strain LLPA10 lawns. Plaque hybridizations were conducted essentially as described by Benton and Davis (1977). Particulate phage and DNA dot blots were conducted on

Table 2.2. *Pseudomonas aeruginosa* strains and bacteriophage used in this study.

Designation	Trait	Source	Reference
Bacteria			
LLPA10	Nal^r	Lakewater	This study
LPL5	Phage LLPP5^r	Lakewater	This study
RM273	Nal^r (A910)	Strain PAO1	Saye *et al.*, 1987
RM272	Nal^r (A5), øDS1^r	Strain PAO1	Saye *et al.*,1987
Phage			
LLPP5	*Myoviridae*, Temperate	Lakewater	This study
LPE	Heterogenous	Lakewater	This study
F116L	*Siphoviridae*, Temperate	R.V. Miller	Holloway, 1960
DS1	*Siphoviridae*, Temperate	R.V. Miller	Saye *et al.*, 1987
D3	*Myoviridae*, Temperate	R.V. Miller	Holloway, 1960
E79	*Myoviridae*, Virulent	R.V. Miller	Slayter *et al.*, 1964

Nal^r = Nalidixic acid resistance.

Nylon membranes (Biotrans) using a microfiltration apparatus (Schueller and Schell Products). Hybridizations were performed at 65 ^{o}C and filters were washed according to membrane manufacturers recommended procedure. The protocol is estimated to retain hybridized sequences that are at least 75% similar.

Detection of Prophages.

The occurrence of prophage DNA sequence corresponding to the viruses studied (listed in Table 2.2) in bacterial colonies was detected by hybridization of total DNA from the colonies to labeled phage DNA *in situ*.. Bacteriophage DNA was isolated from glycerol gradient purified phage by the method of Silhavy *et al.*, (1984). The recovered DNA was further purified by drop dialysis against Tris-EDTA-Sodium acetate buffer (pH 7.5) at 4 ^{o}C for 2 hours. Phage DNA was labeled with ^{32}P by nick translation (Rigby *et al.*, 1977) using [α-^{32}P]dCTP (BRL, Gaithersburg, MD) as the isotope donor. Hybridizations were carried out at 65°C for 24 hours. Filters were washed according to the high stringency procedure described by Sayler *et al.*, (1985). Positive signals were detected by autoradiography. Colonies that probed positive for phage DNA were restreaked and inoculated into 5 ml LB broth cultures containing 1 ug ml^{-1} mytomycin C. The culture filtrates were

subsequently assayed for phage particles by plaque formation on *P. aeruginosa* LLPA10.

RESULTS.

Distribution of Pseudomonas in Lakewater.

Pertinent physical and chemical characteristics of the freshwater environment used for this study are presented in Table 2.1. The data represent the extremes of conditions measured at the sampling site several times across the seasons in the past two years. The lower values generally occured in winter (November to February) while the higher values occured in the warmer months (June to August). Various values were recorded between these extremes.

The total viable heterotrophic bacterial density of the water profile (assayed on YEPG) fluctuated around an average of 2.5×10^4 cfu ml^{-1} (Table 2.3). Acridine orange direct counts (AODC) conducted by the method of Hobbie *et al* (1977) on the same sample gave a density of about 2.6×10^6 cells per ml with a 33.5% deviation.

Bacteria capable of growing at 37 ^oC on rich media (LB) constitute about 1% of the heterotrophic bacterial population. Pseudomonads assayed

Table 2.3. Bacterial Colony Forming Units per ml of Environmental Samples.[a]

Growth Media	Lakewater	Sediment	Soil	Sewage
LB at 37 °C	2.45×10^2	2.86×10^4	5.45×10^6	1.89×10^6
% Deviation :	10.20	14.44	2.56	8.46
YEPG at 22 °C	2.49×10^4	3.75×10^7	1.10×10^6	3.16×10^6
% Deviation :	4.02	17.33	5.00	3.79
PIA at 22 °C	7.0×10^1	1.3×10^3	1.76×10^4	5.29×10^4
% Deviation :	28.57	69.23	6.53	0.095

[a]Values recorded are means of four counts representing duplicates of two samples from each environment.
LB = Luria-Bertani agar ; YEPG = Yeast Extract Peptone Glucose agar ; PIA = *Pseudomonas* Isolation Agar. See text for composition of media, or reference.

on PIA occur at a density of 50 - 70 cfu ml^{-1} in the colder season and at about 1.72×10^3 +/- 64% cfu ml^{-1} during warmer and rainy sampling periods. The fluorescent *Pseudomonas* population varied according to season and mode of detection. Compared with two other media tested, PIA (Difco) gave the lowest but most reproducible counts that also correlated with the detection and enumeration patterns obtained with the DNA probe specific for this group of organisms (Festl *et al.*, 1986).

Distribution of *P. aeruginosa*, determined by growth on LB and PIA at 42 ^{o}C varied between 0 and 6 cfu ml-1 for the water samples examined. Usually, 100 ml aliquots of water had to be filtered to recover *P. aeruginosa* strains from this environment.

Distribution of *Pseudomonas aeruginosa*-specific Bacteriophage in Lakewater.

The average density of particulate, *P. aeruginosa* PAO-infecting, plaque-forming phage in the freshwater samples studied was determined to be 4.8×10^{-2} per ml. This value represents the mean of counts from several 25 liter volumes of lakewater. In some cases, no detectable plaque forming units were found in concentrated samples from the same site. This may indicate an uneven distribution or occurrence of these particles, or else an undetermined discrepancy in the assay protocol.

The variety of plaque morphologies is represented in the population of free phage recovered (Figure 2.1). The most common plaques were clear with entire margins. However, some of the plaques became turbid with prolonged incubation.

Enrichment of lakewater with a locally isolated *P. aeruginosa* strain LLPA10 produced a mixed population of phage consisting of about four morphologically distinct virions (Figure 2.2). When the DNA isolated from this heterogenous population of phage was digested with an endonuclease, a continous, unresolved digestion pattern was obtained, confirming the heterogeneity of the phage population (Figure 2.3).

In order to determine the possible identity of the plaque forming particles recovered from lakewater, the isolated plaques were probed with genomic DNA from isolated (phage LLPP5), mixed phages (LPE, named for **L**ake **P**hage **E**nrichment) and previously characterized *Pseudomonas* bacteriophages (Table 2.2). The results (Figure 2.4) indicate that about 10% of the 33 plaques examined relate to phage LLPP5, 20% relate to phage F116L and none relate to phage D3. A majority of the plaques (46.7%) share some homology with one or more of the phages present in the enriched phage population, LPE. Figure 2.5 show the result obtained when plaques recovered from sewage were probed with the same phage DNA probes.

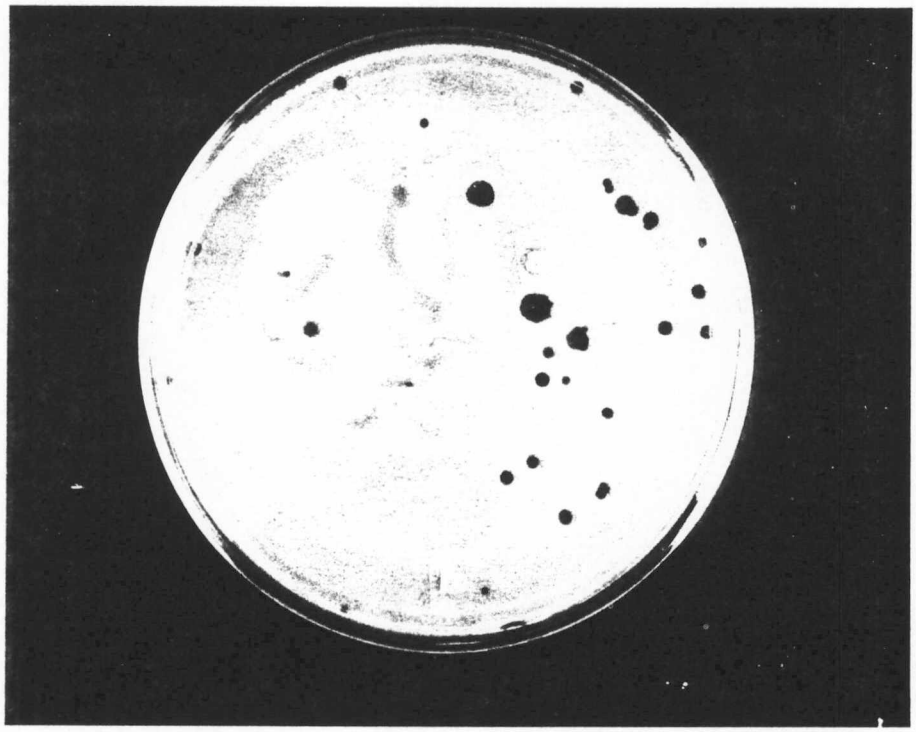


Figure 2.1. Plaque morphology of free phage particles recovered from 20,000x concentrated lakewater. The bacterial lawn was made of indicator host strain, *P. aeruginosa* RM273. Distinct plaque types are seen, including clear, irregular margin plaques (a), clear, entire margin plaques (b) and turbid plaques (c).

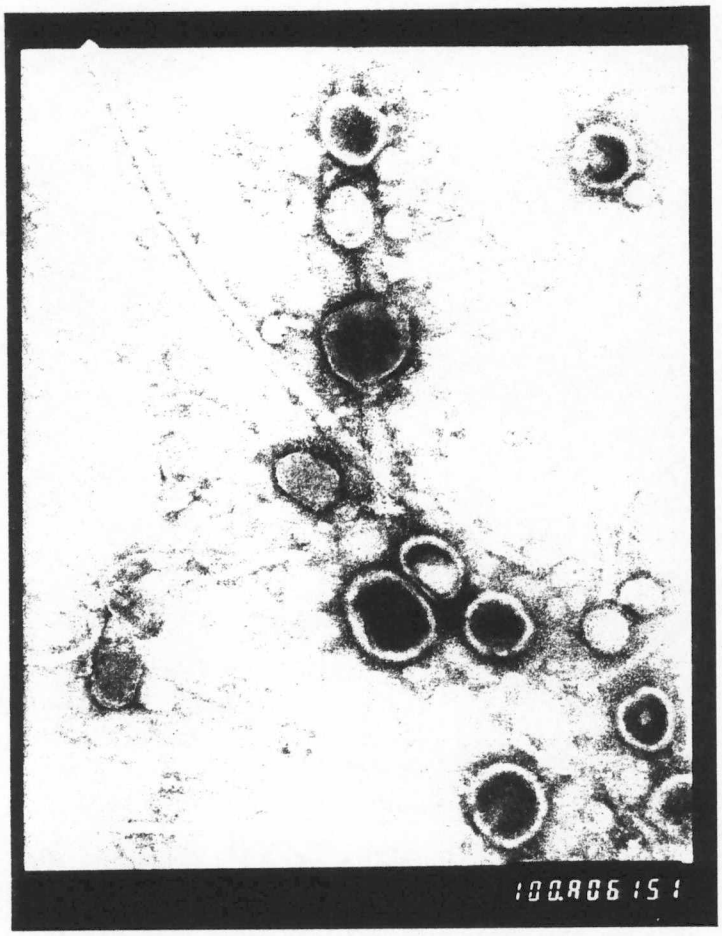


Figure 2.2. Heterogenous phage population isolated from lakewater by enrichment with *Pseudomonas aeruginosa* LLPA10, a lakewater strain. Negative staining with phosphotungstate. Bar represents 100 nm.

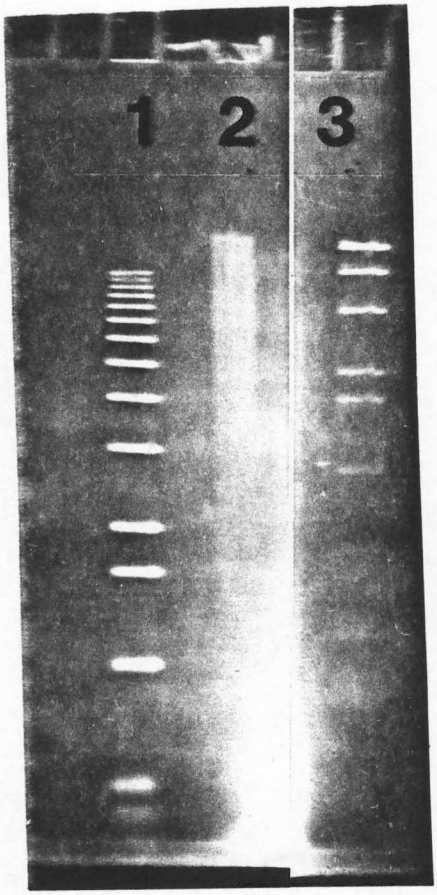


Figure 2.3. Restriction pattern of DNA isolated from heterogenous phage population, LPE, and pure isolate, phage LLPP5. Lane 1 : One kilobase sizing standard ladder ; Lane 2 : LPE digested with Sal 1 ; Lane 3 : LLPP5 digested with Sal 1. Agarose gel (0.5%), run at 75 volts for 5 hours.

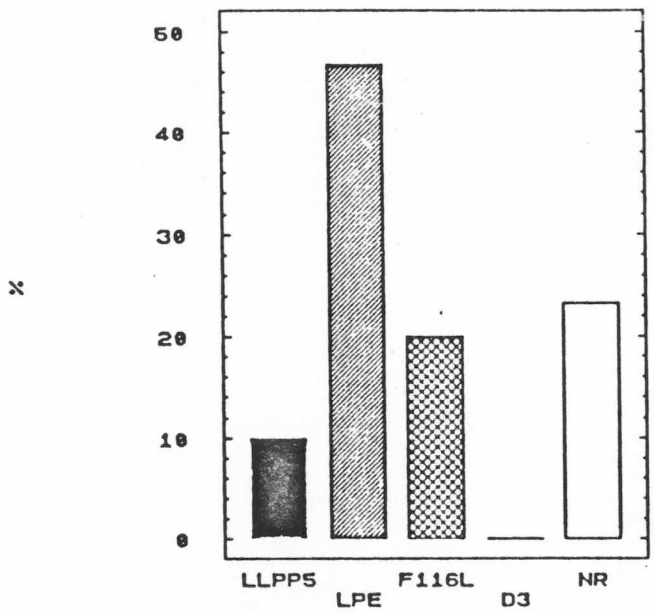


Figure 2.4. Distribution of free phage particles in lakewater. Thirtythree plaques were probed with radiolabeled phage DNA. About 23% of the plaques did not show similarity to any of the phage DNA used (NR). See Table 2.2 for the characteristics of phages LLPP5, LPE, F116L, and D3.

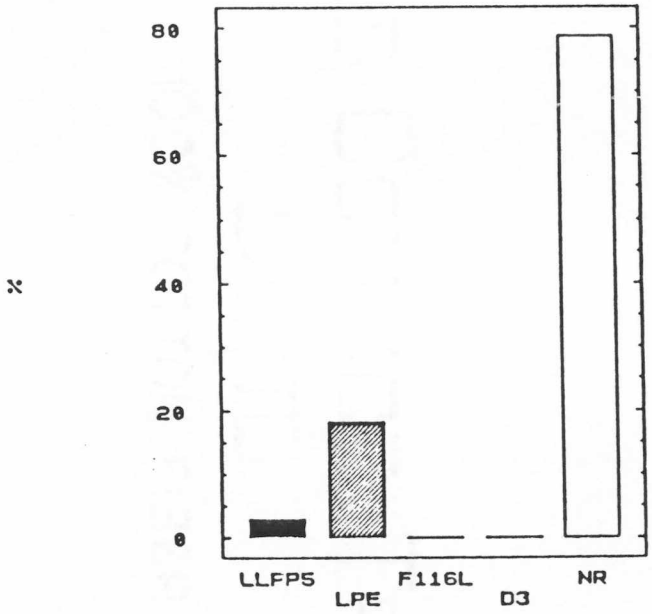


Figure 2.5. Distribution of free phage particles in sewage. Thirtytwo plaques were probed with radiolabeled phage DNA. A majority (78%) did not show similarity to any of the phages studied (NR). See Table 2.2. for the characteristics of phages LLPP5, LPE, F116L, and D3.

Distribution of Prophage DNA Sequences in Lakewater Bacteria.

Distinct autoradiograph signals are produced by prophage-containing bacterial colonies probed with ^{32}P labelled phage DNA (Figure 2.6). When bacterial colonies isolated from lakewater are probed with DNA from a variety of phage particles (listed in Table 2.2), the data obtained (Figures 2.7, 2.8 and 2.9) show that phage LLPP5-related sequences are most common, with about 40% of pseudomonads showing positive signals. Phage F116L-related sequences were found more often than either phage D3 or DNA sequences from the enriched phage population, LPE.

Distribution of *Pseudomonas aeruginosa* Bacteriophages in other Environmental Habitats.

The lakewater environment studied represents one that receives run-off drainage from soil, industrial, and domestic effluents, and occasionally, sediment turnover as a result of dredging. For this reason, samples of soil, sediment and sewage contributing possible phage loading were subjected to similar investigations conducted for the freshwater samples. The bacteria densities of these samples are shown in Table 2.2. The distribution pattern of prophage sequences in these bacterial populations are presented in Figures

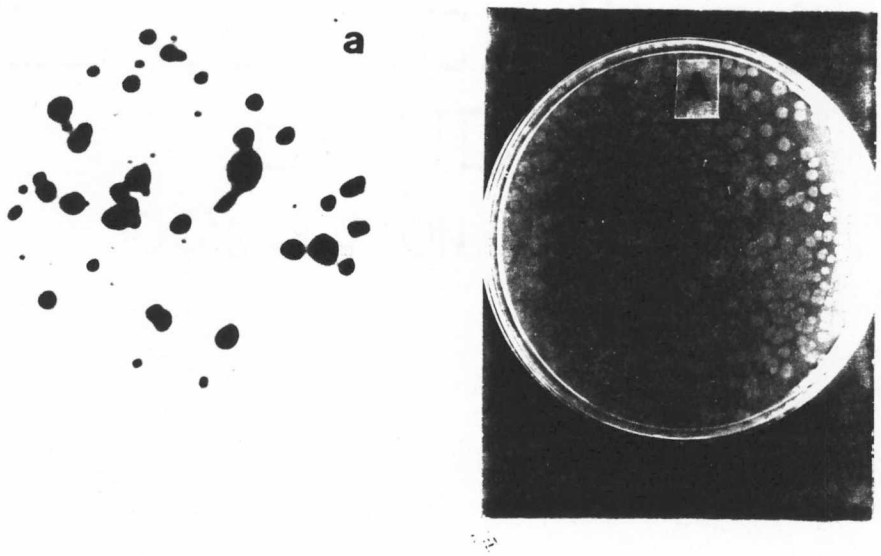


Figure 2.6. Detection of prophage-containing bacterial colonies in a population, by hybridization to ^{32}P-labeled phage DNA. Autoradiograph signals from *P. aeruginosa* LLPA10 colonies (panel **A**) harboring phage LLPP5 are shown in panel **a**.

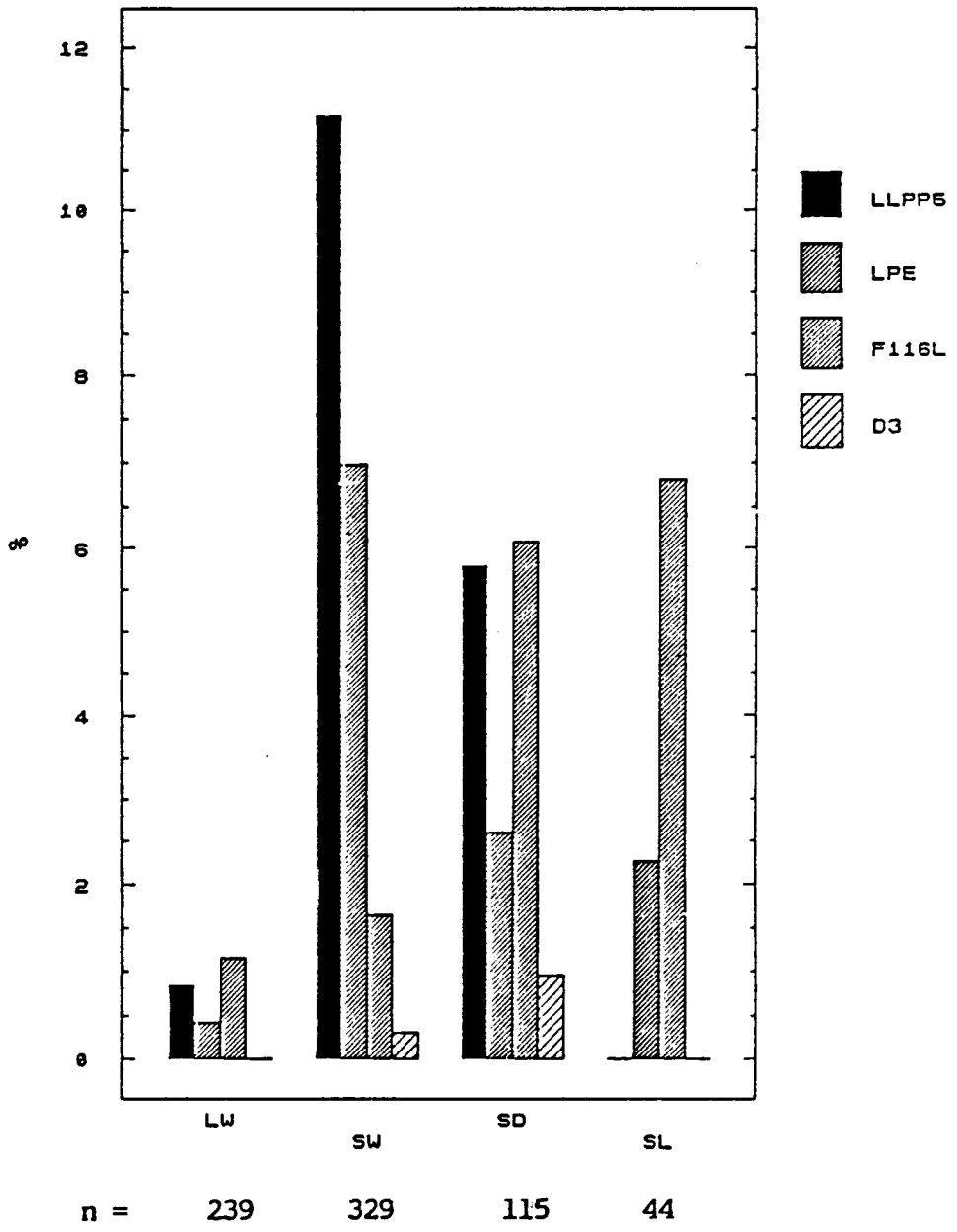


Figure 2.7. Prophage DNA sequences detected in bacterial colonies isolated on YEPG at 22 °C. Colonies recovered from lakewater (LW), sewage (SW), sediment (SD), and soil (SL) were probed with radiolabeled phage DNA. n = number of colonies probed. See Table 2.2 for description of phage.

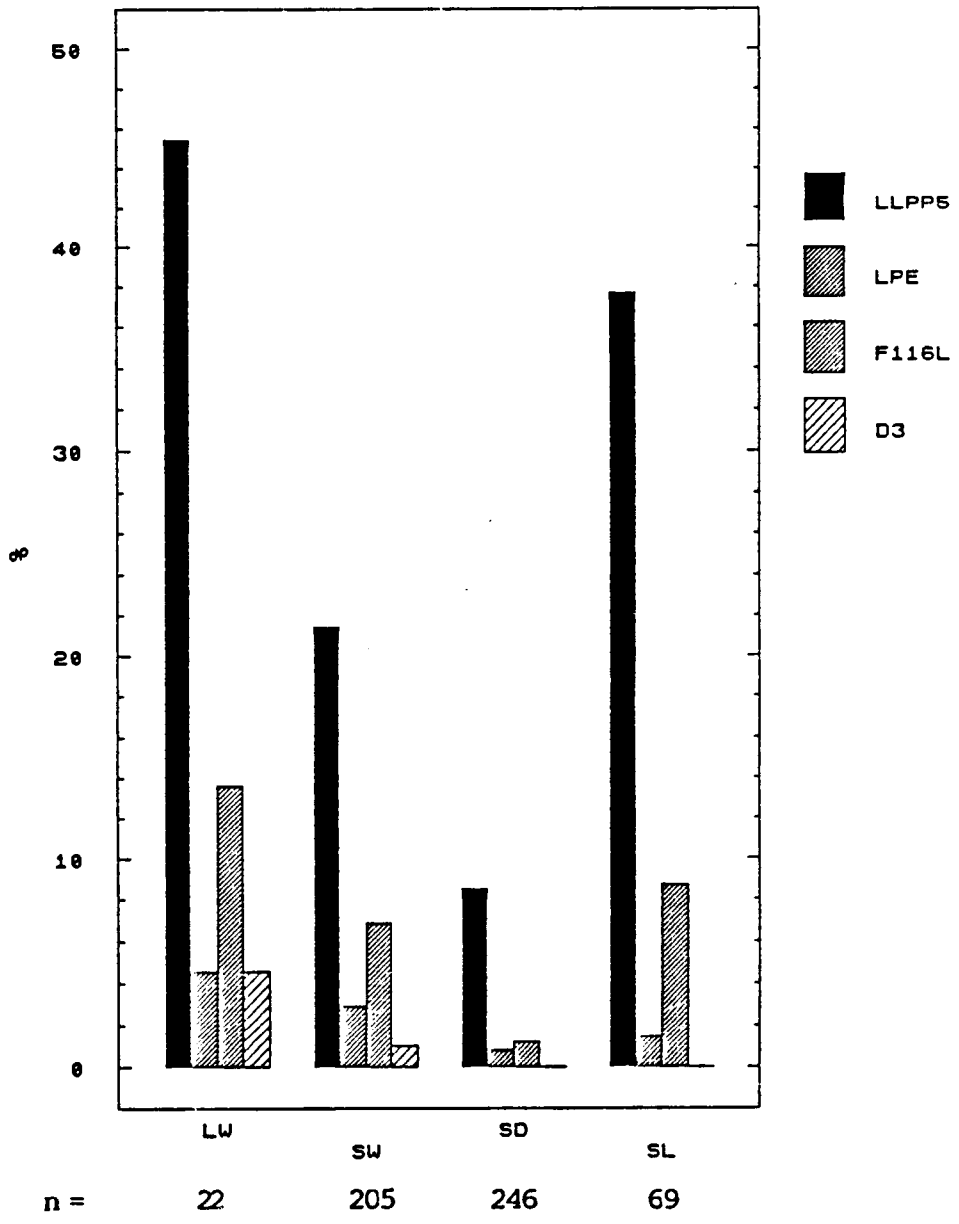


Figure 2.8. Prophage DNA sequences detected in bacterial colonies isolated on LB agar at 37 °C. Colonies recovered from lakewater (LW), sewage (SW), sediment (SD), and soil (SL) were probed with radiolabeled phage DNA. n = number of colonies probed. See Table 2.2 for description of phage.

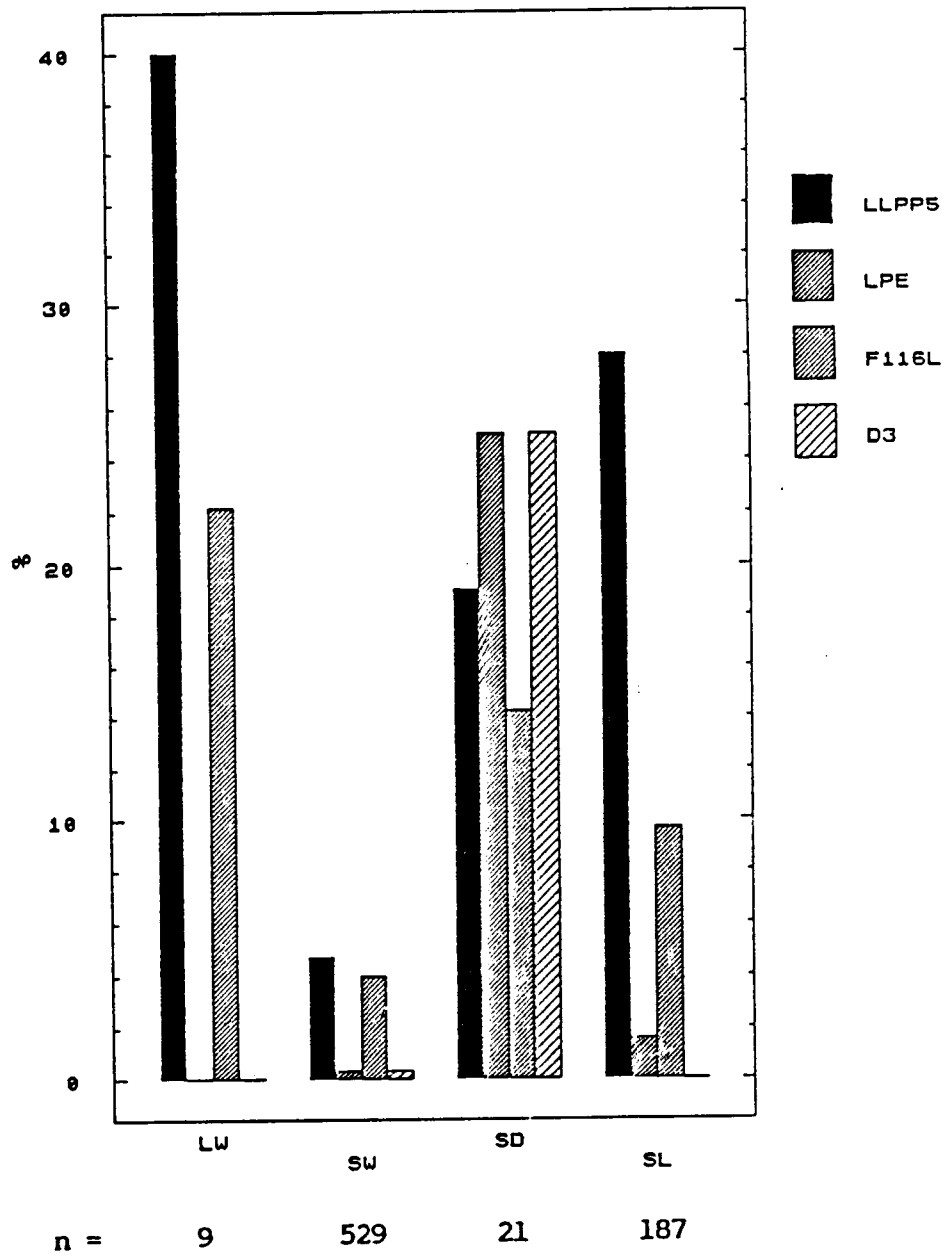


Figure 2.9. Prophage DNA sequences detected in bacterial colonies isolated on PIA at 22 °C. Colonies recovered from lakewater (LW), sewage (SW), sediment (SD), and soil (SL) were probed with radiolabeled phage DNA. n = number of colonies probed. See Table 2.2 for description of phage.

2.7 to 2.9. The occurrence of *Pseudomonas aeruginosa* bacteriophage existing as free particles was only detected in the sewage samples and these occurred at a density of 1.55×10^2 +/- 10 pfu ml^{-1} of sewage. Inability to detect free phage particles in soil and sediment may be due to poor efficiency of extraction (Lanning and Williams,1982), rather than a complete absence of these particles in the habitat. Out of 33 plaques from sewage examined with labelled DNA probes, 1 plaque was positive for phage LLPP5, and 6 were positive for phages represented in the enriched population. No phage F116L , nor phage D3 related plaques were found in this phage population.

DISCUSSION.

Since their discovery, several advances have been made in understanding the molecular biology of bacteriophages. Phage have become indispensable tools for examining fundamental biological processes, including the molecular mechanisms of DNA forms and functions. However, the study of bacteriophage ecology has not received comparable attention and may be considered to be in its infancy. One of the reasons for this discrepancy is believed to be the lack of suitable methods for ecological studies involving phage (Goyal, 1987).

To study phage ecology, it is necessary to know the geographical

(seasonal and spatial) distribution of phage and the quantitative relationship of phage and host bacteria in natural ecosystems. The effect of environmental factors on the physiology of both phage and bacteria should also be investigated. These can only be accomplished when suitable methods of recovery, enumeration, and identification are available for study of phage distribution. The two currently accepted methods for phage recovery from aquatic habitats are concentration and enrichment, while enumeration is often limited to plaque assays and occasionally direct electron microscopy counting.

There are two major limitations for the concentration and plaque assay protocols for studying phage ecology in water systems. First, the efficiency of concentration is poor because phage may loose parts of their host recognition appendages like tail fibers during the filteration process. The estimated population of phage in the original sample is thus underestimated, sometimes by several orders of magnitude (Ackerman and Nguyen, 1983). Secondly, the plaque assay technique is only as efficient as the specificity of phage for the host cells used as lawns. Some studies have utilized a mixture of indicator strains to expand the detection range for a group of phage particles (Primrose *et al.*, 1982). However, such multiple strain lawns may introduce other uncharacterized interactions that may cloud the detection assay. The choice of indicator strain is less clear when dealing with

environmental bacterial isolates. In an attempt to reduce some of these limitations in the present study, a preliminary scheme for detecting phage DNA in environmental samples was tried. Intact virions, or their extracted DNA, or lysogenic cells were seeded and serially diluted in lakewater. DNA from aliquots of each dilution was quantitatively immobilized on hybridization membranes. The membranes were then probed with a specific phage DNA. Figure 2.10 shows the results of the detection of phage F116L, DS1, and D3, and also the lysogenic carrier of phage DS1, *P. aeruginosa* RM272 introduced into lakewater. The detection limit for virions containing DNA that is homologous to the genomic probe was about 10^4 pfu. About 10^7 cfu of lysogenic bacteria hybridized significantly to the phage DNA probe. The sensitivity of detection was an order of magnitude lower in lakewater than in SM buffer. This reduction in detection efficiency was found to be pronounced if the phage were incubated in lakewater for prolonged periods. When phage LLPP5 was incubated in lakewater for 24 hours, the phage DNA was no longer detectable by dot blot hybridization (Figure 2.11) even though there was evidence of phage presence by plaque assay (Table 2.4).

When concentrated, unseeded lakewater was probed with phage LLPP5 DNA, no hybridization signals were detected. The reduced detection of phage DNA in intact particles in lakewater compared with the detection in SM buffer is speculated to be due to an interference from humic

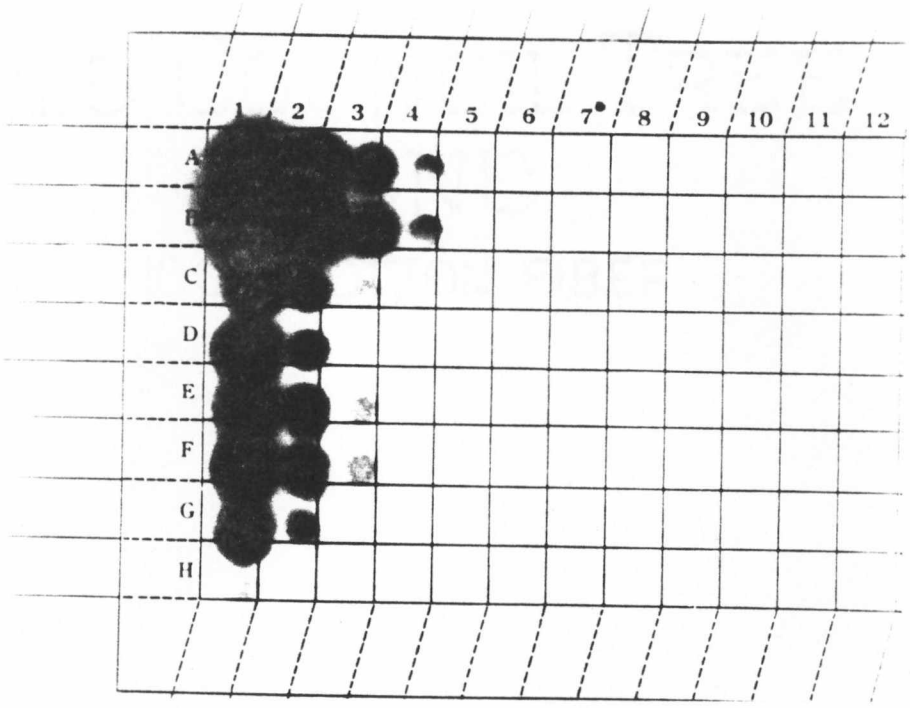


Figure 2.10. Detection of phage DNA in lakewater.

Row :

A : Phage F116L in SM buffer.
B : Phage DS1 in SM buffer.
C : Phage D3 in SM buffer.
D : Phage DS1 in natural lakewater
E : Phage DS1 in filtered lakewater.
F : Phage DS1 in autoclaved lakewater.
G : Phage DS1 lysogenic host strain RM272 in lakewater.
H : Sensitive host strain RM273 in natural lakewater.

All organisms were serially diluted from 10^8 viable units in column 1, to 1 viable unit in column 8. DNA was immobilized on a Nylon membrane and probed with ^{32}P-labeled phage DS1 DNA. Panel shows X-Ray film autoradiograph from hybridized DNA sequences.

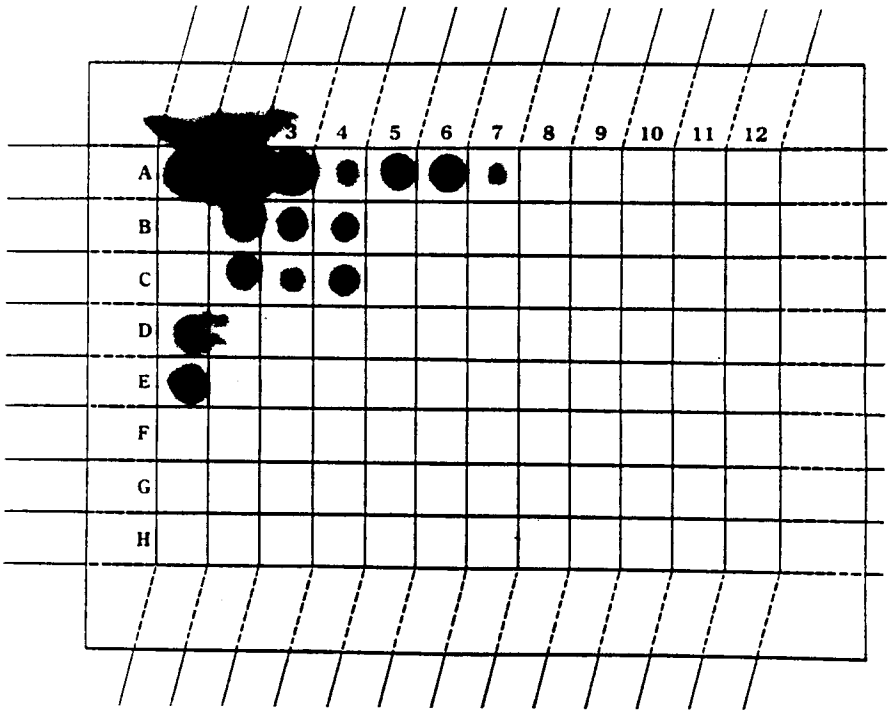


Figure 2.11. Detection of phage LLPP5 DNA from particles introduced into different aqueous media.

Row :

A : LLPP5 in SM buffer, serially diluted from 10^6 in column 1, to 1 pfu in column 10.
B : LLPP5 at 25 ^oC . Columns 1 : In lakewater ; 2 : In LB; 3 : In distilled water ; 4 : In SM buffer.
C : LLPP5 at 37 ^oC. Column content as in Row B.
D : LLPP5 resistant strain isolated from lakewater, 10^8 cfu to 1 cfu / spot.
E : LLPP5 lysogenic host strain. Column concentrations as in Row D.

Phage suspensions were incubated for 24 h and aliquots were immobilized on a Nylon membrane. The membrane was probed with ^{32}P-labeled phage LLPP5 DNA. Panel shows autoradiograph of signals from hybridized DNA sequences.

Table 2.4. Percent Recovery of Plaque Forming Units Incubated in Lakewater for 24 Hours.

Medium	25 °C	37 °C
Lakewater	12.5*	9.8
Luria-Bertani Broth	23.4	14.6
Distilled Water	10.5	8.4
Phage dilution buffer	24.1	21.2

*Phage LLPP5 was seeded into the media at an initial concentration of 5.4 x 10^5 pfu.ml^{-1}. Plaques were assayed on *P. aeruginosa* LLPA10 lawns. Percent recovery represents average of counts from duplicate phage titer plates.

materials that alter the binding characteristics of the hybridization membrane used. This technique therefore appears potentially useful for environmental samples but improvement in membrane manufacture will be necessary to overcome such interference. Nevertheless, DNA probes proved useful for estimating the relative abundance, or distribution of a particular phage within a heterogenous population of phage recovered by enrichment of the habitat with particular hosts. For example, Figure 2.12 shows the detection of phage LLPP5-related sequences in a gross DNA sample isolated from an enriched phage population, LPE. Densitometric assessment of the autoradiograph signals indicate that LLPP5 sequences constitute about 2.05 +/- 0.57% of *Pseudomonas aeruginosa* phage community genomes in lakewater.

The detection of prophage-DNA sequences in bacterial fractions isolated from environmental samples does not necessarily prove the occurrence of lysogenic bacteria. Attempts at mitomycin C induction of a total 38 isolated colonies that probed positive for phage LLPP5 and the heterogenous population, LPE sequences resulted in only five colonies that showed evidence of lysis. The occurrence of plaque forming units (on *P. aeruginosa* LLPA10 lawns) in a culture supernatant could only be demonstrated for one isolate. There are several factors that could limit the detection of plaque forming phage from induced environmental isolates such as the identity of indicator bacterial strain used for detecting plaque formation. Related phage do not necessarily share identical host ranges (Ackermann *et*

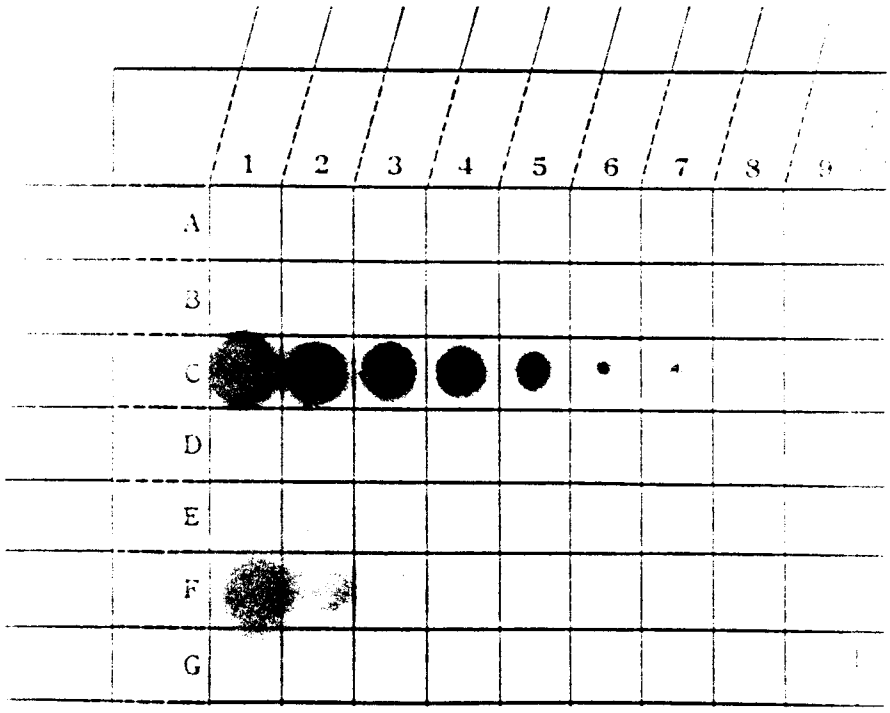


Figure 2.12. Detection of phage LLPP5-related DNA sequences within the phage community genome isolated from the heterogenous phage population, LPE.

Row :

A : DNA from heterogenous phage population spotted in decreasing concentrations from 10 ug to 50 ng per spot.
C : Phage LLPP5 DNA spotted in same concentrations as in Row A.
D : Heterogenous phage particle population, serially spotted from 10^{11} to 1 pfu / spot.

Purified DNA or phage particles were spotted and immobilized on a Nylon membrane. The membrane was probed with ^{32}P-labeled phage LLPP5 DNA. Panel shows autoradiograph of signals from hybridized DNA sequences.

al., 1978). Secondly, not all temperate phage are inducible by one single induction agent (Barksdale and Arden, 1974). The occurrence of phage sequences in bacteria hosts might also have a phylogenetical significance concerning the origin and evolution of bacteriophage. Bradley (1971) and others suggested that phage may have evolved from the bacterial chromosome as means of genetic transfer. A primitive bacteriophage could simply be regarded as a segment of bacterial chromosome producing a protein that could encapsidate DNA joined to the origin of bacterial chromosomal replication. Reanney and Ackermann (1982) in considering the similarities between some bacterial and phage genes, concluded that genomes of temperate phage are part of the vertically inherited 'selfish' bacterial DNA. They suggest that phage genes have probably been a factor in the evolution of host bacterial genomes. Transposition (e.g. IS222 in phage D3 ; Gertman *et al.*, 1986) and DNA fragmentation by endonuclease activity are some of the mechanisms that could aid the interacting processes of phage and bacterial genomic evolution.

The occurrence of phage modules in bacterial host DNA has also been considered a distinct possibility (Reanney, 1976 ; Krylov *et al.*, 1985). This may explain the appearance of viable variants or various grades of defective phage-like bacteriocin particles. There is evidence that indicate that the R-type pyocins of *P. aeruginosa* originated from a common temperate bacteriophage, which by mutation became defective in assembly of normal

virions (Sano and Kageyama, 1984). This is supported by the finding that several *P. aeruginosa* strains produce phage that display immunological cross-reactivity with these pyocins (Kageyama *et al.*, 1979).

Interspecific antagonistic activities appear to be common within the pseudomonads community of lake Loudon freshwater (Figure 2.13). In most cases, none of these activities could be identified as phage mediated. The observation of phage-related DNA sequences in bacterial DNA may therefore be due to the occurrence of molecular determinants of bacteriocin-like activities in the population.

In conclusion, there appears to be a need for more comprehensive methods for assessing the ecological implications of the distribution patterns of bacteriophage in the environment. The distribution of *P. aeruginosa*-specific bacteriophage in the habitat studied indicate a variation that is affected by seasonal and host population changes. It is suggested that future phage ecological investigations be conceived within a general scheme of antagonistic activities such as bacteriocin production by bacteria . This approach may enhance understanding of the molecular basis of phage-host interactions in the environment.

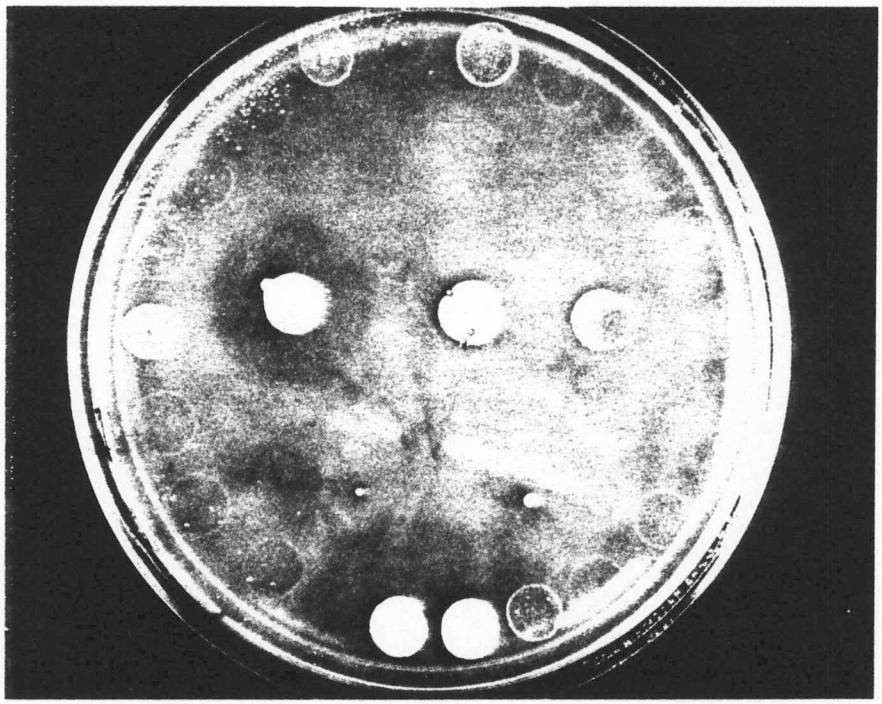


Figure 2.13. Inter-strain antagonistic activity among the pseudomonad community isolated from Lake Loudon.

Five microliter volumes of 26 environmental isolates selected by growth on *Pseudomonas* Isolation Agar were spotted on a lawn of *P. aeruginosa* LLPA10. The plate was incubated at 37 ^{o}C for 48 h. Zones of growth inhibition are seen around colonies producing antagonistic substances.

Summary of Observations.

1. The distribution of *P. aeruginosa* bacteriophages as particulates in Ft. Loudon lakewater was irregular and may occur at transient concentrations up to 5 per 100 ml.

2. The enrichment of Ft. Loudon lakewater with a susceptible bacterial host produced a variety of phage particles.

3. The distribution of *Pseudomonas aeruginosa* in Ft. Loudon lakewater was irregular and may occur at concentrations up to 6 per ml.

4. Molecular hybridization studies with phage DNA and bacterial genomes was capable of quantitative assessment of the distribution and relationship of particulate phage and prophages in environmental samples. Up to 40% of pseudomonads in Ft. Loudon lakewater may contain prophage DNA sequences.

CHAPTER III

CHARACTERIZATION OF BACTERIOPHAGE AND *Pseudomonas aeruginosa* HOST ISOLATED FROM FRESHWATER

INTRODUCTION.

The aerobic pseudomonads are bacteria of significant scientific and industrial merit. They are thought to be among the most active species in biogeochemical processes in nature (Palleroni, 1975). Consequently, this group of organisms has been the focus of attention of several laboratories over the years. However, the taxonomic structure of pseudomonads and the complexity of some subgroups (e.g. the fluorescent pseudomonads) constitute a formidable challenge to the bacterial taxonomist (Woese *et al.,* 1986). The definition of aerobic pseudomonads proposed by Stanier *et al.* (1966) includes the following general phenotypic properties : Unicellular rods,

straight or curved but not helical, measuring 0.5 - 1 um by 1.5 - 4 um ; motile by means of one or more polar flagellar, Gram negative, do not form endospores, stalks, or sheaths, respiratory, never fermentative chemoorganotrophs. The identification of pseudomonads is usually based on these criteria which may be summarized under morphology, pigmentation and nutritional attributes. Phage typing and *in vitro* nucleic acid hybridization data, including ribosomal RNA sequence comparison have also been used (Palleroni, 1975 ; Festl *et al.*, 1986).

Pseudomonas aeruginosa is the type species of the genus *Pseudomonas.* This species is very well defined and in most cases can be readily recognized by a limited number of simple characters such as the production of pyocyanin and fluorescent pigments. Colonies on agar are usually pitted. All strains grow rapidly at 37 ^{o}C, a trait that differentiates *P. aeruginosa* from other fluorescent pseudomonads. The maximum temperature at which growth occurs is 44 ^{o}C, with no growth at 5 ^{o}C. Catalase and oxidase reactions are positive, gelatin is hydrolyzed, but starch is not. All strains can grow with ammonia, valine, lysine, and urea as nitrogen sources and a concentration of 3% sodium chloride is tolerated. Routine identification of *P. aeruginosa* from clinical specimens is a common procedure in hospitals.

The following attributes are considered confirmatory (Lowbury, 1975) :

(a) Blue-green fluorescence after 24 hours growth at 37 ^{o}C on cetrimide-nalidixic acid agar.

(b) Production of a blue, chloroform-soluble, nonfluorescent pigment (pyocyanin) when grown on milk agar.

(c) Gluconate oxidation and slime production in a liquid medium containing potassium gluconate.

(d) Growth at 42 ^{o}C.

(e) Reduction of nitrate to gaseous nitrogen.

(f) Growth with production of red colonies on 1% tetrazolium chloride agar.

In addition to these traits, serological typing, and phage or bacteriocin typing methods are also applied for tracing the sources and routes of transfer of clinically important strains. Reports of the isolation and characterization of *P. aeruginosa* specifically from freshwater are scarce. However, there are numerous investigations into the aquatic distribution of fluorescent pseudomonads in general. The combination of techniques from both clinical and environmental microbiology literature was necessary for the present characterization of *P. aeruginosa* from Lake Loudon.

According to the published estimates of the International Committee on Taxonomy of Viruses (ICTV), there will be about 2,800 listed bacteriophages by January, 1989 (Ackermann *et al.*, 1978). This number continues to grow at about 100 annually, making bacterial viruses the largest of all viral groups (Mathews, 1982). In order to minimize duplication of research efforts, Ackermann *et al.* (1978) proposed guidelines for the characterization of newly discovered, and previously discovered, but uncharacterized bacteriophage. Due to the highly diverse, but consistently simple structure of most bacteriophages, these guidelines are applicable to phage studies in many groups of bacteria. The study of *Pseudomonas aeruginosa* bacteriophages has received considerable attention over the past three decades. Their importance, which is attributed to the significance of genetic organization in their hosts presently approaches that of coliphages. A wide variety of *P. aeruginosa* phages have been characterized to a significant extent (Holloway and Krishnapillai, 1975). This diverse group of phages span most known phenotypes, from tail-less phages to filamentous forms. Both RNA and DNA containing phages have been described, although a majority of these phages contain double stranded DNA genomes. The first step in the characterization of a newly observed phage remains the morphological affiliation. The primacy of morphological criteria in phage taxonomy was established by Bradley (1967) who introduced six basic morphological types

of bacteriophage. These morphotypes have since increased to 18 (Reanney and Ackermann, 1982). The emphasis on morphology has persisted due to the large number of genes presumably involved in determining this property of phage. Other criteria that are used for phage arrangement and nomenclature include host range, nucleic acid structure, strategy of infection and morphogenesis, phylogeny, serology, sensitivity to physical and chemical agents, and host-environmental dependent properties such as adsorption velocity, latent period, burst size, plaque morphology, and efficiency of plating. Obviously, some of these properties are more important than others in providing an adequate description of a new phage. A graded scheme for phage characterization properties has been proposed by Ackermann (1983).

This chapter deals with work done to identify and characterize a specific host and phage system isolated from lake Loudon.

MATERIALS AND METHODS.

Bacteria.

Pseudomonas aeruginosa strains were isolated from lake Loudon freshwater by selective media and temperature cultivation. Triplicates of 1, 10,

50, and 100 ml aliquots of lakewater were filtered on sterile, 0.45 um pore size polycarbonate membranes (Nucleopore). The membranes were incubated on either Pseudomonas Isolation Agar (PIA) , 1% tetrazolium agar medium (Grant and Holt, 1977) or a nalidixic acid containing medium (Gould *et al.*, 1985). Plates were incubated at 42 ^{o}C for 24 hours. Isolated colonies were picked and restreaked onto PIA. Colonies from the original plates were replica lifted onto Nylon hybridization membranes (Biotrans) and probed with plasmid pHF360 which contains a fragment of the 23S ribosomal RNA gene that is specific for fluorescent pseudomonads (Festl *et al*, 1986). Usually, all colonies preselected on PIA are detected by this gene sequence probe.

Fifteen colonies were picked for confirmatory physiological tests. Catalase and oxidase production, gelatin and starch hydrolysis tests were performed according to standard procedures.

Susceptibility to previously characterized *P. aeruginosa* phage F116L, D3, and E79 were investigated by spotting concentrated phage suspensions on freshly poured lawns of test organism. The plates were incubated at 37 ^{o}C and plaque production was scored after 24 h incubation. The strain designated LLPA10 (named for **L**ake **L**oudon ***Pseudomonas aeruginosa***, isolate **10**) was chosen for further investigation because it gave the most reproducible response to phage infection, and it consistently secreted pyocyanin pigments into growth media.

The growth pattern of *P. aeruginosa* LLPA10 in Luria-Bertani (LB) broth was monitored by a Klett-Summerset colorimeter equiped with a red filter. At appropriate intervals, 1 ml samples were withdrawn from the growth medium, centrifuged to pellet cells, washed twice, and resuspended in Phosphate-buffered Saline (PBS) for electron microscope visualization of the cells. The microscope used was a Hitachi (H-600) model, set at 100 kV accelerating voltage. The samples were spotted onto a 400-mesh, carbon-coated, copper grids. Negative staining was achieved with 0.5% phosphotungstate.

Bacteriophage.

The procedure for phage isolation by concentration of lakewater was described in Chapter One. To study the morphology of phage LLPP5 (named for **L**ake **L**oudon *Pseudomonas* **P**hage, plaque **5**) high titer phage lysates were purified by glycerol gradient ultracentrifugation (Silhavy *et al.*, 1984). The purified particles were further concentrated by centrifugation at 35,000 rpm in clear 2 ml capacity ultracentrifuge tubes (Beckman). The transluscent pellets were resuspended in cold 1% ammonium acetate. Five microliter aliquots of phage suspension were spotted onto carbon-coated,

copper grids and negatively stained with 0.5% phosphotungstate or 0.5% uranyl acetate. The electron microscopic conditions were as previously described.

DNA Restriction Analyses.

Extraction of DNA from purified phage particles was performed essentially as described by Silhavy *et al.* (1984). The extracted DNA were further purified by drop dialysis or by Cesium chloride gradient ultracentrifugation (Maniatis *et al.*, 1982). Concentrations of DNA were estimated by ultraviolet light absorbance spectrum measurement, using a DU-70 spectrophotometer (Beckman). One microgram of phage DNA was digested with 10 units of enzyme (BRL) for 2 h at 37 ^{o}C. The restriction fragments were separated in 0.5% or 0.75% agarose gel electrophoresis. DNA bands were visualized under shortwave UV light after Ethidium bromide staining of gels. In order to achieve maximum separation of the larger fragments, the use of a pulse field gel electrophoresis apparatus was employed (Pharmacia, LKB Pulsaphor). For this purpose, a 1% agarose gel was used and the fragments were separated with 170 volts of current pulsating each second in perpendicular directions for 16 hours. The molecular

mass of restriction fragments was estimated by means of a computer program using λ DNA restricted by Hind III and the 1 kilobase size ladder (BRL, Gaithersburg, MD) as standards.

Molecular Relatedness of Phages.

To evaluate the DNA sequence similarity between phage LLPP5 and three previously characterized *P. aeruginosa* bacteriophages, D3, F116L, and E79, a hybridization analysis of restricted phage genomes was conducted. DNA fragments were separated on an 0.75% agarose gel and the fragments were electroblotted onto a hybridization membrane (GeneScreen) using a Trans-Blot Cell apparatus (Bio-Rad, Richmond, CA). The membrane immobilized DNA was probed with ^{32}P-labeled, Sal 1 digestion fragments of phage LLPP5 DNA. Hybridization was carried out at 65 ^{o}C for 24 - 48 h. The membranes were washed in a solution containing 0.3 M sodium chloride and 0.03 M sodium citrate at 65 ^{o}C for 2 hours. Hybridization signals were detected by autoradiography.

Host Range and Adsorption Mechanism.

The host range of phage LLPP5 was examined by spotting a known concentration of particle suspension on lawns of various *P. aeruginosa* strains (Table 3.1). The plates were incubated at 37 ^{o}C. Production and morphology of plaques were recorded after 24 h incubation.

To investigate the mechanism of adsorption of phage LLPP5 to host bacteria, *P. aeruginosa* LLPA10, host cells were grown to a density of approximately 10^{8} cfu ml^{-1}. The cell culture was centrifuged to pellet cells, which were washed and resuspended in 1/10th volume of PBS. Purified phage were added to the cells at various multiplicity of infection values, ranging from 0.1 to 200. The infected cells were incubated for 5 minutes at 37 ^{o}C after which 5 ul aliquots were immobilized, stained with 0.5% phosphotungstate and examined under a transmission electron microscope as previously described.

Lysogenization.

Continued incubation of a confluently lysed lawn of *P. aeruginosa* LLPA10 by phage LLPP5 frequently yields healthy phage resistant colonies. Continued incubation of a lysed broth culture also resulted

Table 3.1. Sensitivity of *P. aeruginosa* LLPA10 and Host Range of Phage LLPP5.

Host strain	LLPP5	D3	DS1	F116L	E79	LPE
LLPA10	S	R*	S	S	S	S
LPL5	R	R*	S	S	R	S
PAO381	S	S	S	S	R	S
RM273	S*	S	S	S	S	S
RM272	S	S	R	R*	S	S
RM2140	S*	S	S	S	R	S

S = Sensitive ; S* = reduced sesitivity or plaquing efficiency after cultivation of phage on LLPA10. ; R = resistant. ; R* = few turbid plaques are seen.

in growth turbidity approximately 5 hours following obvious lysis. When these phage resistant colonies are isolated and purified by repeated streaking, about 20 to 40% of them probe positive for phage DNA and they yield phage without obvious lysis in broth culture. To further investigate the nature of this phage-host association, a purified colony of phage resistant bacteria was grown to a cell density of approximately 10^8 cfu ml^{-1}. The cells were harvested and washed three times with PBS by repeated pelleting at 5,000 rpm. Total cellular DNA was isolated from this culture by a modification of the procedure described by Anderson and McKay (1982). Vortexing was avoided to minimize shearing of chromosomal DNA. When a fraction of this DNA was purified by cesium chloride gradient ultracentrifugation, no extrachromosomal band was observed. Increasing quantities of the genomic DNA (1, 25, 50, and 100 ug) were subjected to restriction by Sal 1 endonuclease. The restriction fragments were blotted and probed with phage LLPP5 DNA to ascertain the possible location of prophage in the host genome. Hybridization conditions were as previously described.

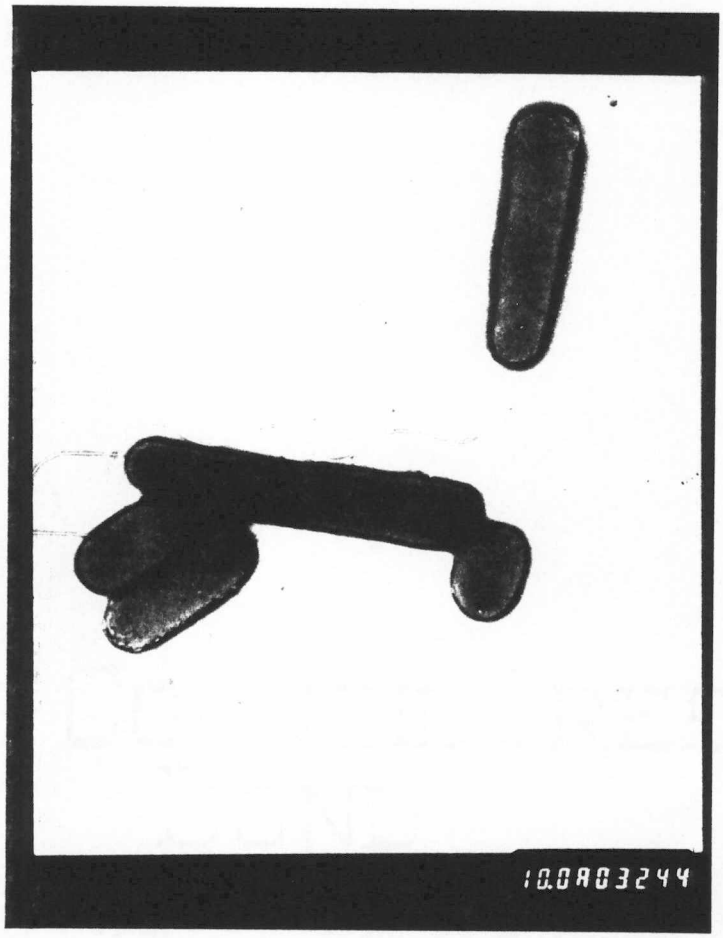
UV induction of Phage Production from Lysogenic Culture.

The method of Miller and Ku (1978) was used for studying the induction pattern of phage in a lysogenized culture of *P. aeruginosa* LLPA10.

An attempt to substitute a sunlamp (Sylvania) for the pure UV light source was unsuccessful presumably because of the high level of heat generated by the lamp which resulted in a high proportion of inactivated cells and phage. Visible light may also counteract the effect of UV light, due to photoreactivation.

RESULTS.

The lakewater isolate, *Pseudomonas aeruginosa* LLPA10 exhibits charateristic traits of the species. This strain grows at 42 ^{o}C and produces copious amounts of chloroform-soluble pyocyanin under various growth conditions. It is catalase and oxidase positive, hydrolyses gelatin, and does not ferment glucose. *P. aeruginosa* LLPA10 has a polar flagellum and no visible pili (Figure 3.1). There is no trace of an extrachromosomal DNA present in the organism, when investigated by the method of Anderson and McKay (1982). In mid exponential growth phase, this organism has a doubling time of approximately 150 minutes in Luria-Bertani broth. As the phase of growth approaches stationary, individual cells become shorter and



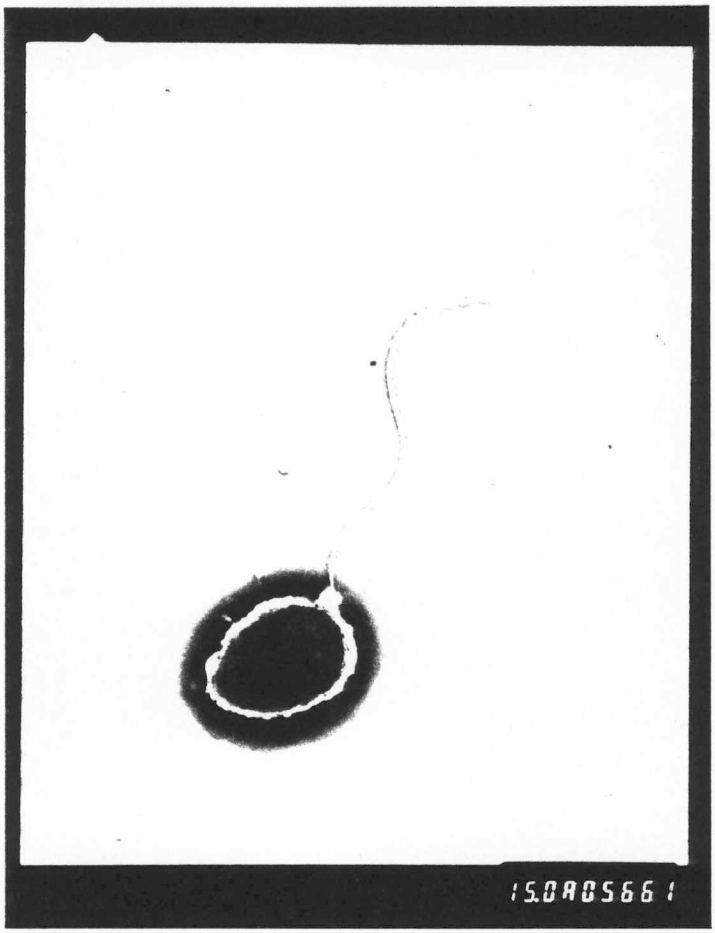
(A) *P. aeruginosa* LLPA10 cells taken from an exponential growth phase batch culture.

Figure 3.1. *P. aeruginosa* LLPA10 isolated from Lake Loudon. Morphological details were observed by negative staining with phosphotungstate. Bars represent 100 nm.



(B) *P. aeruginosa* LLPA10 cells at late exponential growth phase.

Figure 3.1 (continued)



(C) *P. aeruginosa* LLPA10 cells at stationary growth phase, showing reduced cell dimensions compared to cells in exponential growth phase (panel A).

Figure 3.1. (continued)

rounder, with thicker cell walls (Figure 3.1c). Strain LLPA10 is susceptible to all previously characterized *P. aeruginosa* phages tested (Table 3.1).

The results of an evaluation of several electron micrographs indicate that this phage has an isometric (octahedral) head of about 67.5 nm in diameter, measured in the horizontal axis (Figure 3.2). A 10 nm collar separates the head from a contractile sheath that measures about 12 nm in thickness. The sheath covers a non-flexible tail core that is about 125 nm long. Four tail fibers can be observed, that appear to be attached to a base plate. These morphological features tend to prompt an instant categorization or classification of phage LLPP5 in the ICTV-approved phage family *Myoviridae,* along with the T-even phage group represented by coliphage T2 as the type species (Brown, 1986). Among the previously characterized *P. aeruginosa* phages, isolate LLPP5 resembles phage 7v described by Feary *et al.* (1964), phage PB-1 of Bradley (1967), and phage E79 as described by Slayter *et al.* (1964).

The molecular affiliation of phage LLPP5 could only be compared to that of phage E79 in the present study. E79 is probably the most well characterized, virulent *Pseudomonas aeruginosa* phage. It is a non-transducing, female specific virus (Holloway, 1975). Apparently, phage LLPP5 is closely related to E79 at the molecular, genomic level. These two phages have considerable DNA sequence similarity as determined by

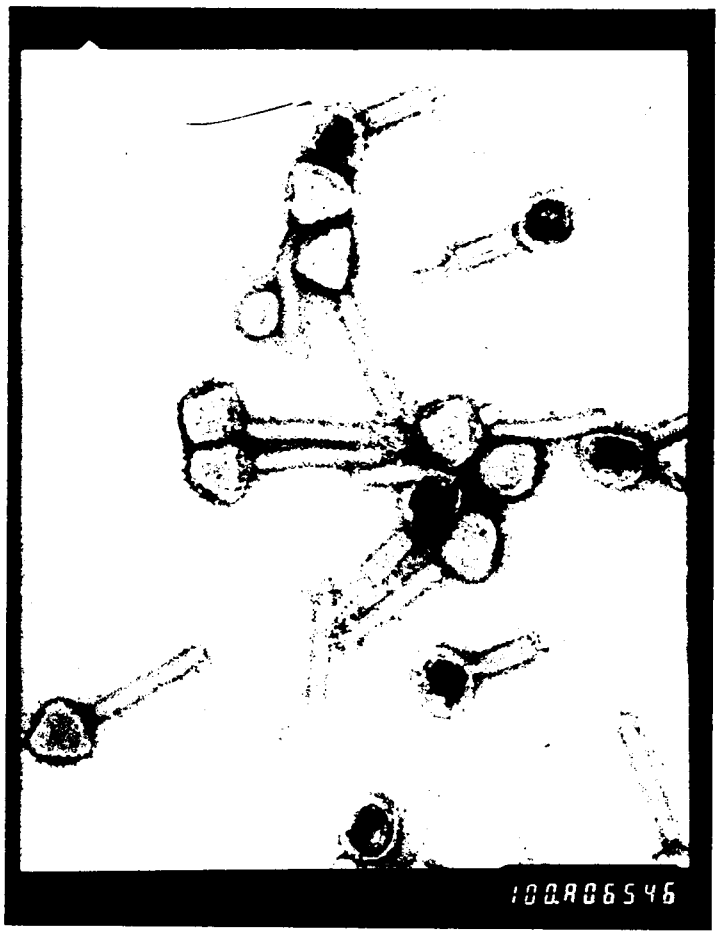


Figure 3.2. Phage LLPP5 showing morphological details.

Apparent are an isometric head, an inflexible, contractile-sheathed tail, and four tail fibers. Purified phage suspension was stained with 0.5% uranyl acetate. Bar represents 100 nm.

cross hybridization (Figure 3.3), and by resistance to various restriction enzymes including Bam H1, Pst 1, Sma 1, and Sst 1 (Figure 3.4). In addition, the two phages appear to be homoimmune since E79 will not form plaques on a host strain LPL5 (named for **L**ake ***Pseudomonas*** **L**ysogenic for plaque **5**) carrying LLPP5 prophage (Table 3.1). However these two phages differ in that phage LLPP5 grows readily on an F plasmid carrying host strain, *P. aeruginosa* PAO381 ($FP2^+$) which is resistant to infection by E79. The genome of phage LLPP5 is considerably smaller than that of E79. Estimates of E79 genome puts its size at about 120 to 180 kilobases (Holloway and Krishnapillai, 1975). By extrapolation of the molecular weights of restriction fragments, phage LLPP5 genomic DNA is estimated to be 78 +/- 5 kilobases. The restriction pattern of phage LLPP5 differs from those of phage F116L and phage D3 (Figure 3.5). There is no detectable DNA sequence similarity between these two phages and phage LLPP5 (Figure 3.6).

The temperance of phage LLPP5 was inferred by a high proportion of phage resistant cells that develop from sensitive *P. aeruginosa* LLPA10 cells incubated with this phage. The evidence that such phage-resistant cells contain LLPP5 prophage come from colony hybridization studies that indicate the presence of phage LLPP5 DNA sequences in about 20% of all phage-resistant colonies recovered. These prophage-positive colonies yield phage spontaneously in turbid broth

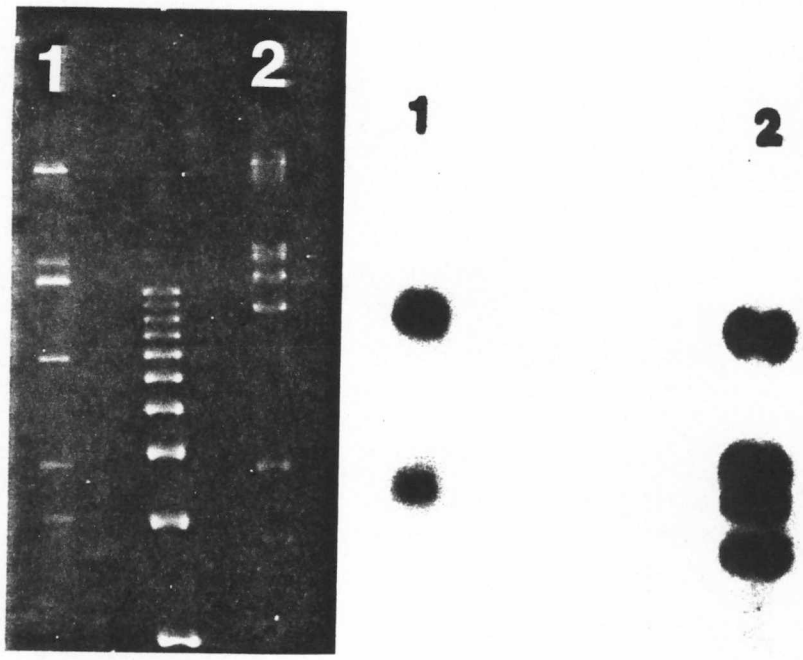


Figure 3.3. DNA sequence similarity between phage LLPP5 and phage E79.

Endonuclease Sal 1 fragments of phage DNA were separated in a 1% agarose gel run at 170 volts per second for 16 h in a Pharmacia LKB pulsaphor apparatus. The fragments were electro-transfered onto a Nylon membrane (Biotrans) and probed with ^{32}P-labeled Sal 1 digested LLPP5 DNA. Left panel : Agarose gel, lane 1 : LLPP5 x Sal 1 ; Lane 2 : E79 x Sal 1 ; Middle lane : One kilobase size marker. Right panel : Autoradiograph of signals from hybridized DNA molecules.

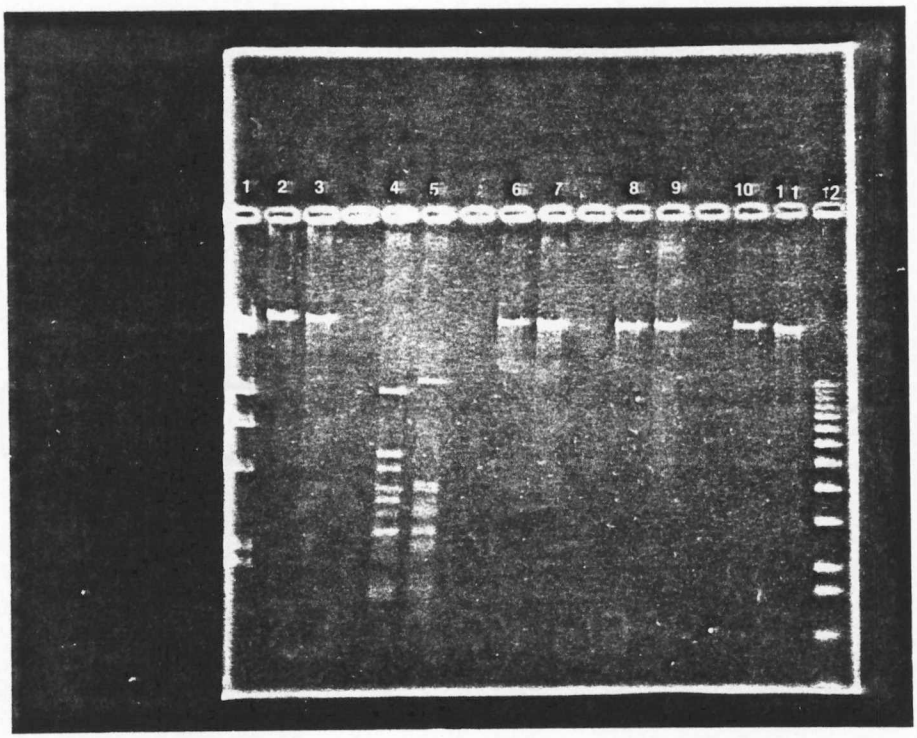


Figure 3.4. Endonuclease recognition of phage LLPP5 and phage E79 genomes.

Lane 1 : λ x Hind III control. Lanes 2 & 3 : Bam H1. Lanes 4 & 5 : Cla 1. Lanes 6 & 7 : Pst 1. Lanes 8 & 9 : Sma 1. Lanes 10 & 11 : Sst 1. Lane 12 : One kilobase size marker. Lanes 2, 4, 6, and 8 are phage LLPP5 DNA. Lanes 3, 5, 7, and 9 are phage E79 DNA. Fragments were separated on an agarose gel, run at 75 volts for 5 hours.

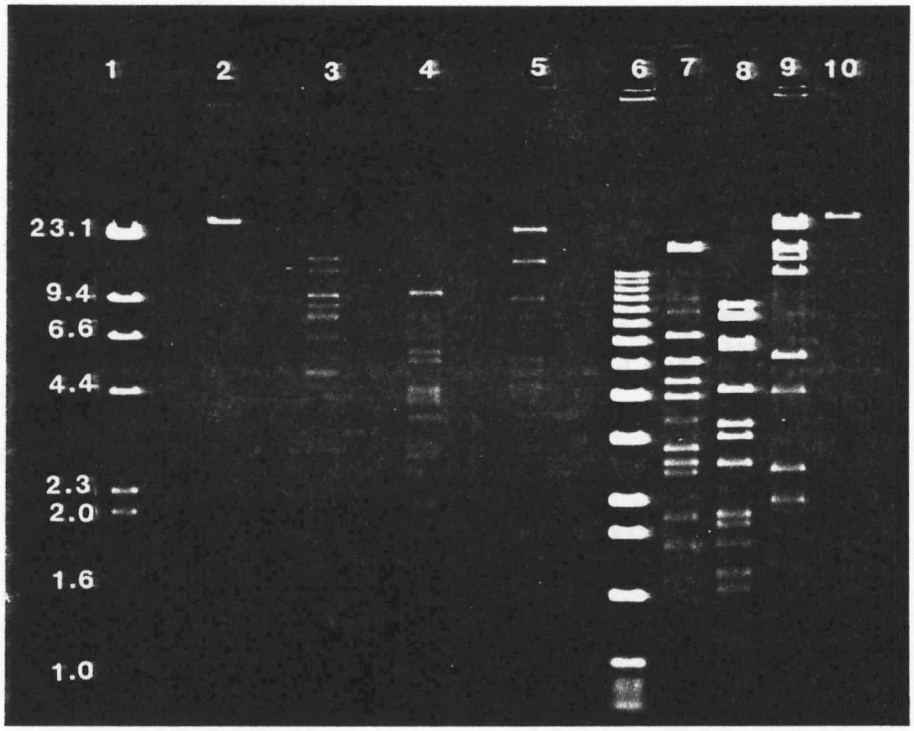


Figure 3.5. Endonuclease restriction pattern of phage LLPP5 genome.

Lane :

1 : Phage λ by Hind III
2 : Phage LLPP5 DNA
3 : Phage LLPP5 by Hind III
4 : Phage LLPP5 by EcoR1
5 : Phage LLPP5 by Sal 1
6 : 1 kb size marker
7 : Phage D3 by Sal 1
8 : Phage DS1 by Sal 1
9 : Phage E79 by Sal 1
10 : Heterogenous phage (LPE) DNA

Endonuclease digestion of phage DNA was carried out at 37 ^{o}C for 2 h. Fragments were separated in 0.5% agarose gel run at 75 volts for 5 hours.

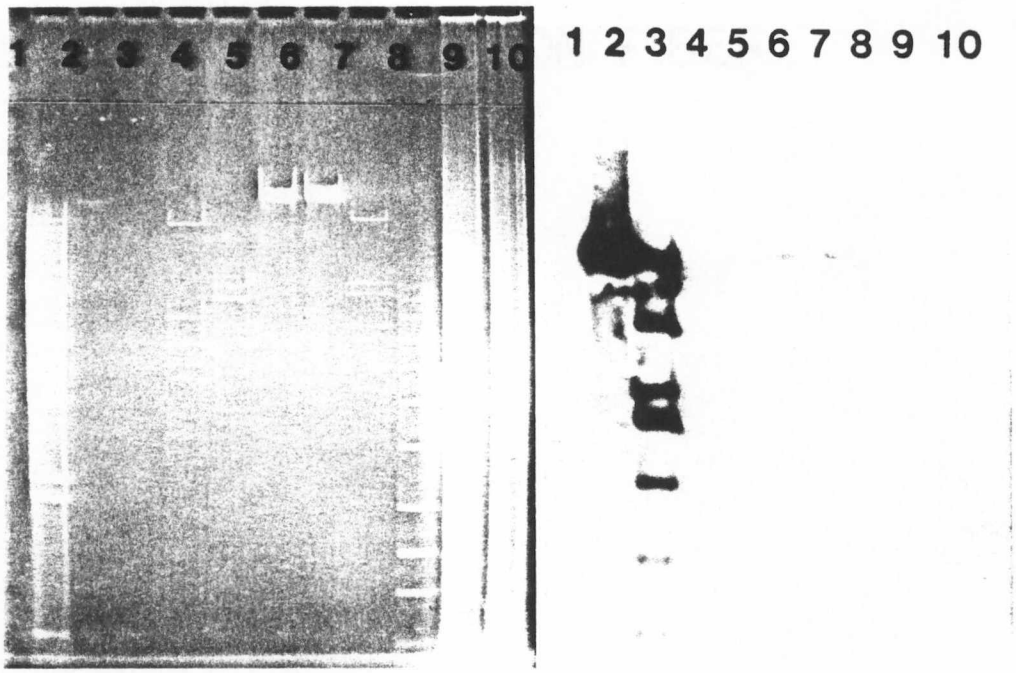


Figure 3.6. Absence of DNA sequence similarity between phages LLPP5, F116L, and D3, and between LLPP5 and pRMS149, and the genomes of *P. aeruginosa* RM273, and PAO381.

Lane :

1 : λ by Hind III
2 : LLPP5 DNA
3 : LLPP5 by Sal 1
4 : D3 by Sal 1
5 : DS1 By Sal 1
6 & 7 : LPE DNA
8 : pRMS149 by Sal 1
9 : RM273 by Sal 1
10 : PAO381 by Sal 1.

Endonuclease generated fragments of phage DNA were separated in a 0.75% agarose gel (left panel). The DNA fragments were electrotransfered onto a Nylon membrane (Biotrans) and probed with ^{32}P-labeled phage LLPP5 DNA. Right panel shows autoradiograph of signals from hybridized DNA molecules.

cultures, and plaques when they are plated as lawns without external source of phage. DNA dot blots, Genomic digests, and Southern hybridization of DNA from these lysogenized hosts confirm the presence of phage LLPP5 DNA sequences in the host bacteria, that are absent in the non-lysogenized, phage-sensitive hosts (Figure 3.7 and Figure 3.8). It seems improbable that phage DNA exists as an autonomously replicating plasmid in the host, but the exact integration site of the phage genome into the host chromosome could not be ascertained by the techniques used (Figure 3.8).

Under the influence of ultraviolet radiation, phage production from lysogenized hosts is increased (Figure 3.9). This UV induction is maximal at a dose of 800 microwatts per second, when the phage titer obtained is approximately one order of magnitude higher than the titer from spontaneous phage yield by the cells.

The mechanism of adsorption of phage LLPP5 to host cells appears to be similar to those of previously characterized, contractile tail phages (Bradley, 1967). However, the function of its short tail fibers are not immediately apparent in the adsorption processes observed (Figure 3.10).

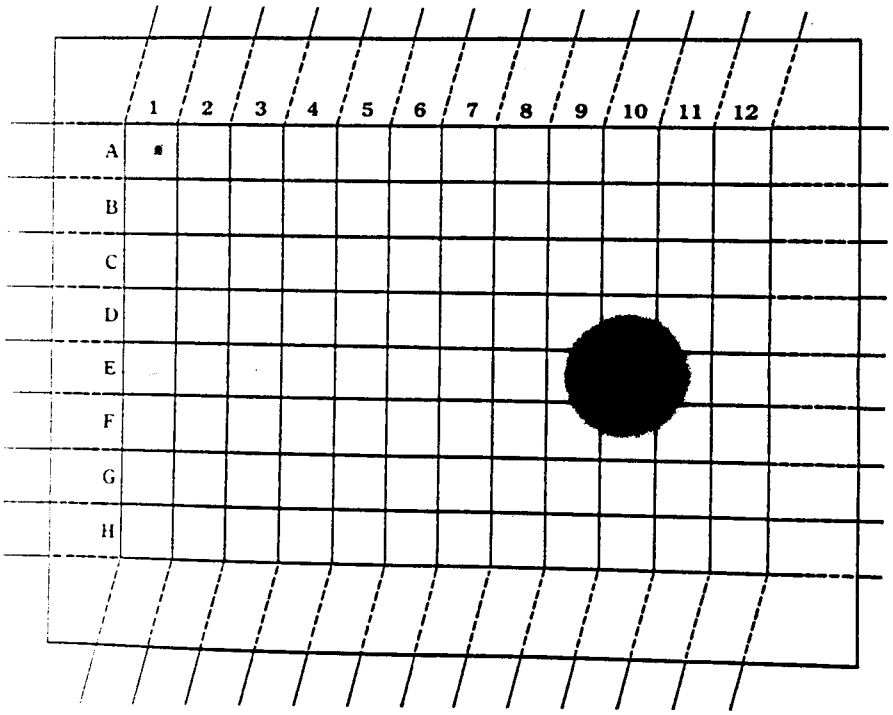


Figure 3.7. Detection of phage LLPP5 prophage DNA in lysogenized host strain, LPL5.

Row :

A : LPL5 purified DNA spotted in decreasing concentrations from 100 ug in column 1 to 0.01 ug in column 10.
B : Sensitive host strain, LLPA10 purified DNA spotted in concentrations identical to those in Row A.
C : Phage LLPP5 purified DNA spotted from 1 ug in column 1, to 0.1 ug in column 4. 10 ug was spotted in column 10.

Purified DNA solutions were immobilized on a Nylon membrane and probed with radiolabeled phage LLPP5 DNA. Panel shows autoradiograph of signals from hybridized molecules.

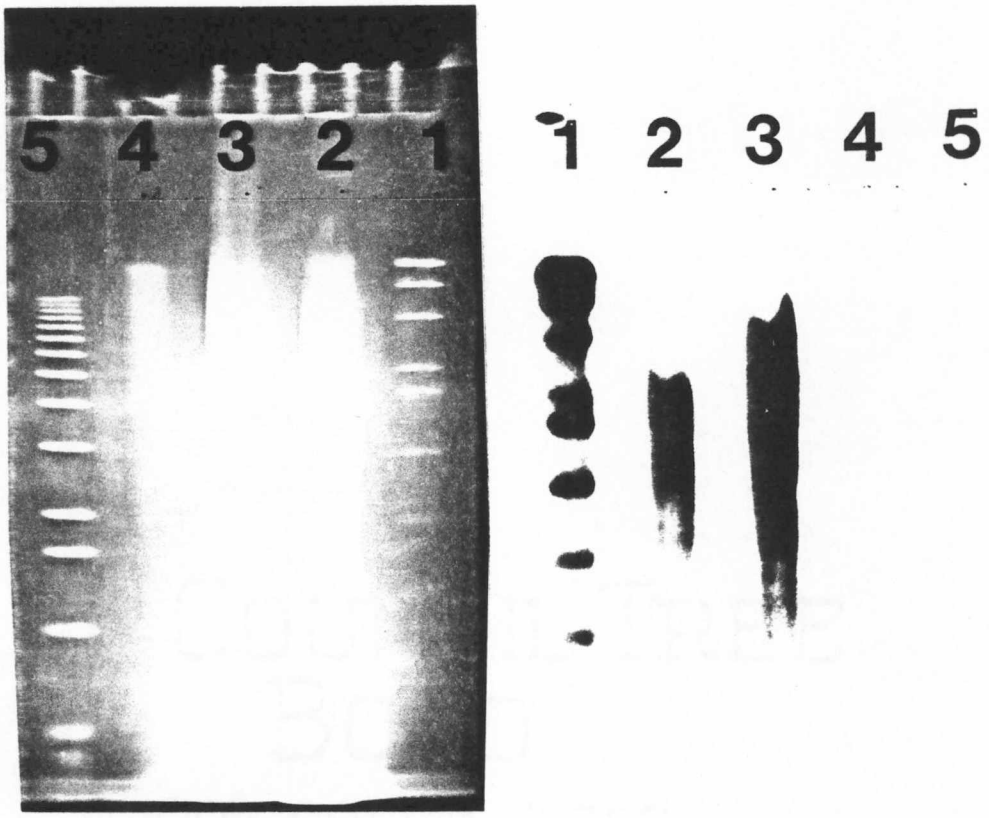


Figure 3.8. Detection of Phage LLPP5 DNA in lysogenized host strain, LPL5.

Lane :

1 : LLPP5 by Sal 1
2 : LPL5 by Sal 1 (100 ug DNA)
3 : LPL5 by Sal 1 (50 ug DNA)
4 : LPE by Sal 1 (1 ug DNA)
5 : One kilobase size marker (BRL).

Endonuclease Sal 1 generated fragments of phage and chromosomal DNA were separated on 0.8 % agarose gel, run at 75 volts for 5 hours (left panel). Gel-separated DNA fragments were electro-transfered onto a Nylon membrane (Biotrans) and probed with ^{32}P-labeled LLPP5 DNA. Right panel shows autoradiograph of signals from hybridized DNA molecules.

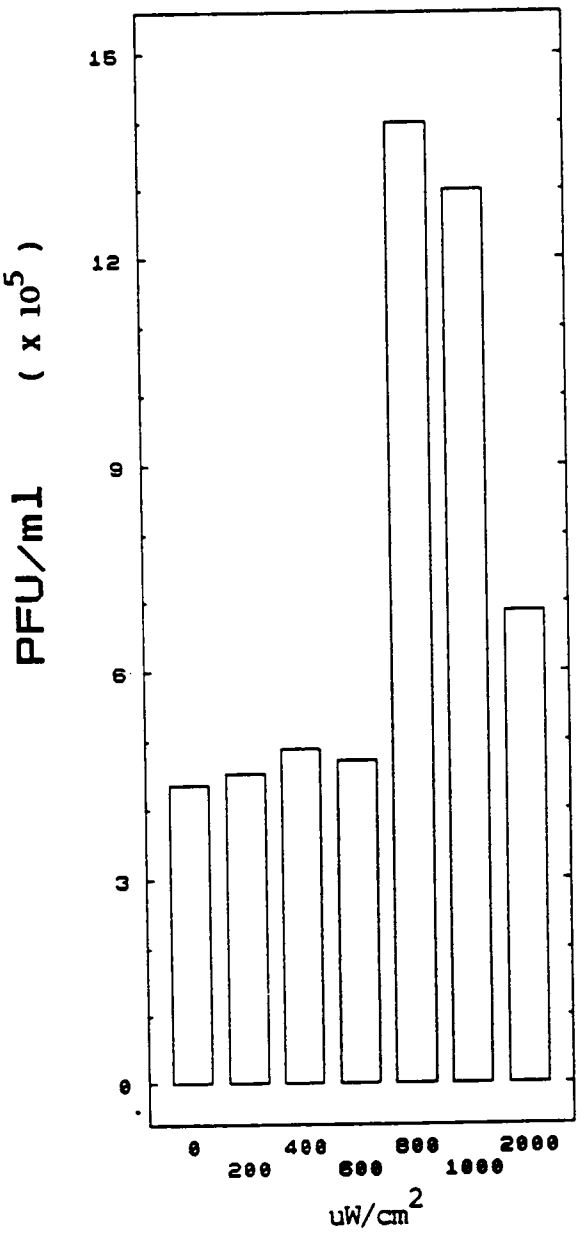


Figure 3.9. Effect of ultraviolet radiation on phage production by *P. aeruginosa* LPL5 culture. Plaque forming units were titered on lawns of *P. aeruginosa* LLPA10.

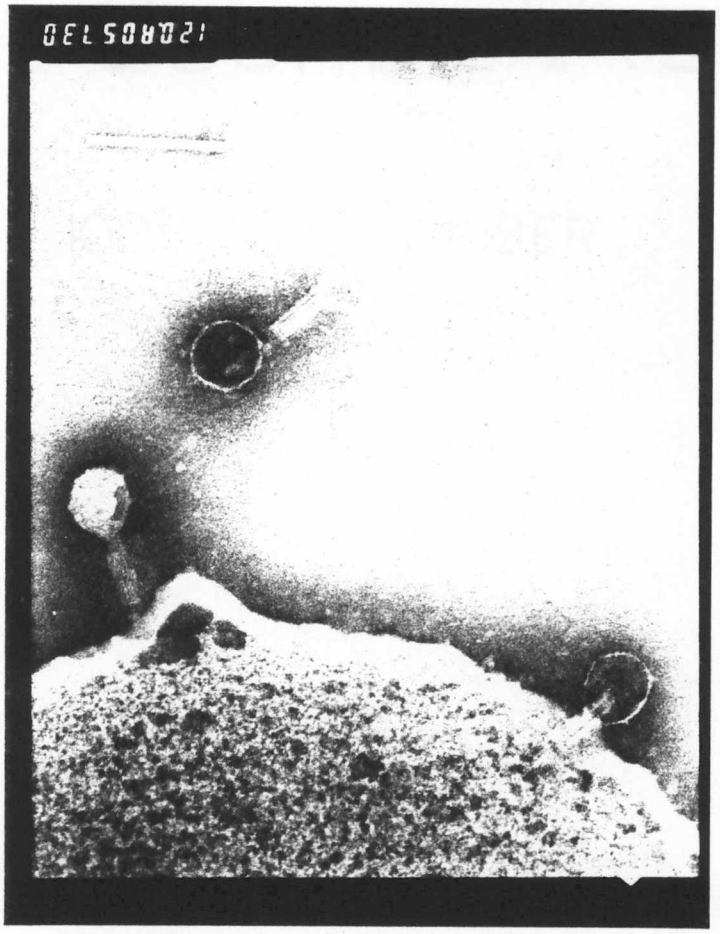


Figure 3.10. Adsorption of phage LLPP5 to *P. aeruginosa* LLPA10 cells.

Adsorption of phage was allowed to occur for five minutes. Visualization was by negative staining with phosphotungstate. Bar represents 100 nm.

DISCUSSION.

The present investigation was undertaken in an attempt to identify and characterize as much as possible, the nature of *Pseudomonas aeruginosa* strain LLPA10 and its specific bacteriophage strain LLPP5, both isolated from a freshwater environment. This preliminary characterization is deemed necessary partly because of the ubiquitous distribution of the host strain, and because of the wide variety of *P. aeruginosa* phages that have been previously characterized. Information on the molecular attributes of this phage-host couple is also essential for a correct interpretation of all kinds of interactive behavior between the bacteria and phage, such as population variability (Chapter IV) and genetic exchange (Chapter V), that are possible in lakewater.

From all indications, salient characteristics *P. aeruginosa* LLPA10 are typical for the species. To what extent the observed features are influenced by the *in vitro* nature of investigation remains an unresolved ecological issue in studies of this kind. At the present state of knowledge, the true nature of this organism *in situ* can only be inferred from its behavior under laboratory conditions.

Because all bacteriophages are obligately parasitic and the kinetics of adsoption to host cells is rapid and highly specific, any phage that can be isolated as a particulate from a habitat such as lakewater is not likely to be a transient occurrence. The observed characterisitcs of bacteriophage LLPP5 grown on a host indigenous to its habitat should therefore be representative of the *in situ* characteristics. The morphological and genetic characteristics of phage LLPP5 does not diverge appreciably from some previously characterized *P. aeruginosa* phages. The resemblance of LLPP5 to phage E79 that was originally isolated and described by Holloway *et al.* (1960) is apparent (Figures 3.3, 3.4, and 3.5). While E79 is characterized by an obligately virulent nature, bringing about a marked reduction of bacterial growth at almost any phase of the growth cycle (Holloway *et al.*, 1960), phage LLPP5 readily enters a prophage association with a substantial fraction of the host population. Similar E79-resembling, but less virulent phage were reported by Feary *et al.* (1963), and Bradelyand Robertson (1968). It is not unlikely that these phages have diverged by means of simple mutations involving additions and deletions to the 'original' genome. The spatial and temporal scales of such divergence can only be estimated by future detailed investigation of DNA sequence homology that exist between these strains.

The lysogenic status of hosts carrying prophage genome corresponding to phage LLPP5 warrants further investigation. The classical

criteria for lysogeny, namely, immunity of carrier cells to infection by phage identical to the carried strain (Lwoff, 1953 ; Jacob and Wollman, 1959) is met by the LLPP5-Host system. A currently held criterium for true lysogeny requires the demonstration of integration of the phage genome into the host chromosome (or the closely associated bacterial membrane replicatory system) (Barksdale and Arden, 1974). The implications of the observation that phage LLPP5 DNA sequences are represented at no particularly discrete location of the host carrier genome needs further clarification.

Transposon-like phages (Krylov *et al.*, 1984) and phages that carry transposon sequences shared by the host chromosome (e.g. phage D3, as reported by Gertman et al., 1986) have been reported for *P. aeruginosa*. If a phage genome may integrate into the host chromosome at multiple sites, the kind of result shown in Figure 3.8 would be observed. However, in each of the previously characterized cases, not more than one site of integration was inferred by DNA hybridization results (Krylov *et al.*, 1984). To further investigate and clarify the result shown in Figure 3.8, it will be necessary to isolate individual restriction fragments of the phage DNA. These separate fragments may then be used to 'walk' the host chromosome in an attempt to delimit the possible sites of integration of the phage genome into the bacterial genome.

Summary of Observations.

1. Based on the variety of tests performed, *Pseudomonas aeruginosa* LLPA10 isolated from Ft. Loudon lakewater is a typical strain of the species.

2. The *P. aeruginosa*-specific bacteriophage LLPP5 isolated as a particulate from Ft. Loudon lakewater has typical morphological features of members of the phage family *Myoviridae*.

3. Phage LLPP5 is related to previously characterized phage E79, and its genome is estimated to be 78 +/- 5 kb in size.

4. Phage LLPP5 shows the ability to lysogenize susceptible bacterial hosts.

5. Based on molecular hybridization studies, the LLPP5 prophage appears to be integrated into the lysogenized host chromosome.

CHAPTER IV

DYNAMICS OF INTERACTION BETWEEN PHAGE AND *Pseudomonas aeruginosa* IN FRESHWATER

INTRODUCTION.

Two important factors regarding the fate of industrial and environmentally applied bacteria are survival under environmental stress and the potential for genetic modification and exchange (Liang *et al.*, 1982 ; Rissler, 1984). The occurrence and activities of bacteriophage in ecosystems where bacteria proliferate may directly affect the outcome of those two fatal events. The growth of obligately virulent phage threatens the survival and effectiveness of any introduced bacteria while temperate phage may precipitate lysogenic conversion (Bartell and Orr, 1969 a ; Freeman, 1952 ; Uetake *et al.*, 1958) or genetic transduction (Saye *et al.*, 1987). One approach

to reduce or eliminate the effects of bacteriophage interaction with industrial organisms is to select or engineer phage resistance (Limsowtin and Terzaghi, 1976 ; Marshall and Berridge, 1976 ; Hershberger and Rosteck,1985 ; Sanders, 1987). This approach may be limited to closed systems. In complex, poorly characterized environments, it may be more reliable to better understand phage-host interactions with the aim of influencing the association towards desirable equilibrium *in situ*.

Prominent features of the apparent coexistence of bacteria and phage distinguish their interactions from established patterns of more studied predator-prey associations found among eukaryotes. Such features have been the subject of many theoretical and a few laboratory investigations (Campbell, 1961 ; Horne, 1970 ; Stewart and Levin, 1984 ; Lenski and Levin, 1985 ; Noack, 1986). The relevance of the predictions based on those studies rely heavily on the assumption of continous cell proliferation under homogenous conditions. Several parameters of natural freshwater habitats may directly affect the population and activities of bacteriophage. These include the metabolic state of both host and non-host bacteria, predation, unproductive infections, adsorbtion to particulates, and other physical-chemical factors like temperature, pH, and light. Few investigations have dealt with the establishment of equilibrium and the threshold population of bacteria susceptibility to phage (Anderson, 1957 ; Wiggins and Alexander,

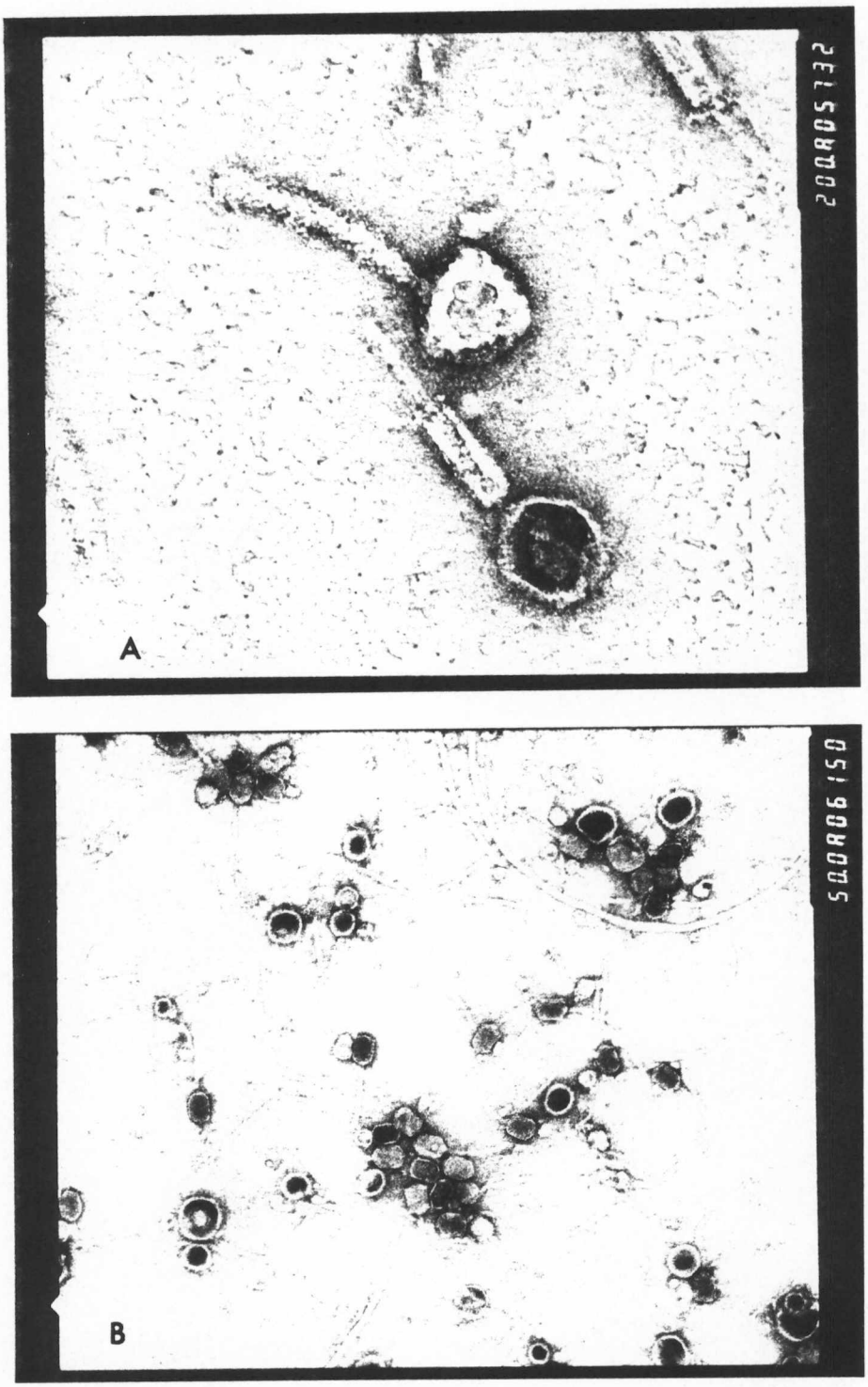
1985). Although the potential significance of lysogeny is often acknowledged, experimental data to characterize the phenomenon in natural environments is generally lacking. Bacteriophage temperance is held to be advantageous over phage virulence because lysogenized bacteria tend to survive better in oligotrophic environments (Stewart and Levin, 1984). Temperance may be an adaptation of phage for survival when host populations fluctuate unfavorably due to other factors prevalent in nutrient poor habitats (Shilo, 1979). This chapter examines some factors that may affect the maintenance of bacteriophage in freshwater, and the stability of interaction between phage and host populations. These factors are considered in the context of evolution of carrier state and phage resistant phenotypes in the host population.

MATERIALS AND METHODS.

One of the major limitations of the microcosm approach to phage ecology studies is the presence of unquantifiable bias traceable to incompatibility of test organisms and habitat. This limitation is eliminated in the present study by the use of bacteria and viruses that were recently isolated from lakewater in which they were re-introduced for subsequent experiments.

The strains of phage and bacteria used are shown in Figure 4.1. Phage LLPP5 (Figure 4.1a) was isolated by routine concentration of lakewater on an hydroxyapatite slurry (Primrose and Day, 1977). Phage LLPP5 belongs to the family *Myoviridae*, according to the specifications of the International Committee on the Taxonomy of Viruses (ICTV) (Brown, 1986). It forms turbid margin plaques on *P. aeruginosa* PAO and susceptible lakewater strains. Phage LLPP5 is not sex-specific. A heterogenous phage population (Figure 4.1b) consisting of at least four morphologically distinct particles was isolated from the same site by enrichment of 200 ml aliquots of freshwater with *P. aeruginosa* LLPA10 (shown infected in Figure 4.1e). The enrichment procedure was carried out as described by Grimont *et al.* (1978). Clarified phage lysates were purified by glycerol gradient ultracentrifugation (Silhavy *et al.*, 1984). For microcosm studies and DNA isolation, purified phage were routinely resuspended and diluted in SM buffer (Maniatis *et al.*, 1982). Protocols for the isolation, purification, and radiolabelling of phage DNA are detailed in Chapter II.

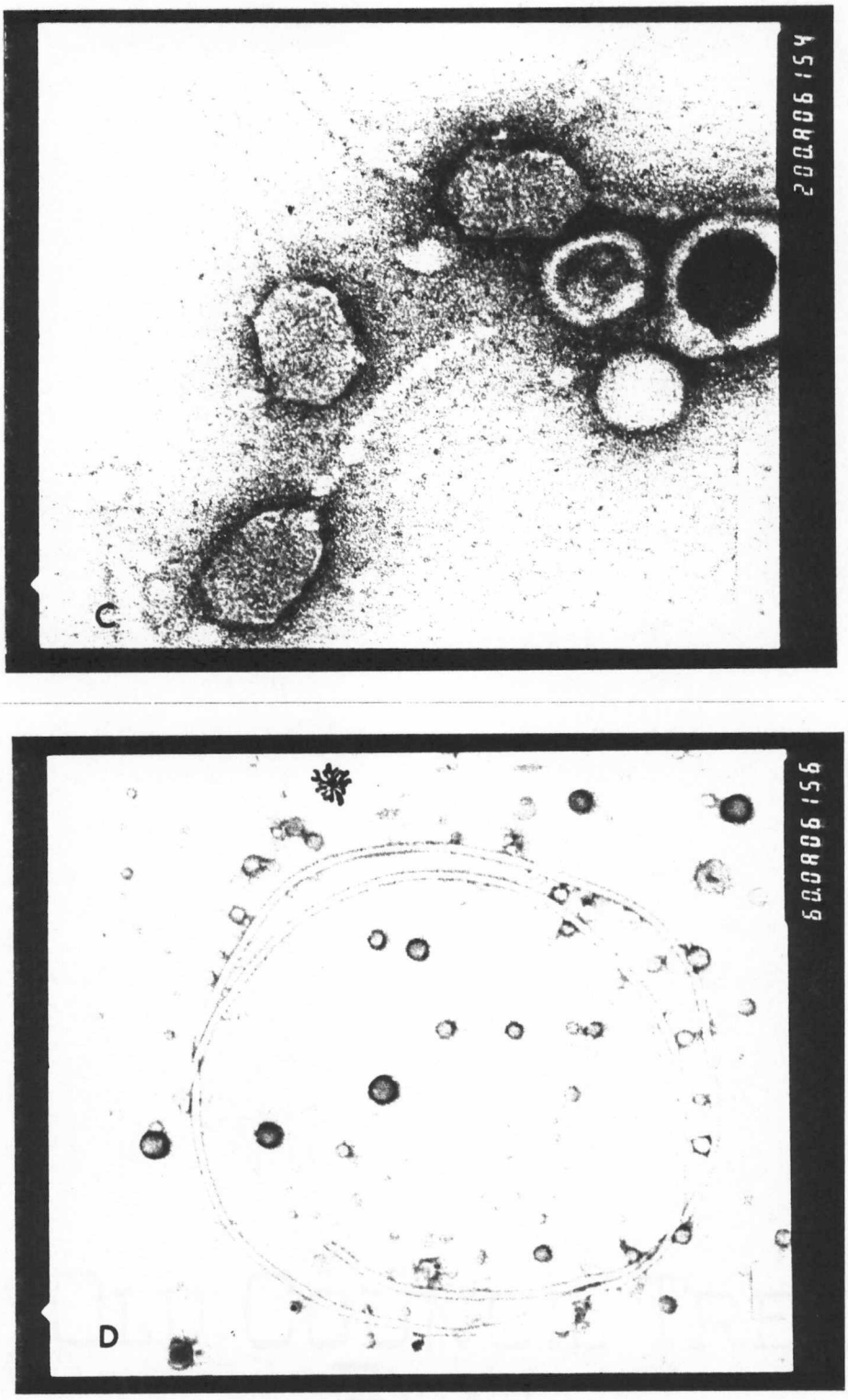
Pseudomonas aeruginosa strains were isolated from lakewater by means of selective media (Grant and Holt, 1977 ; Gould *et al*, 1985, and *Pseudomonas* Isolation Agar (PIA) ,Difco). Isolated pure colonies were subjected to taxonomic tests and susceptibility to previously characterized *P. aeruginosa*-specific bacteriophages. Strain LLPA10 was chosen for the



(A) Phage LLPP5
(B) Heterogenous phage population, LPE

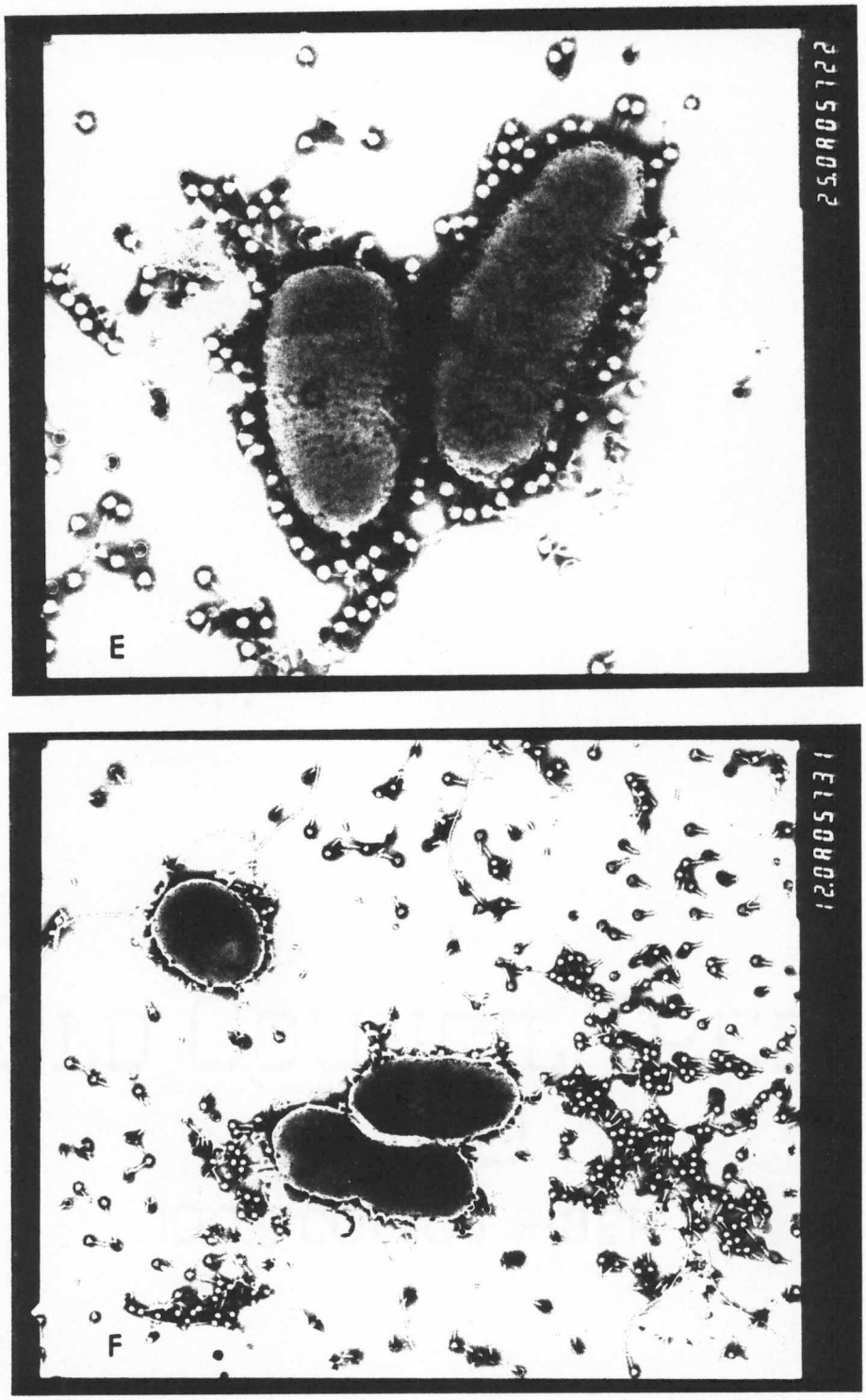
Figure 4.1. Organisms used in this study.

Visualization was by negative staining with phophotungstate. Bars represent 100 nm.



(C) Prominent λ-type phage from LPE
(D) Prominent f-2 type phage from LPE

Figure 4.1 (Continued)



(E) Phage-sensitive *P. aeruginosa* LLPA10 infected with phage LLPP5 at a multiplicity of infection of approximately 200.

(F) Phage-resistant P. aeruginosa LPL5 infected with phage LLPP5 at a multiplicity of infection of approximately 200.

Figure 4.1. (Continued)

present study because it gave the most reproducible response to phage infection and it consistently produced a characteristic blue-green pigment when cultivated at 37 - 42 ^{o}C. Spontaneous Nalidixic acid resistant mutants (up to 1,000 ug $ml^{-1)}$ of this strain were isolated on Luria-Bertani (LB) gradient plates amended with 500 ug ml^{-1} Nalidixic acid. This antibiotic was routinely used for selecting strain LLPA10 from the wild type population. Phage LLPP5 resistant colonies were isolated from confluently lysed lawns or by continous incubation of lysed broth cultures which later became turbid with growth. Phage resistant colonies that contain prophage were identified by DNA hybridization to phage genome and by phage production in media containing 1 ug ml^{-1} mitomycin C. All colonies that were resistant to phage LLPP5 (With or without prophage) were susceptible to lysis by the heterogenous phage population (LPE), which produced clear plaques on all *P. aeruginosa* strains tested.

To study bacteriophage persistence in lakewater, duplicate100 ml microcosms of autoclaved, filtered, autoclaved and filtered , or natural lakewater were set up in sterile 250 ml polyvinyl carbonate flasks. The flasks were inoculated with phage LLPP5 or the heterogenous phage population

(LPE) and incubated with occasional agitation at 22°C in an Envirotrol chamber (Labline). Two 1.0 ml samples were taken from each microcosm at 0, 6, 24, 48, 72, and 96 hours after inoculation. The time point samples were serially diluted in SM buffer and plaque forming units (pfu) were titered on *P. aeruginosa* LLPA10 using the soft agar overlay method (Silhavy *et al.*, 1984). Total aerobic heterotrophic bacteria and pseudomonads were enumerated by plating aliquots diluted on phosphate buffered saline (PBS) on Yeast Extract Peptone Glucose (YEPG) agar and PIA respectively.

To study the interaction between bacteriophage, host bacteria, and the natural microbial population, duplicate 100 ml microcosms were set up Table 4.1. At 0, 12, 24, 48, 72, and 96 h and 45 days after inoculation, 3.0 ml samples were taken from each microcosm and passed through a 0.45 um pore size acrodisc filter (Gelman). The filtrates were used for pfu enumeration on *P. aeruginosa* LLPA10 lawns. Other 1.0 ml samples were taken at each time point for estimation of bacterial colony forming units (cfu) on YEPG, PIA, and LB agar plates. LB and PIA plates were incubated at 37 °C for 24 h while YEPG plates were incubated at 22 °C for 10 days. All plates with LB and PIA plates containing distinct colonies were replica lifted onto Nylon membranes (Biotrans). The filters were then probed for hybridization to phage LLPP5 DNA. Colonies that probed positive were restreaked and inoculated into 3.0 ml LB broth amended with 1 ug ml^{-1} mitomycin C to check for phage

Table 4.1. Bacterial and Bacteriophage Content of Microcosms.

Microcosm #	Bacteria[a]	Phage[b]	Lakewater[c]
1	LLPA10	-	Sterile
2	LLPA10	-	Natural
3	LPL5	-	Sterile
4	LPL5	-	Natural
5	LLPA10 + LPL5	-	Sterile
6	LLPA10 + LPL5	-	Natural
7	LLPA10	LPE	Sterile
8	LLPA10	LPE	Natural
9	LLPA10	LLPP5	Sterile
10	LLPA10	LLPP5	Natural
11	LPL5	LPE	Sterile
12	LPL5	LPE	Natural
13	LPL5	LLPP5	Sterile
14	LPL5	LLPP5	Natural
15	LLPA10 + LPL5	LPE	Sterile
16	LLPA10 + LPL5	LPE	Natural
17	LLPA10 + LPL5	LLPP5	Sterile
18	LLPA10 + LPL5	LLPP5	Natural
19	-	-	Sterile
20	-	-	Natural

[a]Bacteria were inoculated at approximately 2×10^5 cfu ml^{-1}.
[b]Phage LLPP5 was inoculated at an initial multiplicity of infection of 0.1 while LPE was inoculated at an inital MOI of 10.
[c]Sterile lakewater was produced by autoclaving.

production. Phage decay rates (K) in lakewater were calculated as :

$$K = [\ln C_o / C_t] / t$$

where C_o is the concentration of phage at time zero and C_t is the recovered concentration at t hours. Half life of phage ($t_{1/2}$) is equated to 0.693 / K.

RESULTS.

The heterogenous phage population (Figure 4.1b) contains several phage types, including a prominent B2 type (Figure 4.1c) and a filamentous, F1 type (Figure 4.1d). Figure 4.1e depicts the infection of *P. aeruginosa* LLPA10 with phage LLPP5 at a ultiplicity of infection (MOI) of approximately 200 in SM buffer, incubated for 5 minutes. Figure 1f shows phage LLPP5 resistant *P. aeruginosa* LPL5 under similar conditions as in Figure 4.1e. There is an apparent inhibition of adsorbtion, resulting in a phage-free halo around the cells, presumable due to a slime layer production induced by the lysogenic state of the cells.

The relative persistence of phage LLPP5 in lakewater is illustriated in Figures 4.2 and 4.3. Decay rates of phage LLPP5 were significantly higher in natural lakewater than in either autoclaved or filtered lakewater. Linear regression analysis of the temporal recovery patterns of pfu confirmed that natural lakewater significantly reduced the population of detectable phage. The half life of phage LLPP5 in natural lakewater was 2.63 h compared to the values in autoclaved ($t_{1/2}$ = 6.33 h) ; autoclaved and filtered ($t_{1/2}$ = 5.55 h) , and filtered lakewater ($t_{1/2}$ = 26.97 h), within the first six hours of inoculation. Although bacteriophage introduced into filtered lakewater consistently suffered less decay than in autoclaved or natural lakewater, this observation was not statistically significant over a period of 96 hours. The heterogenous phage population suffered less decay in all the media tested (Figures 4.4). In some instances, more pfu were detected with prolonged incubation. This observation is not easily clarified and may only be explained as a temporary, non uniform response of different phage types to host recognition during titration assays. This explanation is partially supported by the delayed effect LPE has on the growth kinetics of host strain under optimum conditions as shown in Figures 4.5 and 4.6. Whereas it takes phage LLPP5 only 2 hours to produce apparent lysis in a growing culture of *P. aeruginosa* LLPA10 infected at an MOI of 1.0, the heterogenous phage population did not significantly affect the growth kinetics of strain LLPA10 prior

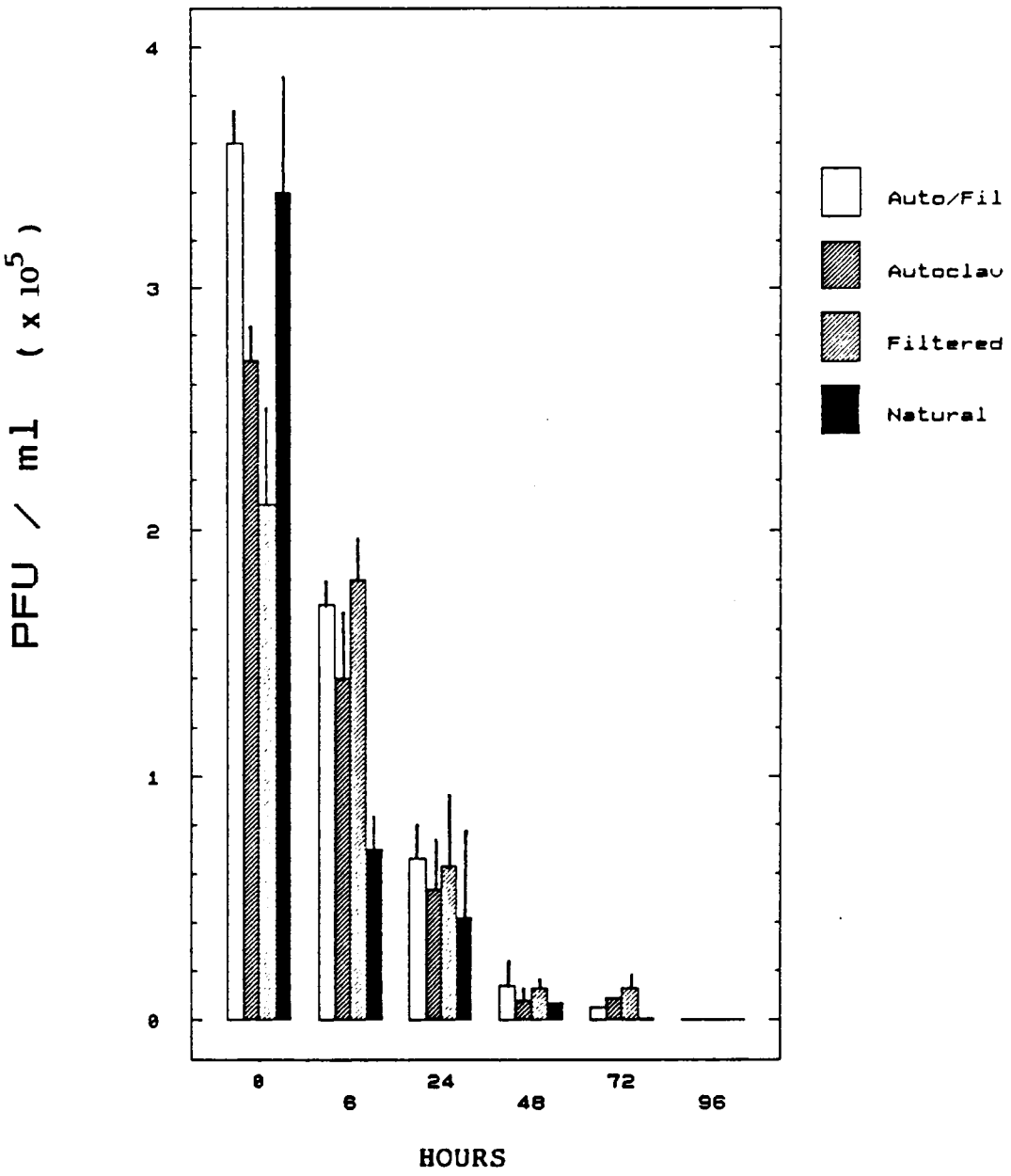


Figure 4.2. Survival of phage LLPP5 in lakewater microcosms. Phage was inoculated at an initial density of approximately 3 x 10^5 pfu ml^{-1}. Microcosms were incubated at 22 ^oC with occasional agitation. Auto / Fil = Autoclaved and Filtered lakewater.

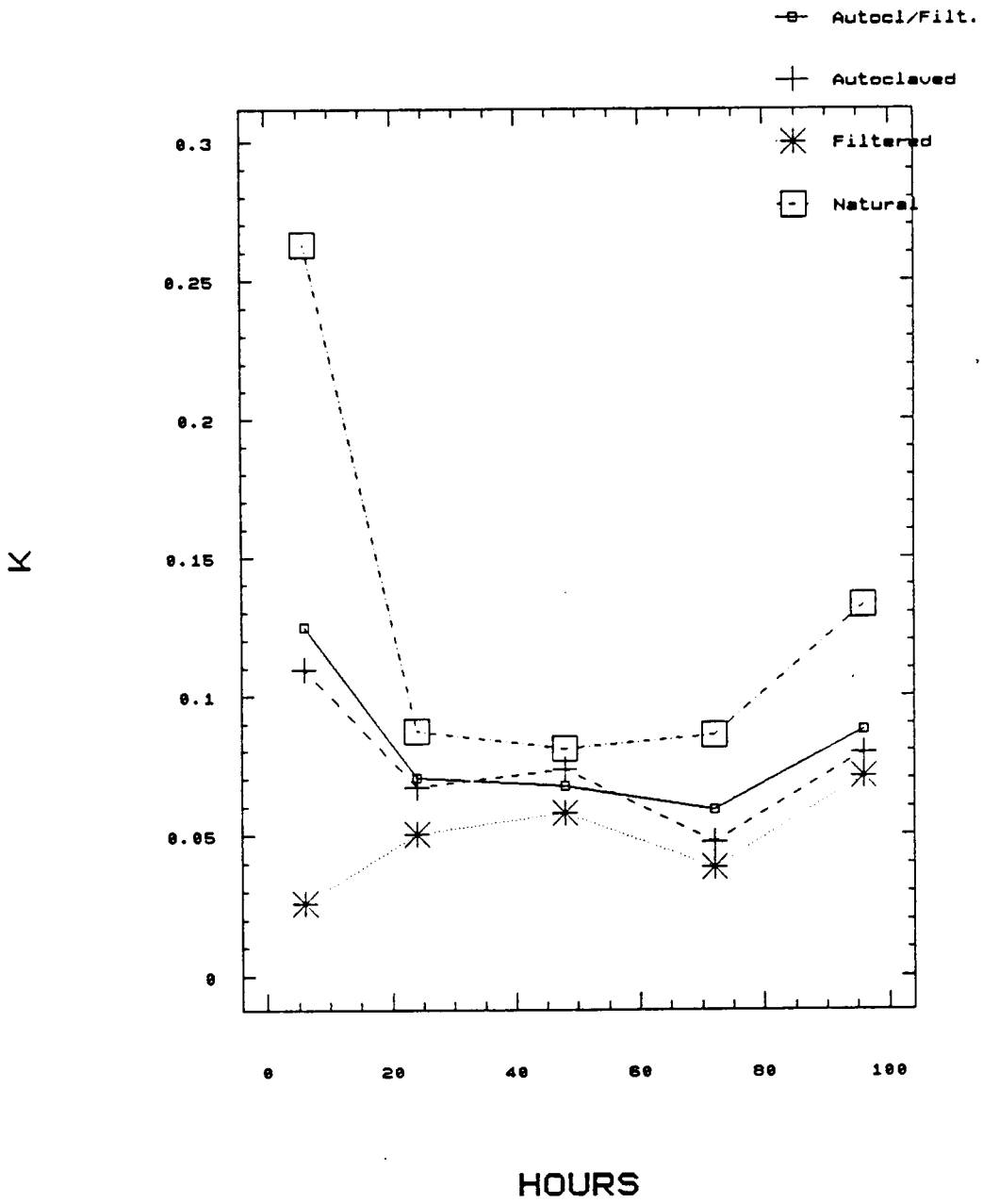


Figure 4.3. Decay rates of phage LLPP5 in lakewater microcosms. K values are calculated from data plotted in Figure 4.2.

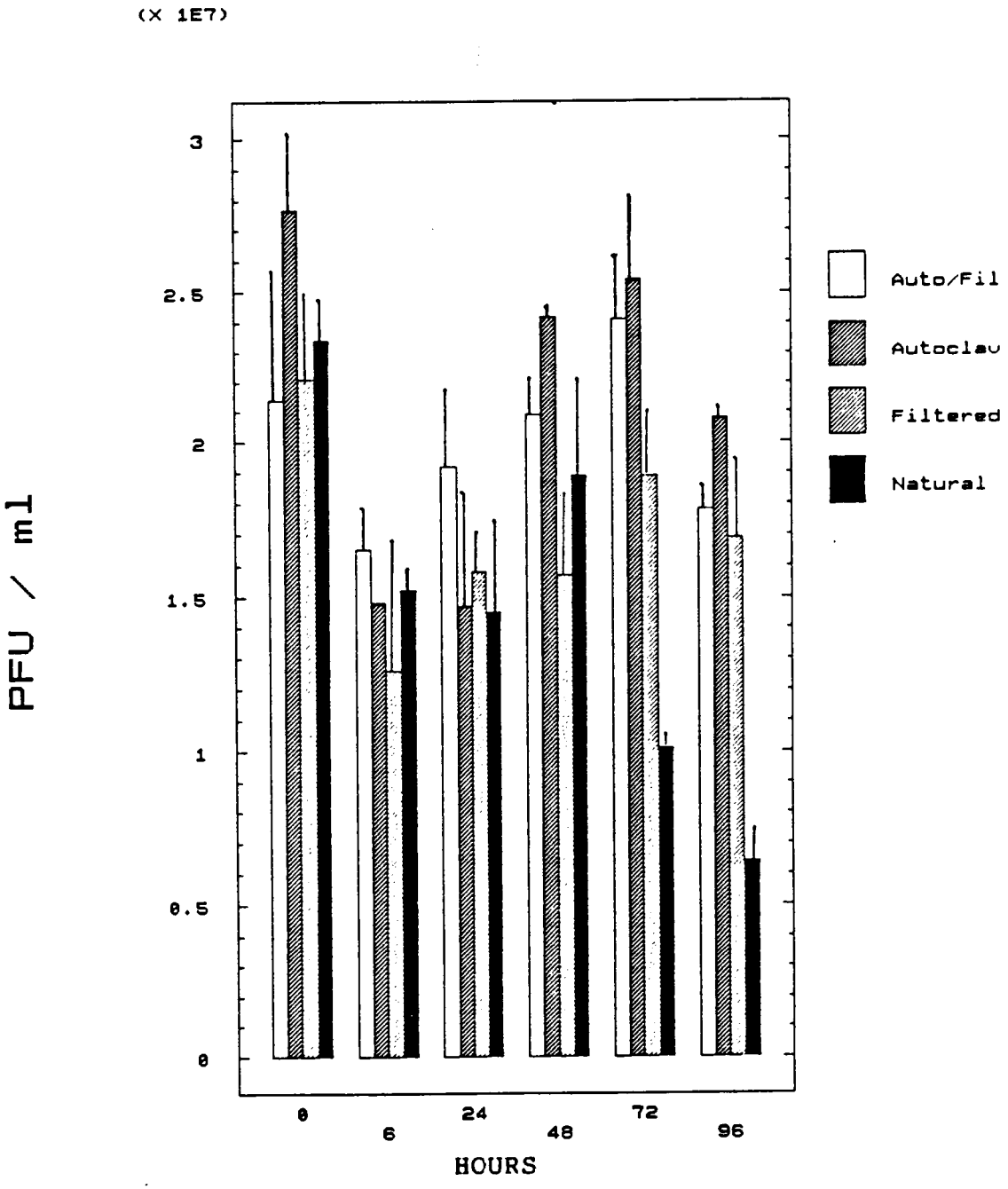


Figure 4.4. Survival of plaque forming units in heterogenous phage population (LPE) in lakewater microcosms. Phage were inoculated at an initial density of approximately 3 x 10^7 pfu ml^{-1} and incubated with occasional agitation at 22 ^oC. Auto / Fil = Autoclaved and Filtered lakewater.

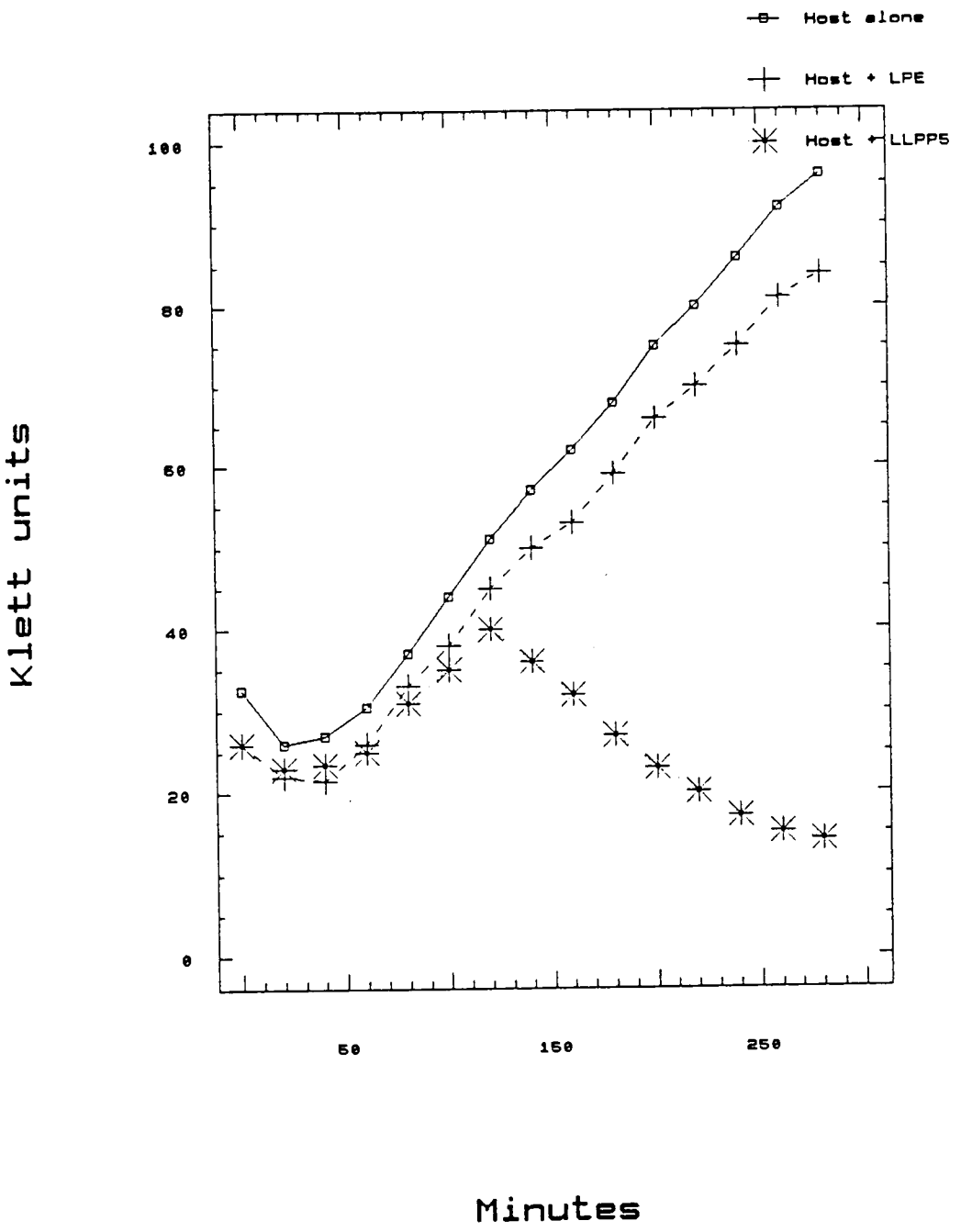


Figure 4.5. Effect of phage infection on the growth kinetics of *P. aeruginosa* LLPA10. Bacterial cultures were infected in Luria-Bertani broth at a multiplicity of infection of 1.0 for both LLPP5 and LPE. Flasks were incubated at 37 ^{o}C with constant shaking at 100 rpm.

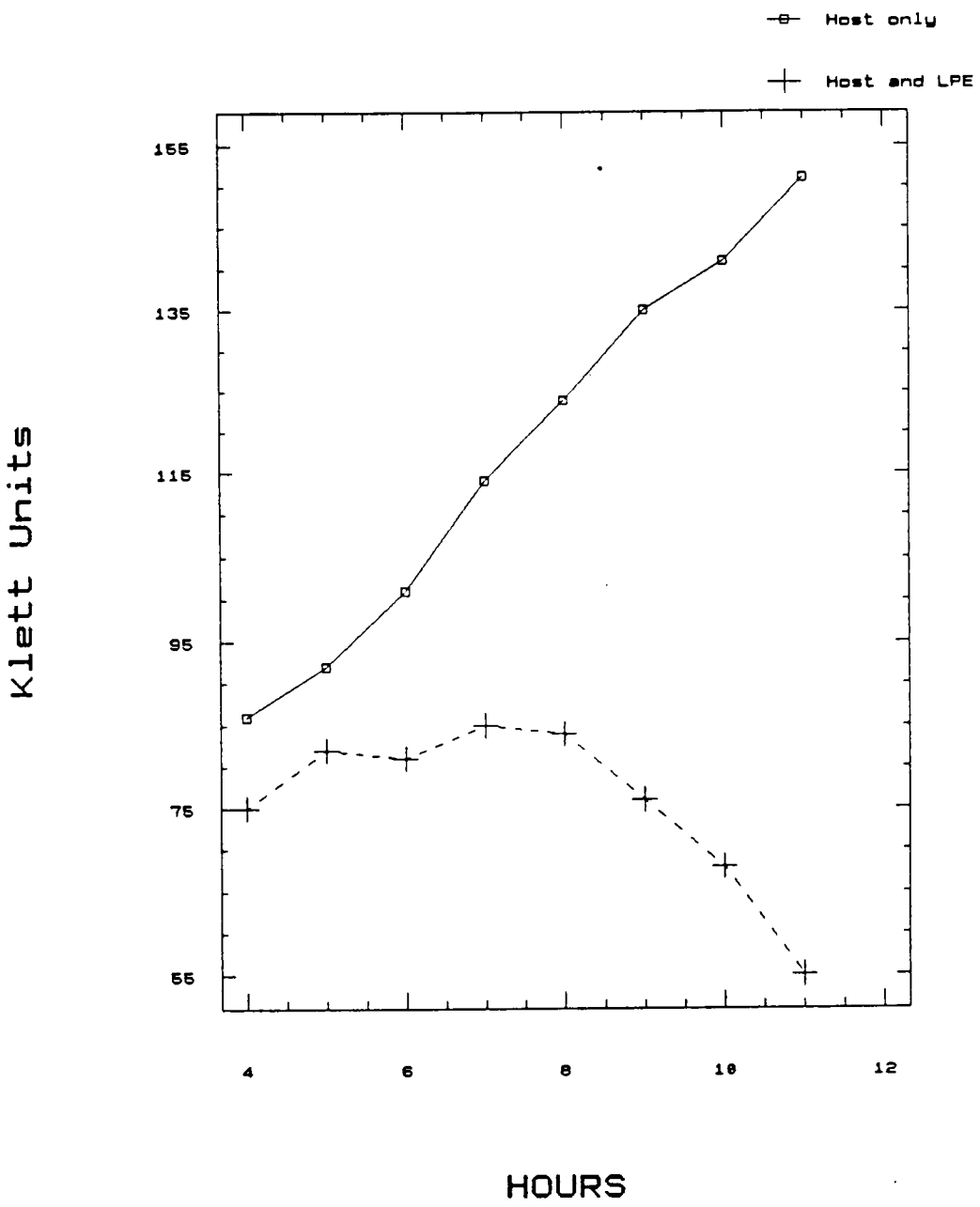


Figure 4.6. Effect of heterogenous phage population on growth kinetics of *P. aeruginosa* LLPA10. The data is from a continuation of the experiment recorded in Figure 4.5.

to 5 - 8 hours of incubation at the same MOI (Figure 4.6).

Figures 4.7 to 4.12 show the survival pattern of *Pseudomonas* introduced into lakewater, in the absence or presence of phage. The *Pseudomonas* population of the sample site usually ranged between 7.0 x 10^1 and 1.0 x 10^3 cfu per ml when assayed on PIA. However, transient populations may reach 10^4 to 10^5 cfu per ml especially after rainstorms. The embankment soil typically contains 2.0 x 10^4 to 1.0 x 10^5 pseudomonads per gram while the sediment harbors about 10^3 pseudomonads per ml. When *P. aeruginosa* LLPA10 was introduced into natural or sterile lakewater at an initial density of 2.0 x 10^5 cfu ml^{-1}, an initial population decline was observed within 12 hours of inoculation. Growth in autoclaved lakewater recovered and stabilized at 10^7 cfu per ml after 45 days. In natural lakewater, the initial recovery did not last beyond 48 h and was soon followed by a decline to 1.45 x 10^5 cfu ml^{-1} (Figure 4.7 and Table 4.2).

In the presence of phage LLPP5, the growth pattern of *P. aeruginosa* LLPA10 was affected (Figure 4.8). In natural lakewater net bacterial population was static at about the inoculation density for a minimum of 96 hours. In the sterile lakewater control, an initial sharp decline which corresponded to an increase in MOI. was observed. After 45 days, host bacterial population in the presence of phage LLPP5 stabilized at about 2 x 10^4 cfu per ml in both natural and sterile lakewater microcosms

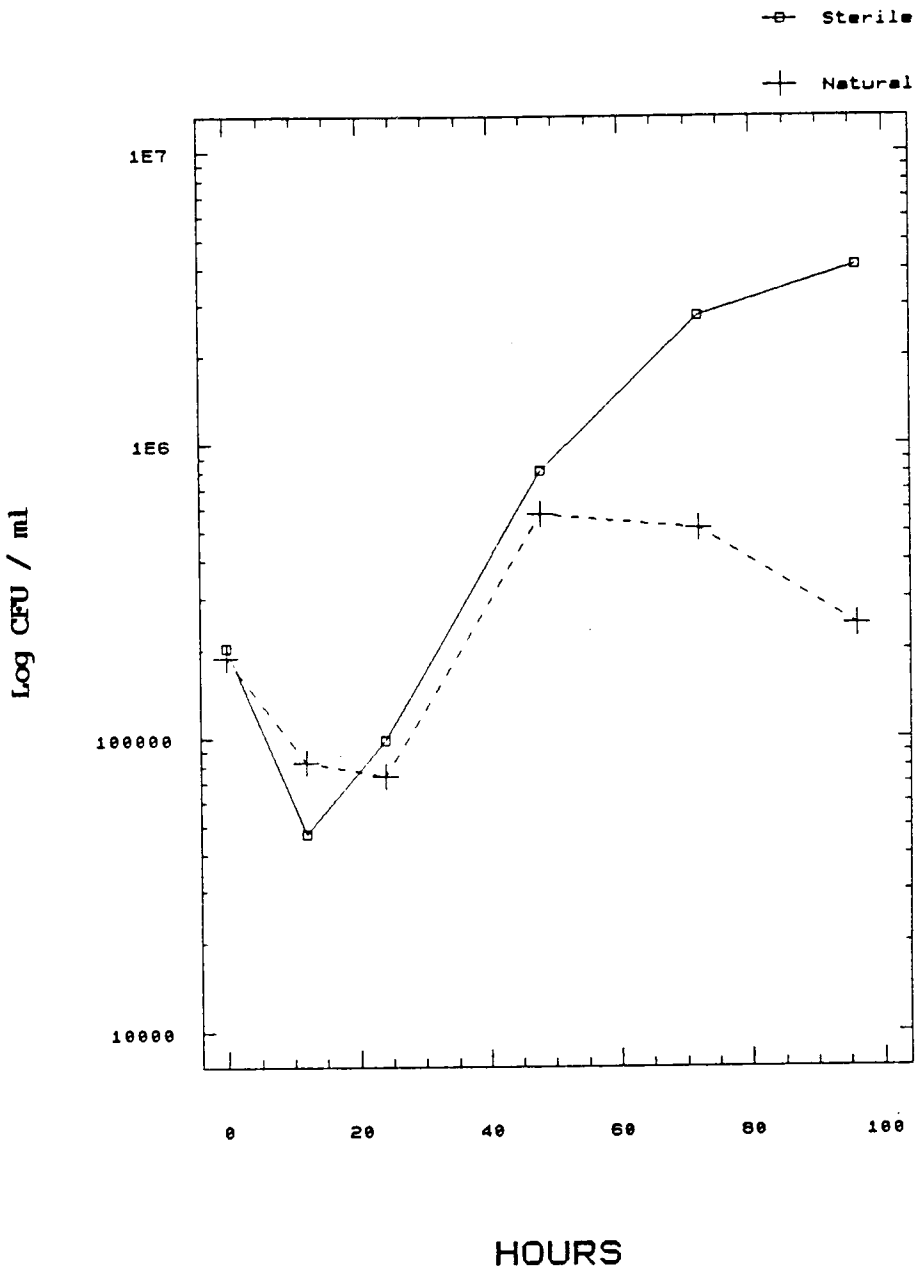


Figure 4.7. Survival of sensitive host strain LLPA10 in freshwater microcosms. See Table 4.1 and Materials and Methods section for experimental set up.

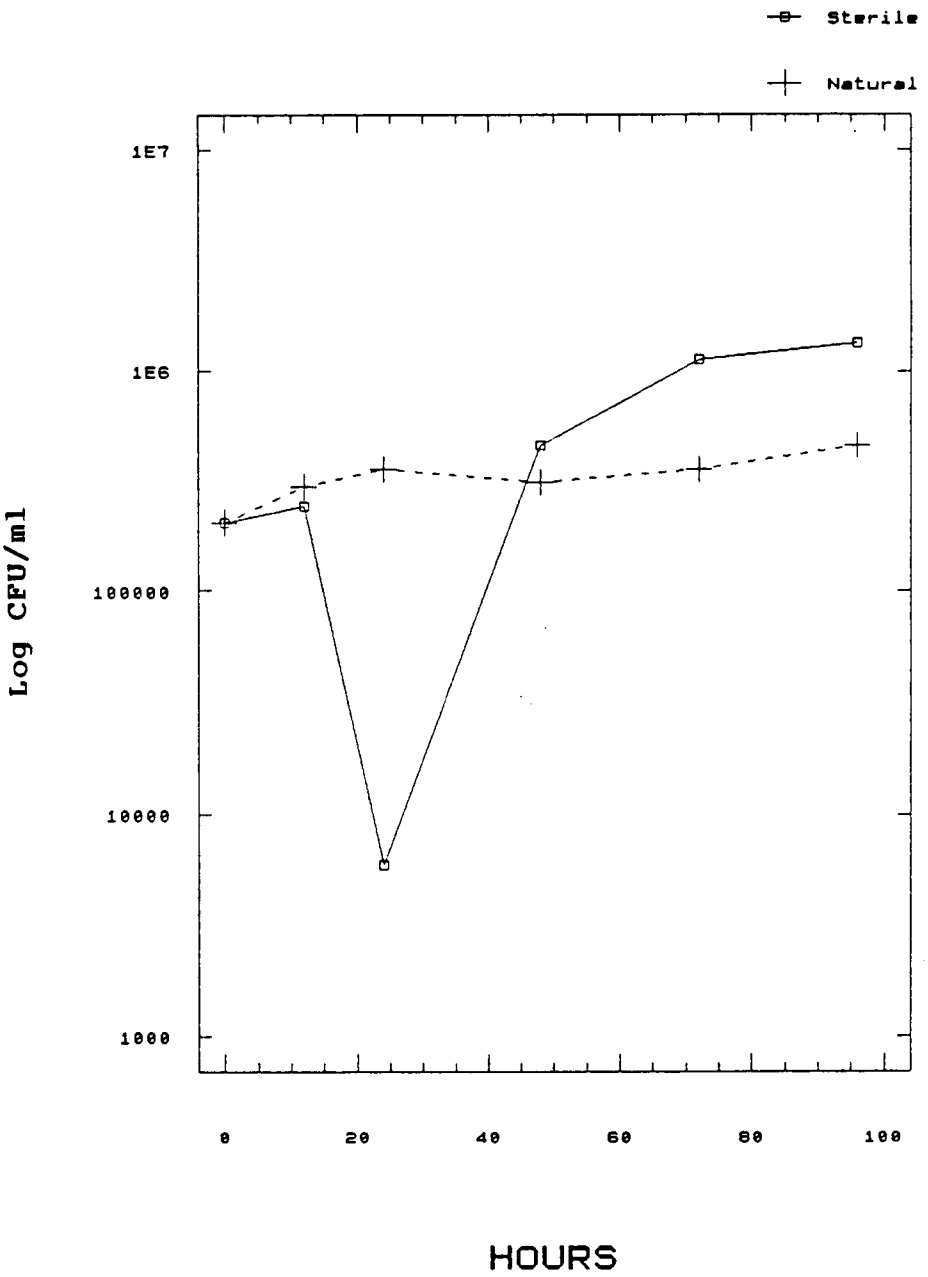


Figure 4.8. Survival of phage-sensitive strain LLPA10 in the presence of phage LLPP5.

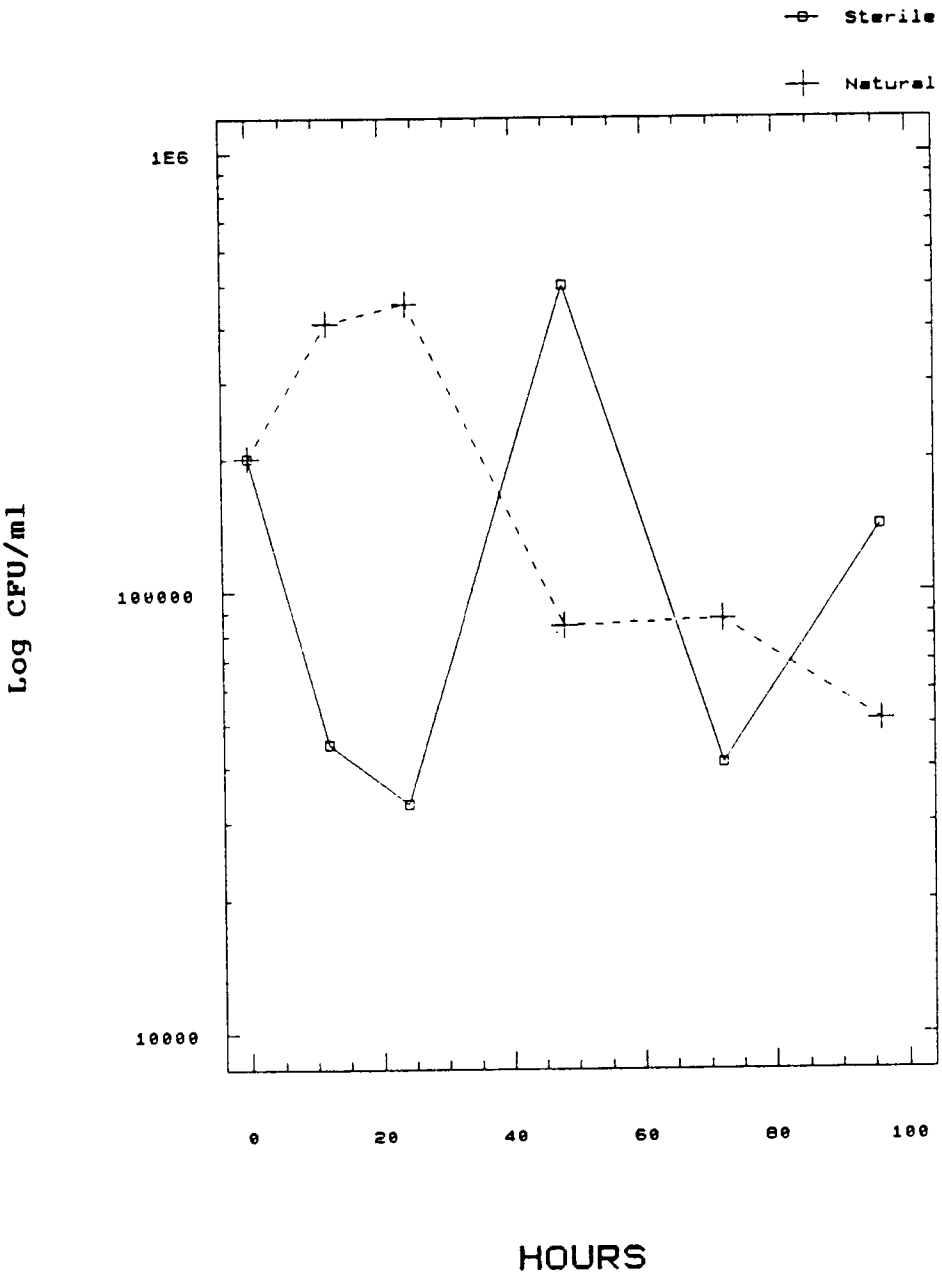


Figure 4.9. Survival of phage-sensitive strain LLPA10 in the presence of heterogenous phage population (LPE).

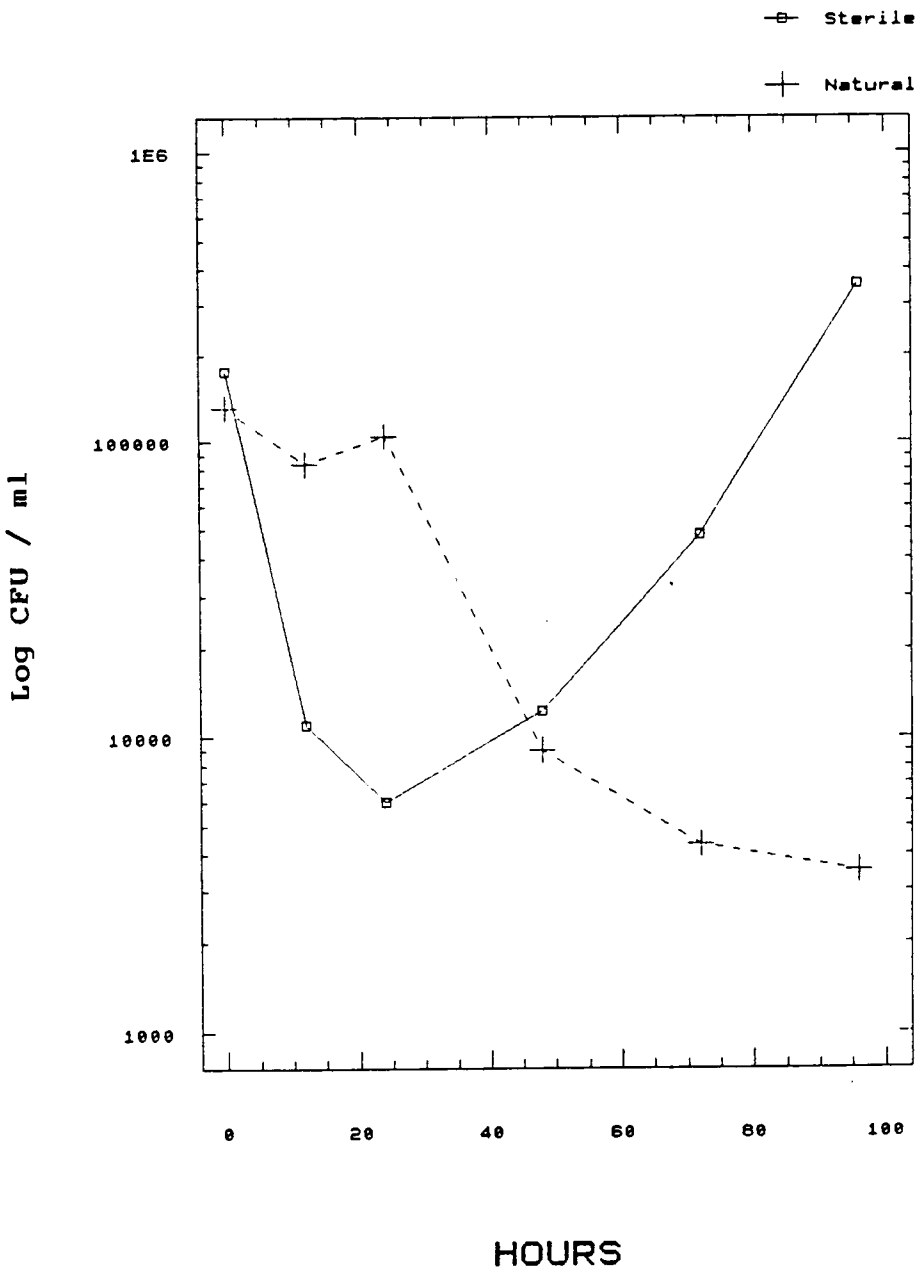


Figure 4.10. Survival of lysogenic strain LPL5 in freshwater microcosms.

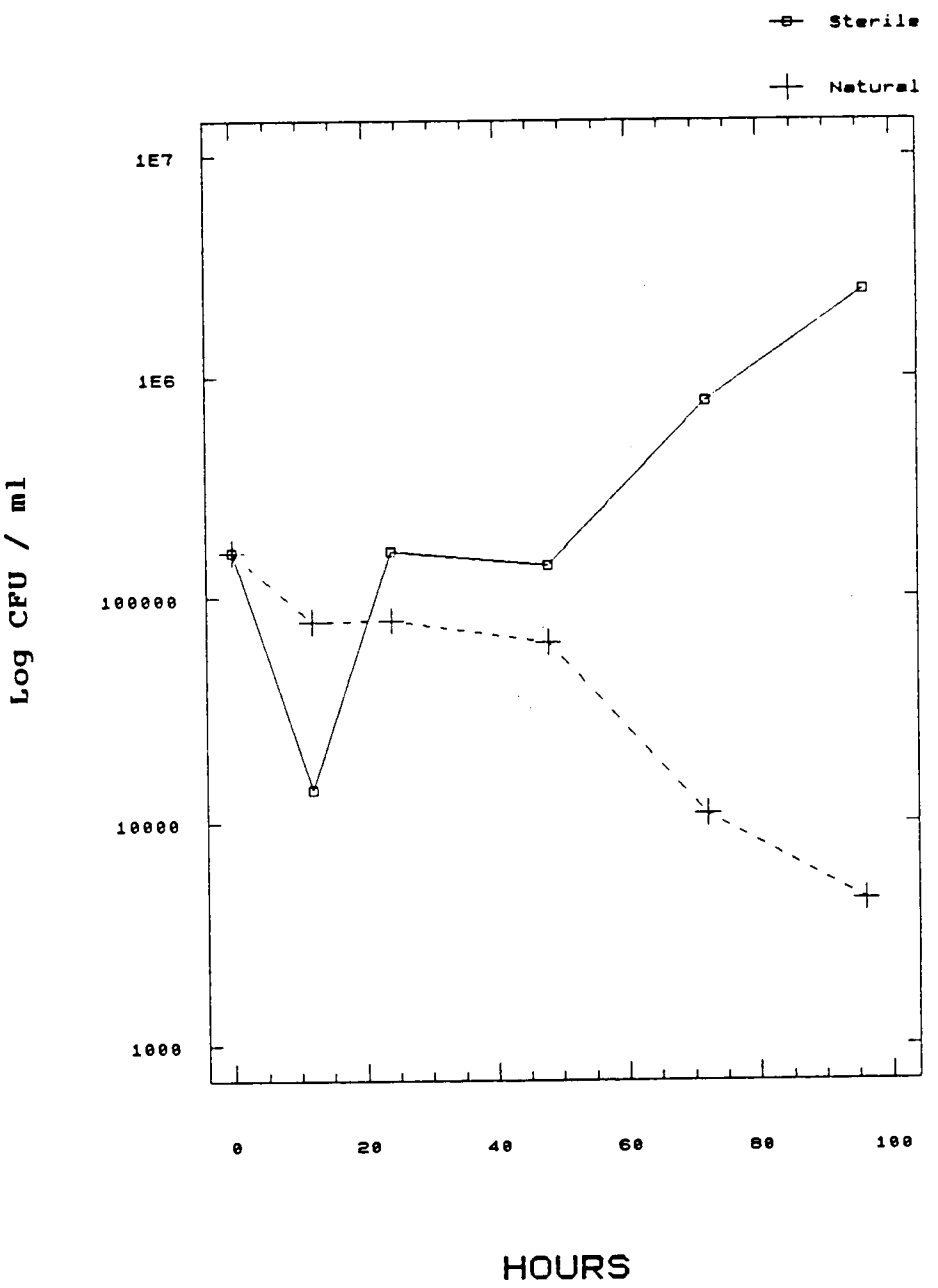


Figure 4.11. Survival of lysogenic host strain LPL5 in the presence of phage LLPP5.

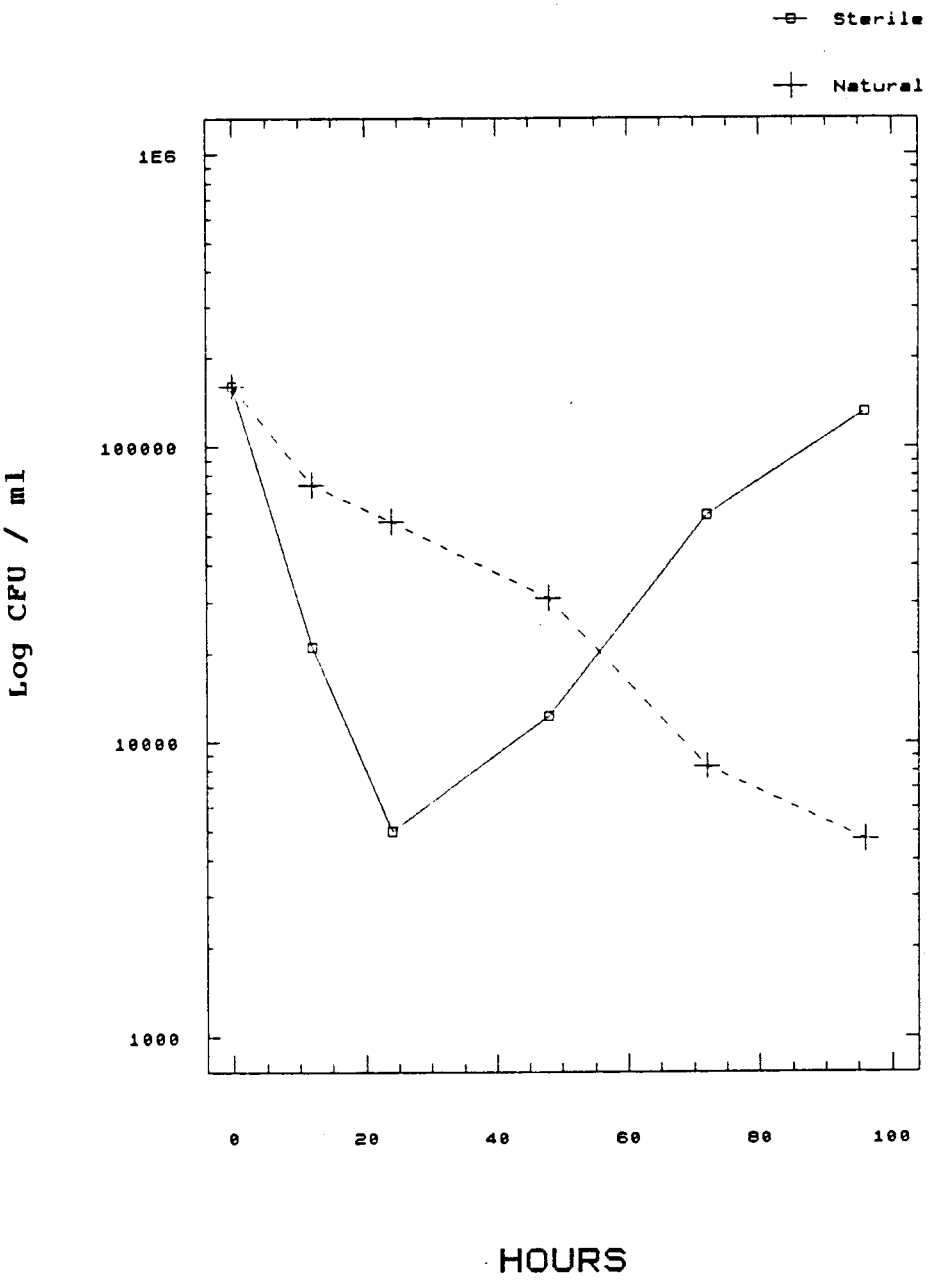


Figure 4.12. Survival of lysogenic host strain LPL5 in the presence of heterogenous phage population (LPE).

Table 4.2. Bacterial Densities and Multiplicity of Infection in Microcosms after 45 days Incubation.

Microcosm #	Content	Lakewater	
		Sterile	Natural
1 & 2	LLPA10	1.0×10^7	1.45×10^5
3 & 4	LPL5	3.76×10^7	1.80×10^3
5 & 6	LLPA10 + LPL5	2.12×10^7	1.12×10^5
7 & 8	LLPA10 / LPE	1.90×10^7 (3.18)[a]	9.92×10^4 (2.5)
9 & 10	LLPA10 / LLPP5	2.13×10^4 (2,742)	2.06×10^4 (8.9)
11 & 12	LPL5 / LPE	6.0×10^5 (6.15)	1.59×10^3 (0.2)
13 & 14	LPL5 / LLPP5	2.39×10^7 (0.006)	2.90×10^2 (ND)
15 & 16	LLPA10 + LPL5 / LPE	2.01×10^7 (3.55)	4.4×10^4 (28.2)
17 & 18	LLPA10 + LPL5 / LLPP5	2.31×10^5 (216.9)	2.53×10^4 (2.2)

[a]Multiplicity of infection in parenthesis.
ND = Phage below detection limit.
Bacterial densities are expressed per ml of lakewater.

(Table 4.2). However, the MOI in the sterile lakewater microcosm remained at about 300 times that found in natural lakewater (Figures 4.13 to 4.18). This observation is substantiated by the recovery pattern of prophage harboring colonies recovered from these microcosms (Figure 4.19). In natural lakewater, about 45% of the colony forming pseudomonads had become phage LLPP5 carriers within 12 hours of incubation. In the sterile lakewater control, prophage carrying colonies were not observed before 72 h of incubation.

In the presence of the heterogenous phage population, *P. aeruginosa* LLPA10 survived (Figure 4.9). In natural lakewater, the host population was rapidly reduced to a density of 5.1 - 9.9 x 10^4 cfu per ml (Figure 4.9 and Table 4.2). In sterile lakewater, an oscillating host population density was observed with a resting density at 45 days of 1.9 x 10^7 cfu per ml (Table 4.2). The MOI in both natural lakewater and its sterile control were approximately equal after 45 days even though the initial MOI were inversely proportional to bacterial survival (Figure 4.14).

The survival of *P. aeruginosa* strain LPL5 lysogenized with phage LLPP5 is shown in Figure 4.10. In natural lakewater, a decline of cell density to 3.5 x 10^3 cfu per ml occured within 96 h of inoculation, and this density was maintained after 45 days (Table 4.2). In the sterile lakewater control, a rapid decline of cell density to about 8 x 10^4 cfu per ml occured after 24 hours. This was followed by a recovery and proliferation to 2.12 x 10^7 cfu per ml at the

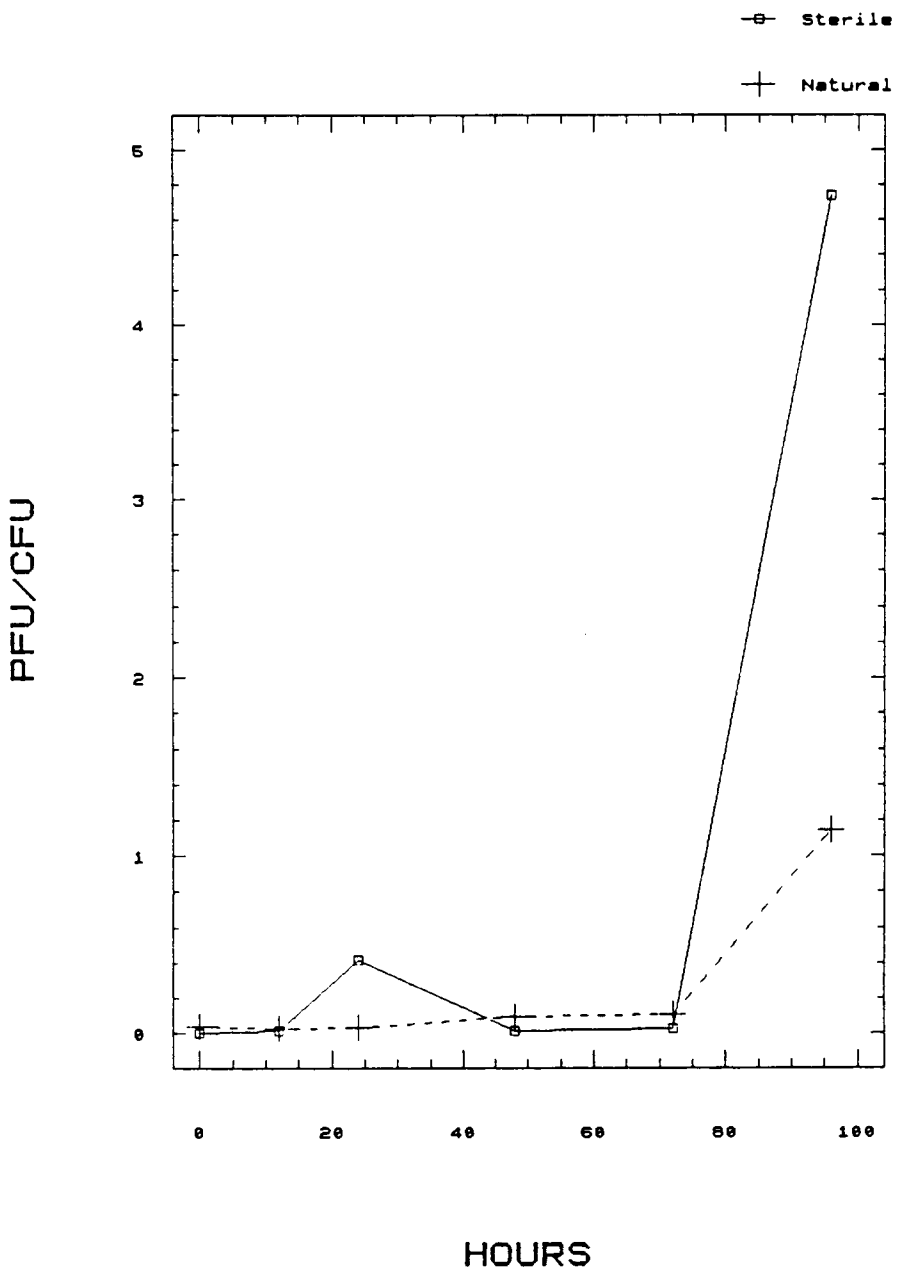


Figure 4.13. Multiplicity of infection in microcosms dosed with sensitive bacterial strain and phage LLPP5.

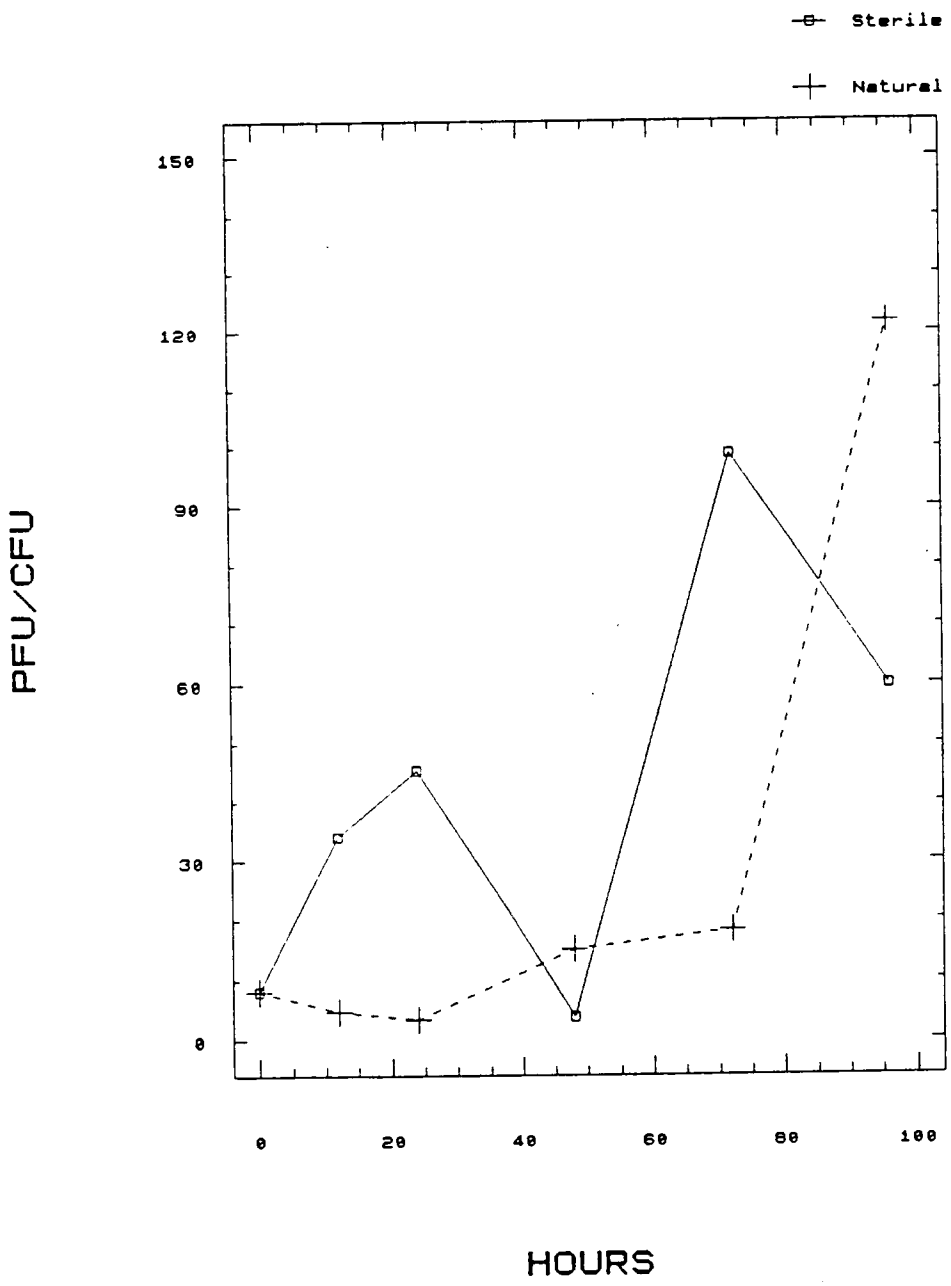


Figure 4.14. Multiplicity of infection in microcosms dosed with sensitive bacterial strain, LLPA10 and the heterogenous phage population (LPE).

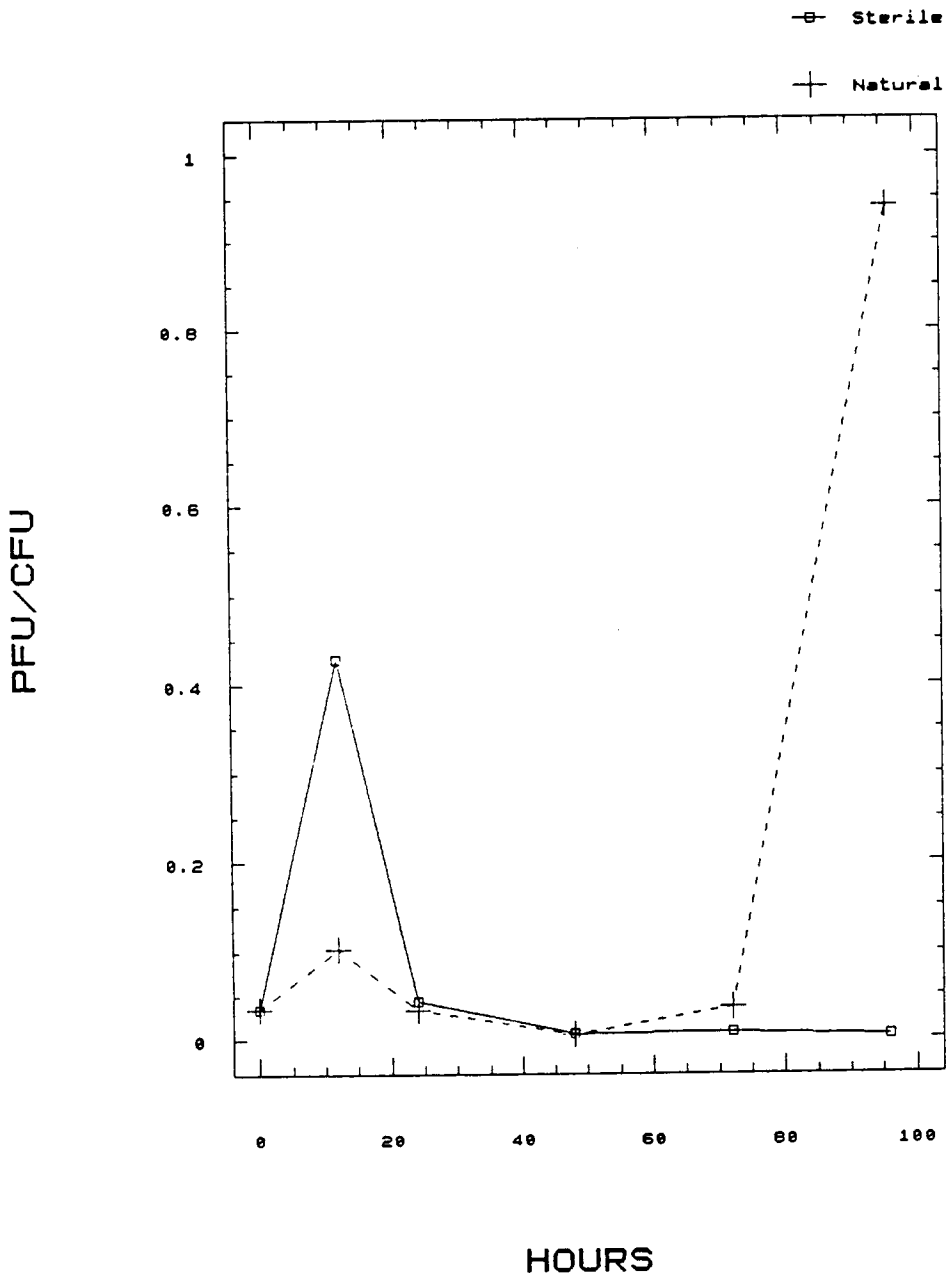


Figure 4.15. Multiplicity of infection in microcosms dosed with lysogenic bacterial strain, LPL5 and phage LLPP5.

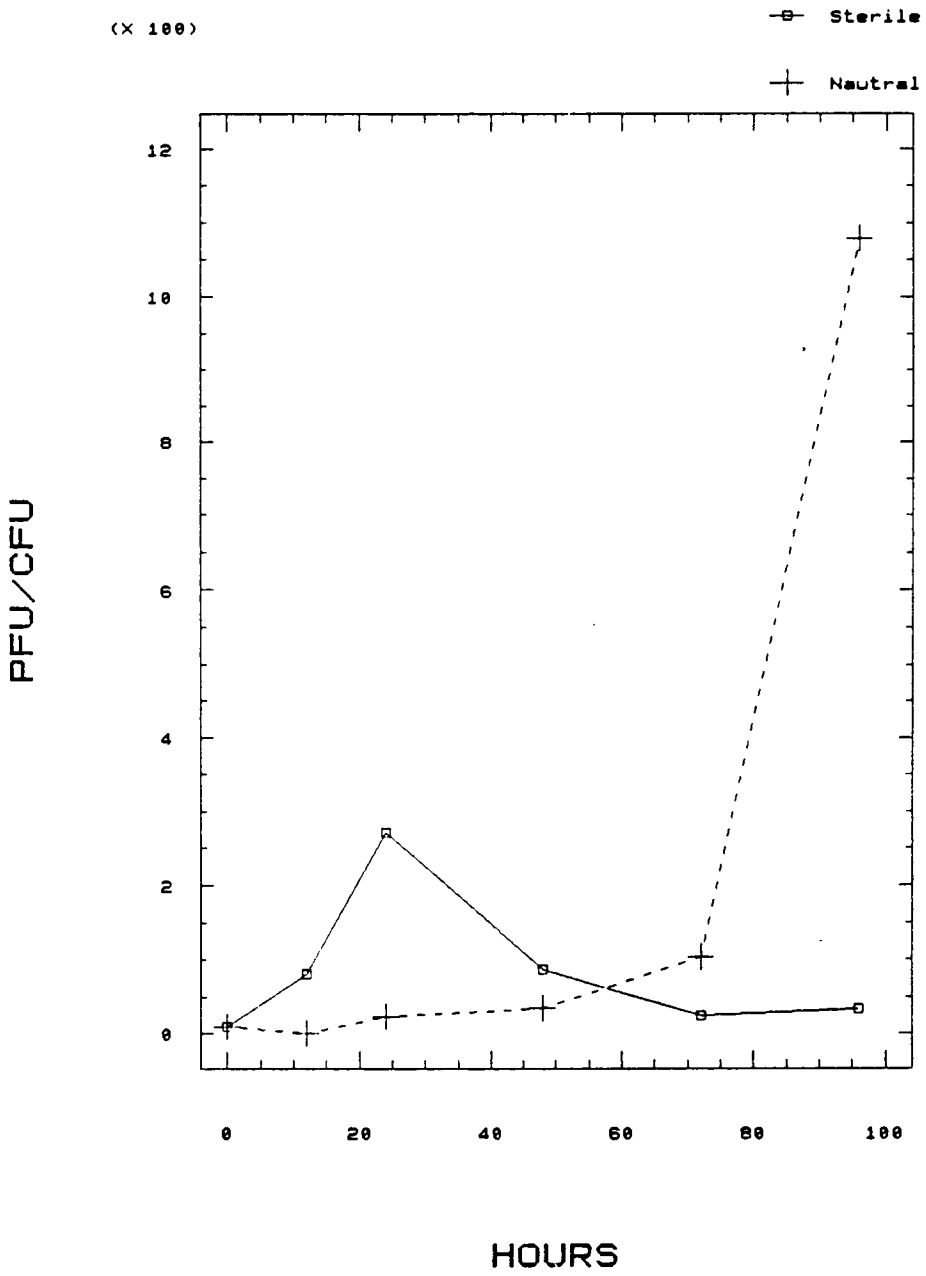


Figure 4.16. Multiplicity of infection in microcosms dosed with lysogenic bacterial strain, LPL5 and the heterogenous phage population, (LPE).

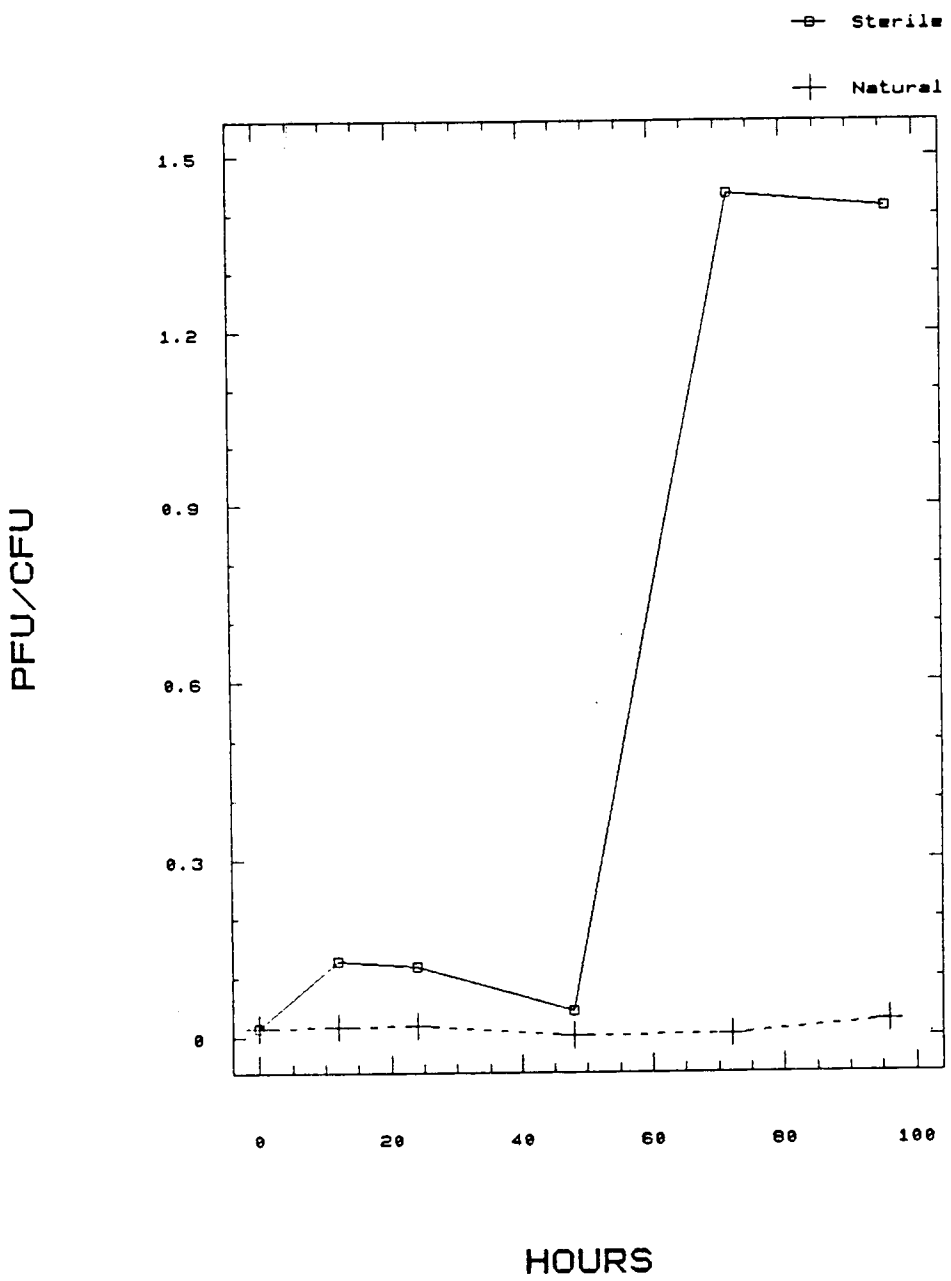


Figure 4.17. Multiplicity of infection in microcosms dosed with both sensitive strain (LLPA10), and resistant, lysogenic strain (LPL5), and phage LLPP5.

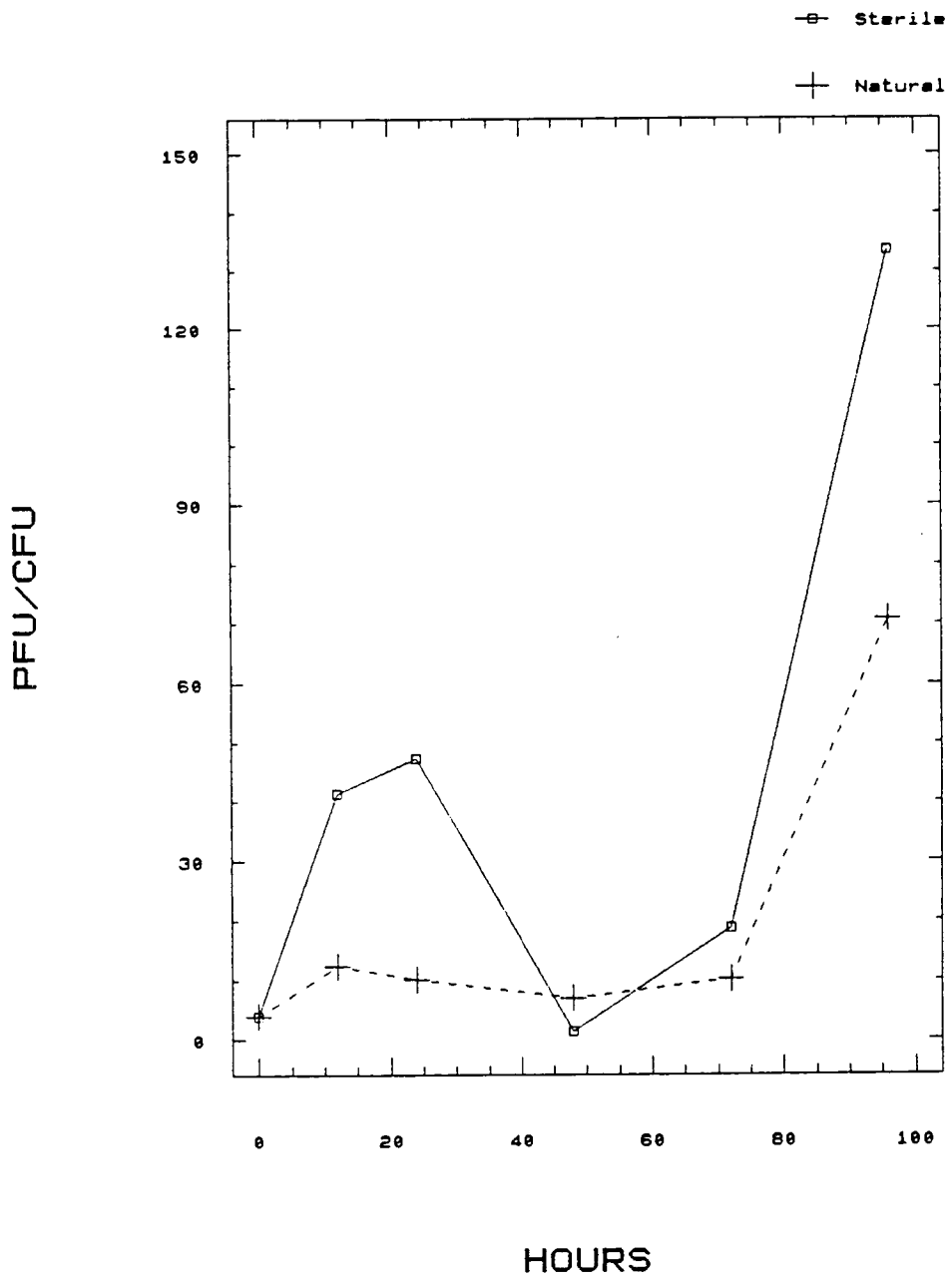


Figure 4.18. Multiplicity of infection in microcosms dosed with both sensitive strain (LLPA10), resistant, lysogenic strain, (LPL5), and the heterogenous phage population, LPE.

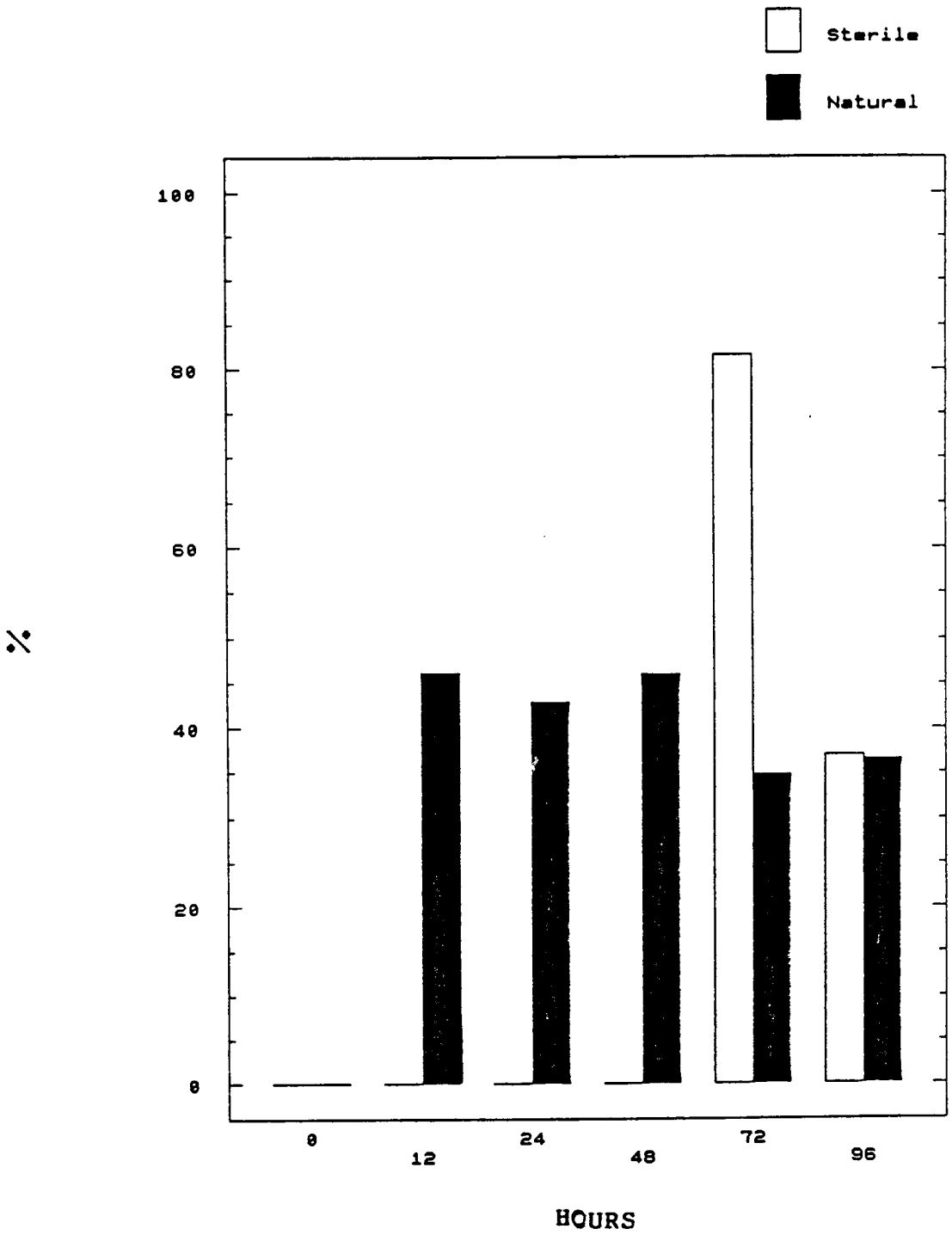


Figure 4.19. Proportion of total colonies harboring LLPP5 prophage, when sensitive strain, LLPA10 is incubated with phage LLPP5 particles.

end of the incubation period. Interaction of extraneous LLPP5 virions with its lysogenized host appears to aid the survival of the bacteria in lakewater at the initial stages of incubation (Figure 4.10 compared to Figure 4.11). In sterile lakewater, the MOI increased from an initial 1/30 to about 1/2.5 within 12 hours but stabilized at less than 1/100 soon after (Figure 4.15). At 96 hours in natural lakewater, the MOI was about 1.0 but at the end of 45 days, phage population dropped below the detection limit of 10 pfu per ml (Table 4.2). The host population stayed at about 10^6 to 10^7 cfu per ml in sterile lakewater but declined to about 2.9×10^2 cfu per ml in natural lakewater. Spontaneous release of phage from host bacteria strain LPL5 yields a stable MOI typically about 1/75.

The influence of the heterogenous phage population on the survival of *P. aeruginosa* LPL5 is illustriated in Figure 4.12 and Figure 4.16. The survival of LPL5 incubated with the mixed phage population did not differ appreciably from when the bacteria were incubated alone, suggesting that the population dynamics of this strain is determined by its lysogenized condition. The MOI in microcosms containing LPL5 and the bacteriophages (LPE) did not vary in pattern from the MOI in microcosms containing strain LPL5 and the bacteriophage LLPP5, although there is a substantial numerical difference (Figure 4.15). This particular observation may result from the higher initial pfu concentration of the heterogenous phage population used.

When both phage-LLPP5-sensitive (LLPA10) and -resistant (LPL5) bacterial strains were incubated together with extraneous phage LLPP5 virions, the MOI remained at less than or equal to 1/50 in natural lakewater for at least 96 hours (Figure 4.17). This value increased to about 2.0 after 45 days of incubation (Table 4.2). In the sterile lakewater control, the MOI became higher than 200 within the same period. The bacterial population density remained at about 10^5 cfu per ml. When compared to the patterns of survival when the two strains are incubated separately (Figure 4.13 and Figure 4.15), it appears as if the phage-sensitive strain is preferentially lysed as a continous source of infective phage particles rather than the spontaneous release of phage by the lysogenic strain. The lower MOI found in the presence of the phage-resistant strain (216.88 compared to 2,741.78 ; Table 4.2) could indicate either the occurrence of non-productive, phage-inactivating infections or increased lysogenization. Increase in the rate of lysogenization is also suggested by the increasing proportion of colonies that probe positive for prophage presence (Figures 4.20 and 4.21). These results indicate that introduced lysogens may die off at a rate higher than that at which establishment of carrier state occurs in sensitive strains. It is possible that prevailing MOI is critical for the maintenance of equilibrium between lysis and lysogeny since in the absence of extraneous phage particles, the proportion of colonies containing prophage remains virtually constant in natural lakewater

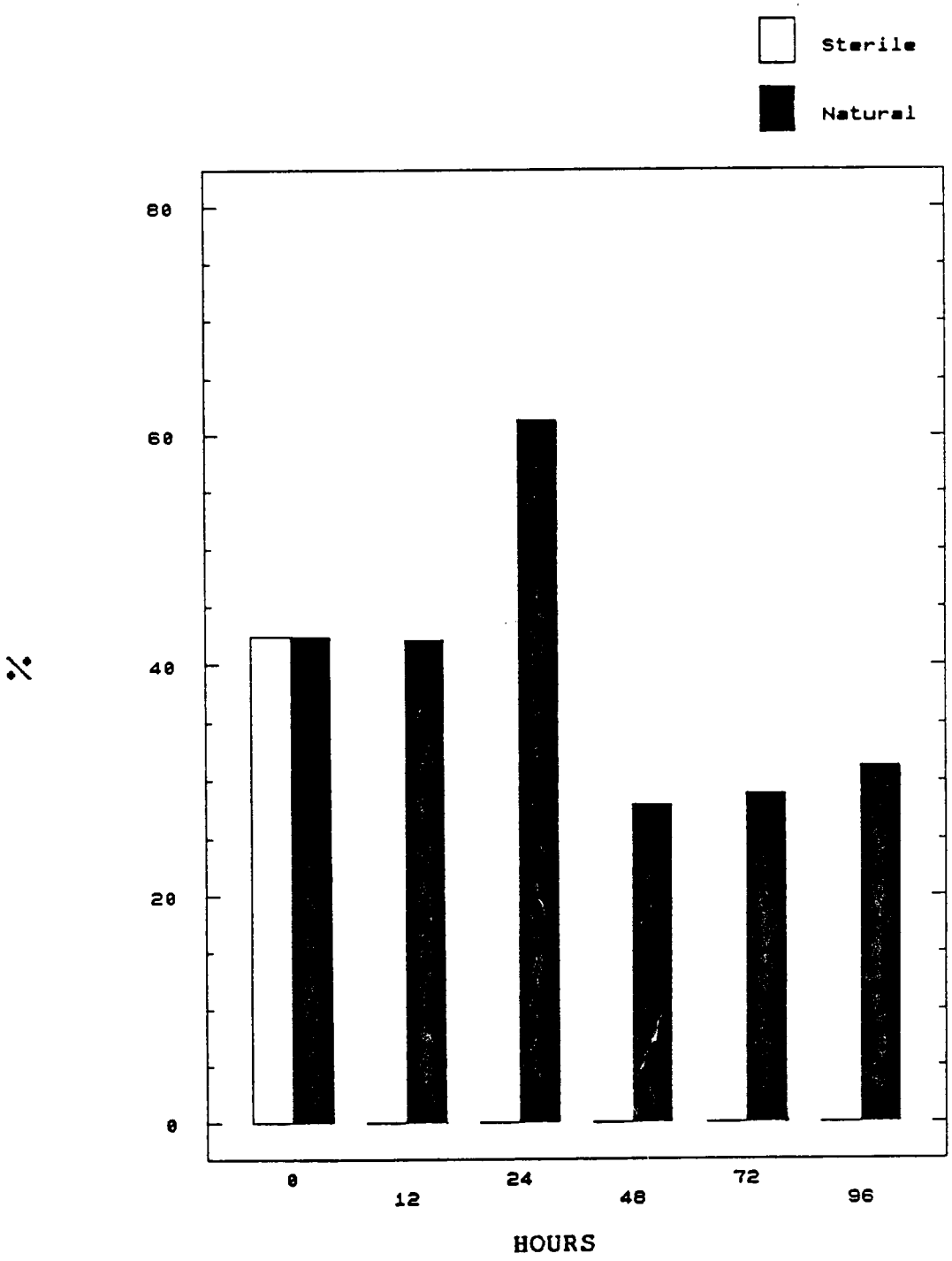


Figure 4.20. Proportion of total colonies harboring prophage LLPP5 in microcosms dosed with both sensitive host strain, LLPA10 and the lysogenic host strain, LPL5. No external phage particles added.

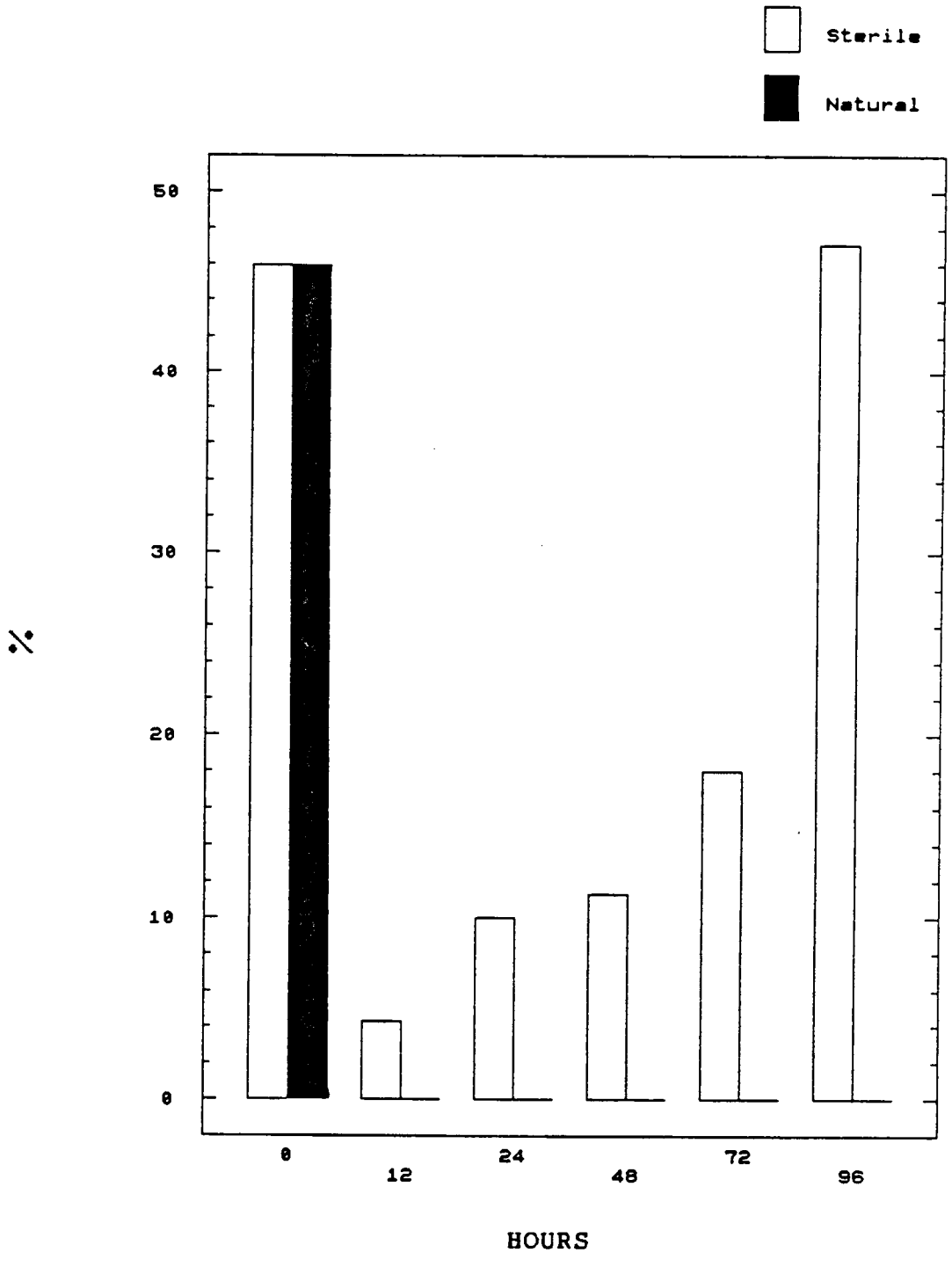


Figure 4.21. Proportion of total colonies harboring prophage LLPP5 in microcosms dosed with both sensitive bacterial strain, LLPA10 and the lysogenic host, LPL5, in the presence of external phage LLPP5 particles.

(Figure 4.20). Conversely, increasing the initial MOI by supplying external phage virtually eliminated prophage containing cells (Figure 4.21).

When *P. aeruginosa* strains LLPA10 and LPL5 were incubated together in the presence of the heterogenous phage population, LPE, the MOI patterns (Figure 4.18) support the earlier indication that the prophage-free strain LLPA10 was preferentially attacked by phage, both in natural lakewater and in the sterile control. In natural lakewater, the MOI in the microcosm containing strain LPL5 with phages LPE was above 1000 (Figure 4.16). This value is compared to 120 when strain LLPA10 was incubated alone with phages LPE (Figure 4.14), and only 70 when both LPL5 and LLPA10 are incubated together with phages LPE (Figure 4.18). After 45 days incubation, the MOI in these three microcosms were not substantially different (Table 4.2). This may indicate a population equilibrium achieved at an MOI of between 3.0 and 6.0. Due to the heterogeneity of the phage population LPE, it was not feasible to ascertain the proportion of lysogenized or phage resistant colonies in the microcosms.

There were no observable changes in the numerical population of heterotrophic bacteria indigenous to lakewater as a result of the inoculations and incubation as assayed on YEPG media.

DISCUSSION.

The persistence of bacteriophage in natural ecosystems may occur through integrative lysogenization of total host bacterial population or by transient carriage in a fraction of the host population (pseudolysogeny). Both of these persistent states of infection may induce characteristic changes in the host phenotype, usually resulting in protection of the carrier cells from subsequent phage infection (Barksdale and Arden, 1974). It has been widely reported that the lysogenic state is more common in nature for several species of bacteria, and that temperate phage result to lysogeny mainly when proliferation of hosts become hampered by nutrient limitation or other external physiological requirements (Holloway, 1960 ; Levin *et al.*, 1977 ; Zachary, 1978 ; Hendrix *et al.,* 1983). This behavior of bacteriophage ensures that host populations are never eradicated and that the phage genome remains preserved in a better protected situation than the virion protein coat. Even obligately virulent phage do not seem to be able to completely eradicate their hosts in nature. A threshold population below which bacteriophage do not appear to affect the numbers and metabolic activities of their hosts have been reported (Wiggins and Alexander, 1985).

The data reported here support the existence of such a threshold at around 10^4 cfu per ml. After 45 days of incubation alone in natural lakewater, the cell density of *P. aeruginosa* LLPA10 fluctuated and stabilized at 2.0 x 10^5 cfu per ml. However, in the presence of phage LLPP5, the population density of the host bacteria varied little and stabilized at about 2 x 10^4 cfu per ml, both in natural lakewater and in its sterile control (Table 4.2). This population density of bacteria was maintained despite a seemingly overwhelming presence of free phage particles in the sterile lakewater microcosm (MOI of 2,742). In the presence of a heterogenous phage population which presumably consists of some temperate and several vigorously virulent phage, the host bacterial density was still maintained at a minimum of 10^4 cfu per ml.

The prophage-carrying derivative strain was maintained at a lower population density than its prophage free parent strain. The threshold of cell lysis by inherent phage appears to be controlled by external MOI. *P. aeruginosa* LPL5 was maintained at a cell density of 10^2 to 10^3 cfu per ml. This reduced threshold may result from restrained ability of the lysogenized hosts to compete effectively with the natural population for enough nutrients to maintain the extra phage genome (Figure 4.10 and Table 4.2). In the absence of the viable, indigenous lakewater population, the lysogenized host survived equally or better than the sensitive host strain. The maintenance of prophage

seems to enhance the survival potential of carrier-hosts in natural lakewater. This observation may be attributed to a phenotype conversion of the phage-carrying cells to a morphology in which is less susceptible to predation by prevailing environmental parameters .

With an initial MOI of 1/25, about 45% of all recoverable pseudomonad colonies from natural lakewater microcosm containing phage LLPP5 with sensitive host strain contained prophage, as early as 12 h after inoculation (Figure 4.19). In the sterile lakewater control, lytic infection predominates at this time point, resulting in an increased MOI (Figure 4.7). Carrier hosts were not observed in this microcosm until 72 h after inoculation, when the prophage containing clones constituted 80% of all recoverable colonies. The correlation between MOI and the proportion of colonies harboring prophage indicate a balance of lytic and lysogenic infection cycles that is influenced either by arrogation of abiotic resources by the hosts or their interaction with other non-phage biotic factors. After 45 days incubation, about 90% of the recoverable cfu had become prophage carriers in the presence of a rather high MOI (Table 4.2). This result may preclude the possibility of the occurrence of aborted infections, but rather a reduction in efficiency of host recognition , resulting in a high population of free pfu in the microcosm. Similar correlations between high MOI and increased lysogenization has been reported for some *Salmonella typhimurium* phages (Boyd, 1951).

Prominent questions remain in aquatic phage ecology concerning the determinants of abundance and persistence of phage, and the extent to which the association between phage and host bacteria is stable. The stability of phage-host coexistence in freshwater can be expected to vary according to the qualitative composition and the level of genetic relatedness among any heterogenous phage population that can be expected to occupy that habitat. The bacteriophage population of a natural environment may be structured according to virulence, temperance, immunity specifications, or promiscuity. When phage LLPP5 was not replicating in lakewater, its density was drastically reduced and soon became extinct (Figure 4.5). However, the heterogenous phage population recovered from the same habitat did not suffer a similar fate although its overall density was also reduced. This result indicate that various bacteriophage recognizing similar hosts may not be equally affected by naturally occuring phage inactivating factors. In any case, total phage extinction can only be prevented in the presence of susceptible bacterial hosts. In natural lakewater, non-obligately virulent phage tend to coexist with their hosts in an equilibrium that is essentially static for brief periods (Figure 4.8 and Figure 4.13). For overwhelmingly virulent phage, there may exist continous oscillating cycles of low and high MOI, leading to equilibria patterns resembling those predicted by mathematical difference equation models (Williams *et al.*, 1987).

Observations of a population threshold for phage reduction of host bacteria are often explained by the evolution of phage resistance within the bacterial population. It is relatively easy to envisage how such a situation might lead to an endless 'arms race' or a 'gene-for-gene-retaliation' between bacteria and bacteriophage. However, certain theoretical models have indicated that this is not necessarily the case (Lenski and Levin, 1985). In the present study, it is apparent that other indigenous, non-host microbial communities of lakewater exert an influence on the establishment of a threshold of phage-host interaction. Resource limitation and competetion may act to predetermine the density at which bacterial metabolic activity can no longer support vegetative proliferation of phage, resulting in reduced burst sizes (Zachary, 1978). At such periods, the phage-carrier state may prevail in the host population. Reduction of host numbers by predators may also contribute to the threshold setting. It is possible that bacterial populations are rapidly reduced by predation that MOI becomes too high for vegetative growth of bacteriophage. However, the survival of *P. aeruginosa* LLPA10 incubated alone in natural lakewater at 10^5 cfu per ml, 45 days after inoculation does not support the significance of cell density reduction by predation . The observation that cell density was about two orders of magnitude higher in the sterile lakewater control may be due to an extra source of nutrients provided by autoclaved cells. Autoclaving was chosen as the means of sterilization in

order to include possible effects of suspended particulates on phage-host interaction.

Earlier investigations have shown that a high proportion of *P. aeruginosa* strains isolated from environmental or clinical samples carry one or more inducible phage genomes (Holloway, 1960 ; Patterson, 1965). The present results show that procession to the carrier state may be promoted under natural conditions, although lysogenized bacteria are not necessarily more stable than their phage-free parental strains. This implied turnover of the phage-carrying status may increase the potential for genetic interaction between an exogenous *P. aeruginosa* and indigenous strains in freshwater habitats.

Summary of Observations.

1. Establishment of lysogeny appears to be promoted in the presence of a viable natural microbial community.

2. Avirulent phage may act to stabilize the population of sensitive bacterial hosts in the environment.

3. Phage did not reduce bacterial population densities below a 'threshold' value of 10^4 cfu ml^{-1}.

4. Oscillatory population dynamics may be a peculiar attribute of virulent phage infection.

5. Lysogenized bacteria are not necessarily more persistent than sensitive phage-free strains, unless there is a low multiplicity of infection by extraneous phage in the environment.

6. Competition with indigenous microbial assemblage appears to contribute significantly to the process and consequence of phage-host interaction in lakewater.

CHAPTER V

TRANSDUCTION OF GENES TO LAKEWATER *Pseudomonas aeruginosa* BY INDIGENOUS BACTERIOPHAGE

INTRODUCTION.

It has been suggested that bacteria adjust to their environments not by random mutation, but by acquisition of genes able to confer some survival advantage from other bacterial strains (Sonea, 1988). The first clue to the existence of gene-transfer processes in bacteria came in 1928, with the *Pneumococcus* virulence-factor interchange observation by Griffith. However, it was not until sixteen years later before the mechanism of the process involved, now known as transformation, was clarified (Avery *et al.*, 1944). Five

such gene-transfer processes have been identified in bacteria. The other four mechanisms of gene acquisition are conjugation, transfection, lysogenization, and transduction (Reanney *et al.*, 1982). The last three of this set involve some form of interaction with bacteriophage. Transfection resembles transformation in that it can be triggered in a population by cell death, and it involves receptor sites and passive penetration of a host cell by genetic material. But transfection occurs only with small liberated replicons, specifically prophages (Streips *et al.*, 1982). Because several prophages may replicate independently of the host chromosome, transfection can take place, theoretically among widely varying strains of bacteria. However, transfection does not appear to be the most common mechanism by which prophages migrate between bacteria in nature. Rather, their transfer almost always occurs when prophages materialize as the intermediate form of mature, temperate phages. Temperate bacteriophage participate in the process of lysogenization, the second form of gene acquisition by bacteria involving viruses. By lysogenization, bacteria often acquire new characteristics that may aid their survival, especially in pathogenesis (Barksdale and Arden, 1974). As an intact particle, a temperate phage can survive for months outside the host cell, travelling widely by means of rivers, sea currents, wind, or animal vectors, until it chances upon an appropriate receptor on a bacterial cell. Temperate phages may thus permit geographically distant bacteria to benefit

by lysogenic conversion, although the bacterial strains must often be closely related or share similar metabolisms for phage reception. During the assembly of a temperate phage particle, a few of the host chromosomal replicon genes, or an entire plasmid may become encapsulated in the phage protein head. This process permits the third kind of gene acquisition by bacteria namely, transduction. Transduced genes may alter the recipients intracellular functions by homologous recombinational replacement of segments of the host chromosome. Transduction has been demonstrated among various species of bacteria and it appears to be the process most responsible for the transmission of resistance to various antimicrobial drugs in certain groups of bacteria (Sonea, 1988).

All the major types of mechanisms of gene transfer described so far have been shown to take place between strains of *Pseudomonas aeruginosa* (Stanisich and Richmond, 1975). However, only transduction and conjugation have been studied extensively in this group of organisms. Transducing phages for *P. aeruginosa* are reported to be readily isolatable from wild type lysogenic strains (Holloway *et al.*, 1960 ; Patterson, 1965). Some of the better characterized transducing phages of this species include F116 (Holloway *et al.*, 1960), and the homoimmune phages F126 and F130 described by Krishnapillai (1971), G101 (Holloway and van de Putte, 1968), B3, and D3 (Holloway *et al.*, 1960). All these phages transfer

chromosomal genes at frequencies ranging from 10^{-6} to 10^{-8} per plaque forming unit. The size of the phage genome appears to greatly influence the efficiency of cotransduction of closely linked markers (Stanisich and Richmond, 1975). Specialized transduction has only been demonstrated for one phage namely D3 (Cavenagh and Mlller, 1986). Phage F116 is the most well studied of the transducing bacteriophages of *P. aeruginosa*, largely because of its relatively wide host range, and resistance to host restriction enzymes (Stanisich and Richmond, 1975).

Perhaps the most important aspect of the ecology of *P. aeruginosa,* from the medical point of view, is the acquisition of antibiotic resistance genes (Lowbury, 1975). Increasingly, evidence is accumulating for the involvement of natural genetic exchange processes in the arrogation of multiple antibiotic resistance determinants on plasmids by bacteria (Trevors *et al.*, 1987). The transduction of such genes, located either on chromosomal, or on plasmid replicons have been demonstrated for *P. aeruginosa* in natural environments (Morrison *et al.*, 1978 ; Saye *et al.*, 1987). The present chapter reports investigations into the ability of naturally occuring bacteriophage to transduce genes from 'introduced' *P. aeruginosa* strains to a freshwater isolate.

MATERIALS AND METHODS.

Organisms.

The characteristics of bacterial strains used as donors and recipients in the transduction experiments are listed in Table 5.1. The characteristics of the bacteriophage under investigation, phage LLPP5 have been described in a previous section (Chapter III). The heterogenous phage population recovered from lake Loudon by enrichment of water sample with the indigenous strain LLPA10 was also investigated for the capacity to mediate genetic transduction. Phage DS1 was used as the control transducing phage (Saye *et al.*, 1987). *P. aeruginosa* strain RM273, a derivative of strain PAO1 was used as the laboratory-characterized recipient.

Growth and Culture Conditions.

Luria-Bertani (LB) broth (1% Tryptone, 0.5% Yeast extract, and 1% sodium chloride, all weight per liter of distilled water, final pH 7.5), or Mueller-Hinton medium (Difco) was used to cultivate bacteria. When required, solidified medium was made by adding agar at 15 grams per l.

Table 5.1. Characteristics of *P. aeruginosa* strains used to evaluate transduction by bacteriophage.[a,b]

Strain	role[c]	Genetic markers									Reference
		ami	*leu*	*pur*	*lys*	*met*	*trp*	*nal*	*stp*	*Hg*	
RM40	D	+	+	-600	-12	-28	-C6	+	-	+	Miller, R.V.
RM2140 (pRms149)	D	+	+	+	+	+	+	+	+	+	Miller, R.V.
PAO381 (FP2)	D	+	-38	+	+	+	+	+	-2	-	Holloway, B.W.
RM273	R	E200	+	+	+	+	+	A910	+	+	Miller, R.V.
LLPA10	R	+	+	+	+	+	+	-	+	+	This study

[a]The genotype symbols and abbreviations used are those of Holloway *et al*, (1979). Wildtypes, (+) ; Mutants, (-).
[b]The characteristics of bacteriophages LLPP5, LPE, and DS1 used were described in Chapter II.
[c]D = Donor ; R = Recipient.

Auxotrophs were selected on *Pseudomonas* minimal medium (PMM) (Miller and Ku, 1978), supplemented with 4 mg ml^{-1} glucose and appropriate growth factors. Amino acids were supplied at the following concentrations. Lysine, 85 ug ml^{-1} ; methionine, 110 ug ml^{-1} ; tryptophan, 50 ug ml^{-1} ; leucine, 60 ug ml^{-1}. Nucleotides were supplied at 20 ug ml^{-1}. Antibiotics and Mercury resistant derivatives were selected on LB-agar and Mueller-Hinton agar supplemented with the appropriate concentrations of the chemical. Nalidixic acid was used at 500 ug ml^{-1} (1.0 mg ml^{-1} for *P. aeruginosa* LLPA10), streptomycin was used at 1,000 ug ml-1, and $HgCl_2$ was used at 10 ug ml^{-1} in double selective plates that also contain nalidixic acid. Higher concentrations of mercury apparently act synergistically with the antibiotic and detection of resistant cells is reduced or eliminated (Loutit, 1970). All liquid cultures and plates were incubated at 37 ^{o}C.

Phage stocks.

Transducing phages were prepared on donor bacterial strains by the confluent plate lysis method, essentially as described by Silhavy *et al* (1984). The soft agar plates were harvested, mixed thoroughly with chloroform and allowed to settle for five minutes. Cell debris were removed by

centrifugation twice at 6000 rpm for 20 minutes each run. The clear supernatant was filtered twice through a 0.45 um pore size acrodisc (Gelman). The particle density of the phage lysate was determined by titeration on *P. aeruginosa* LLPA10 lawns. All phage preparations were stored at 4 ^{o}C.

Transduction by phage lysate.

Recipient bacteria were grown in LB supplemented with 500 ug ml^{-1} nalidixic acid, to a cell density of approximately 10^{8} cfu ml^{-1} (80 Klett units at 660 nm). The cells were pelleted and washed twice with TNM buffer (Miller and Ku, 1978) by centrifugation at 3,000 x g for 10 minutes per run. The washed pellets were resuspended in TNM buffer and infected with transducing virus particles at a multiplicity of infection of 0.01 to 10.0. The infected cells were incubated at 37 ^{o}C for 20 minutes. Unadsorbed phage were removed by centrifugation at 14,000 rpm for 90 seconds in an Eppendorf microfuge. The cell pellets were resuspended in 1.0 ml TNM buffer. One hundred microliter of the cell suspension was plated directly on media selective for transductants. The remaining 900 ul was resuspended in 9.0 ml of TNM and filtered through a 0.45 um pore size cellulose membrane. Approximately 10^{8} cfu of recipient strains were also filtered as a control for

the occurrence of spontaneous mutants. The membranes were transfered onto transductant selective media. All plates were incubated at 37 ^{o}C for 48 to 72 hours.

RESULTS.

Transduction of mercury resistance from *P. aeruginosa* PAO381 to the lakewater strain LLPA10.

Pseudomonas aeruginosa PAO381 harbors the sex factor plasmid FP2. This plasmid has a chromosome mobilizing ability (Cma) and is classified as an Inc-P8 plasmid (Jacoby *et al.*, 1978). The association of mercury resistance determinants with FP2 was first reported by Loutit (1970). The molecular size of FP2 has been estimated to be 88.5 kilobases (Holloway, 1975). The results of the experiment on the possibility of transduction of mercury resistance by lakewater bacteriophage LLPP5, to the lakewater bacteria recipient, *P. aeruginosa* LLPA10 is shown in Tables 5.2 and 5.3. The transfer of mercury resistance to strain LLPA10 was detected at frequencies between 1.67 to 3.89 x 10^{-7} per plaque forming unit. The data shown in Table 5.3 represent the secondary characterization of colonies

Table 5.2. Transduction of *P. aeruginosa* PAO381.

Lysate (pfu ml^{-1})	Recipient (cfu ml^{-1})	MOI	Primary selection medium	Putative transductants per 100 ul	Secondary selection positive	Frequency per 10^{-7} pfu
LLPP5/PAO381	RM273	0.1	L-Hg-N / L-S-N[a]	0	0	<1
1.8×10^9	5.51×10^9	1.0		0	0	<1
		10.0		0	0	<1
	LLPA10	0.1	L-Hg-N / L-S-N	7	3*	1.67
	2.1×10^8			8	7*	3.89
		1.0		0	0	<1
		10.0		0	0	<1
	RM273 (5.51×10^9)	0	L-Hg-N / L-S-N	0	0	<1
	LLPA10 (2.1×10^8)	0		0	0	<1

* See Table 5.3 for the characterization of these transductants.

L-Hg-N = Luria agar supplemented with mercury and nalidixic acid.

[a]L-S-N = Luria agar supplemented with streptomycin and nalidixic acid. No transductants weredetected on this medium.

Table 5.3. Characterization of mercury resistant transductants.

Strain #	MH-Hg	MH-S	L-Hg-N	L-S-N	PIA	PMM	PMM-leu	E79	LLPP5
Donor									
PAO381	+	+	-	-	+	-	+	R	S
Recipient									
LLPA10	-	-	-	-	+	+	+	S	S
Transductants[a]									
1a	-	-	-	-	+	+	+	R	S
2a	-	-	-	-	+	+	+	R	S
3a	-	-	+	-	+	+	+	R	S
4a	+	-	+	-	+	+	+	R	R
5a	+	-	+	-	+	+	+	R	R
6a	-	-	-	-	+	+	+	R	S
7a	+	-	+	-	+	-	-	R	R
1b	+	-	+	-	+	-	-	R	R
2b	+	-	+	-	+	+	+	R	S
3b	-	-	+	-	+	+	+	R	R
4b	+	-	+	-	+	+	+	R	R
5b	+	-	+	-	+	+	+	R	R
6b	+	-	+	-	+	+	+	R	S
7b	+	-	+	-	+	+	+	R	R
8b	+	-	+	-	+	-	-	R	R

[a]Groups a and b are isolates from replicate experiments.

N = Nalidixic acid ; S = Streptomycin ; Hg = Mercury ; MH = Mueller-Hinton ; L = Luria agar ; PIA = *Pseudomonas* isolation Agar ; leu = leucine ; PMM = *Pseudomonas* Minimal Medium.

R = Resistant ; S = Sensitive ; growth (+), no growth (-).

recovered on the original transductant selection plates. The transduction of streptomycin resistance (str-2) and leucine auxotrophy (leu-38), both mapped on the donor cell chromosome at approximately 32 and 48 minutes with respect to the origin of FP2 chromosome mobilization (Holloway, 1975), was not detected among the mercury resistant transductants. Based on other transductional mapping data obtained with generalized transducing phage, including F116L, these genetic loci are not normally co-transducible (Holloway, 1975). To further investigate the character of the transduced genetic molecule, the susceptibility of the recovered transductants to infection by phage E79, a female specific (IncP-2 exclusion), virulent phage was examined. Infection of the transductants by the transducing phage LLPP5 was also scored (Table 5.2). Phage E79 did not form plaques on the donor strain PAO381 at multiplicities of infection under which phage LLPP5 readily produces plaques. Both viruses normally form visible plaques on the recipient strain, LLPA10. The results show that all the putative transductants were resistant to infection by E79, while only 40% of the strains retained sensitivity to the transducing phage LLPP5. These data indicate that the capacity for phage incompatibility exclusion was cotransduced with mercury resistance although this trait remained in transductants that eventually lost resistance to mercury (e.g. strains 1a and 2a, Table 5.2). In addition, it appears that resistance to superinfection by the transducing phage LLPP5 is carried along

with some of the transduced genetic materials.

P. aeruginosa RM273 was tried as a recipient of mercury resistance transduction by phage LLPP5, but there were no detectable transductants in this strain. The frequency of spontaneous resistance to mercury was less tha 10^{-8} for both recipient strains used (Table 5.2).

Transduction of chromosomal streptomycin resistance determinants from *P. aeruginosa* RM40 to the lakewater strain LLPA10.

Table 5.4 reports the result of the experiment conducted to investigate transduction of streptomycin resistance from *P. aeruginosa* RM40 to *P. aeruginosa* LLPA10 by phage LLPP5. Only one colony was detected from the recipient population that acquired resistance to streptomycin. This would indicate a frequency of 2.5×10^{-8} per plaque forming unit. Since this colony retained the ability to synthesize all growth factors normally required by the donor strain, RM40, the possibility exists that the exductant might be a spontaneous streptomycin resistant derivative of the recipient. However, in the control experiment, no such spontaneous mutants were found out of 10^8 cells plated (Table 5.4). The transducing phage DS1 (Saye *et al.*, 1987), used as a positive control in these experiments transduced streptomycin resistance to

Table 5.4 Transduction of *P. aeruginosa* RM40.

Lysate pfu per ml	Recipient cfu per ml	MOI	Primary selection medium	Putative transduct. per ml	Secondary selection positive	Frequency per 10^{-7} pfu
LLPP5 / RM40 2.0×10^9	RM273 5.51×10^9	0.01	L-S-N	1	0	<1
		0.10		0	0	<1
		1.00		0	0	<1
	LLPA10 2.1×10^8	0.10	L-S-N	0	0	<1
		1.00	L-S-N	1	1*	0.2
DS1 / RM40 7.5×10^7	RM273 5.51×10^9	0.10	L-S-N	2	2*	1.33
	LLPA10 2.1×10^8	0.10	L-S-N	39	38*	50.6
	RM273 5.51×10^9	0.00	L-S-N	0	0	<1
	LLPA10 2.1×10^8	0.00	L-S-N	0	0	<1

*None of these transductants required lysine, methionine, or tryptophan in growth medium.
L-S-N = Luria-Bertani agar supplimented with streptomycin and nalidixic acid.

the lakewater recipient at a higher frequency of 5.06 x 10^{-6} per pfu. The transduction of streptomycin resistance to the laboratory recipient strain RM273 was detected at a frequency of 1.33 x 10^{-7} per pfu. The transduction of streptomycin resistance by phage LLPP5 to recipient strain RM 273 was not detected in this study.

Transduction of plasmid Rms149-encoded carbenicillin resistance from *Pseudomonas aeruginosa* RM2140 to the lakewater recipient strain LLPA10.

Phage LLPP5 did not mediate the transduction of pRms149 at detectable frequencies in the present study. However, the data shown in Table 5.5 indicate the potential of the heterogenous phage population recovered from the lake (Chapter II) to transduce this plasmid to lakewater strain LLPA10. The transfer of carbenicillin resistance to the *P. aeruginosa* LLPA10 was detected at a frequency of 1.5 x 10^{-7} per pfu. This frequency is higher than that of 3.0 x 10^{-8} per pfu reported for phage F116L by Saye *et al* (1987). Figure 5.1 shows the plasmid profiles of the donor, recipient, and transductant strains. When *P. aeruginosa* RM273 was tried as the recipient strain in a similar experiment with heterogenous phage, several putative transductant colonies were recovered on the original transductant selective

Table 5.5. Transduction of pRms149.

Lysate pfu ml^{-1}	Recipient cfu ml^{-1}	MOI	Primary selection medium	Putative transductants per ml	Secondary selection positive	Frequency per 10^{-7} pfu
LLPP5 / RM2140	RM273	0.1	L-Cb-N	1	0	<1
3.98×10^9	5.51×10^9	1.0		0	0	<1
	LLPA10	0.1	L-Cb-N	0	0	<1
	2.1×10^8	1.0		0	0	<1
LPE / RM2140	RM273	0.1	L-Cb-N	0*	0	<1
1.50×10^8	5.51×10^9	1.0		0	0	<1
	LLPA10	0.1	L-Cb-N	11	10[a]	1.5
	2.1×10^8	1.0		2	0	<1
RM273 (5.51×10^9)		0	L-Cb-N	0	0	<1
LLPA10 (2.1×10^8)		0	L-Cb-N	0	0	<1

*Several colonies were detected that did not grow on subequent transfer to fresh plates.

[a]Only one of these colonies grew on Luria agar supplemented with streptomycin and nalidixic acid.

L-Cb-N = Luria agar supplemented with carbenicillin and nalidixic acid.

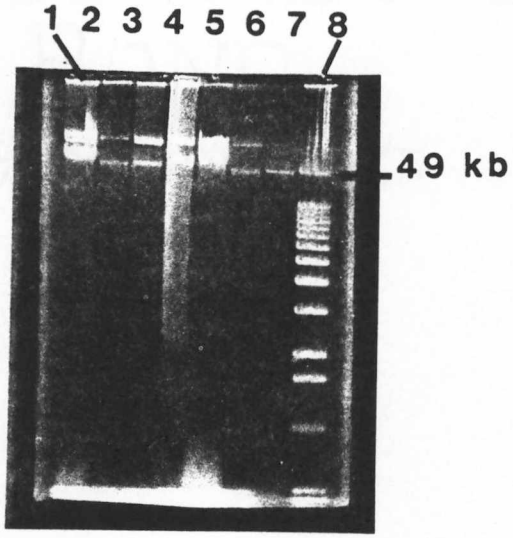


Figure 5.1. Plasmid content of transductants showing pRms149.

Lanes 1 to 4 : transductants
Lane 5 : recipient strain LLPA10
Lane 6 : donor strain RM2140
Lane 7 : transducing phage DNA
Lane 8 : standard DNA molecular size marker.

Panel shows agarose gel (0.75%) run at 75 volts for 5 h.

plates. These colonies did not maintain the transduced traits on subsequent selective subculture, and are not confirmed as regarded as viable transductants. Spontaneous resistance to Carbenicillin was less than 10^{-8} in both of the recipients used (Table 5.5).

DISCUSSION.

The variety of rigorously characterized transducing bacteriophages that now constitute indispensable tools for the molecular analyses of microbial genetic systems were at some point, wild type isolates recovered from one environmental system or the other. It is therefore not surprising that transducing phage exist in various natural ecosystems. What remains to be elucidated is the quantitative estimation of the role assumed by these transducing viruses in the rearrangement processes that lead to the evolution of indigenous bacterial genomes. The current interest in microbial genetic exchange mechanisms stems from concern regarding the effect of the potential release of genetically engineered bacteria into the environment. The process of assessing the implications of such releases has engendered the development of new areas of research involving the molecular determinants of bacterial ecology. The results of the present investigation

demonstrate the transfer of tractable genetic materials from 'laboratory' donor strains to an 'indigenous' lakewater bacterium. Perhaps the more important finding is the mediation of the genetic transfer by bacteriophage also indigenous to the lakewater habitat. Although the present experiments were conducted in the laboratory, the possibility of both chromosomal and plasmid transfer under environmental conditions that prevail in lakewater have been demonstrated (Morrison *et al.*, 1978 ; Saye *et al.*, 1987). Resistance to the toxicity of heavy metal ions and antibiotics can be expected to play vital roles in the ecology of bacteria that occupy habitats prone to contamination by industrial and domestic effluents. The involvement of bacteriophage in the dissemination of such traits within the bacterial population appears now to be more widespread than previously considered. The frequency of genetic transduction mediated by phage LLPP5 (circa 10^{-7} to 10^{-8}) was similar to that previously reported for generalized transducing phages of *P. aeruginosa* (Stanisich and Richmond, 1975). Attempts by previous investigators to demonstrate the transfer of FP plasmids (FP2 and FP39) by the generalized transducing phages F116, F116L, and G101 were reported as unsuccessful (Stanisich and Richmond, 1975). Those results seem to have been influenced by the major limitation found in all transducing systems : the theoretical limit to the length of donor DNA that may be transduced, which is determined by the

genomic size of the transducing phage. In the present study, the genome size of the phage studied (~78 kilobases) is close to the estimated size range of the sex factor FP2 that was transduced (82.5 - 88.5 kilobases). Size difference may also be responsible for the lack of detection of transduction of pRms149 (55 kilobases) by phage LLPP5 when a cell-free source of transducing phage was used. Frequency of transduction is often drastically reduced when the plasmid size is substantially smaller than the genome size of the vector (Saye *et al,* 1987). The inability of phage LLPP5 to recognize the laboratory strain RM273 as a viable recipient is more difficult to explain. However, it was previously noticed (Chapter III) that phage LLPP5 cultivated on *P. aeruginosa* LLPA10 do not plaque efficiently on strain RM273. It is possible that this observed reduction in plaquing efficiency is due to a form of host controlled restriction of phage DNA, in which case, the frequency of detection of possible transductants will be drastically reduced in a recipient strain that exhibit this form of HCM. The molecular semblance of phage LLPP5 to phage E79 (discussed in Chapter III) may contribute to the relatively higher frequency of transduction of FP2 in strain PAO381. It has previously been observed that the virulent phage E79 is capable of detectable transduction, provided steps (such as using recipients carrying IncP-2 plasmids) are taken to prevent transductant killing by superinfection with

phage (Holloway *et al.*, 1979). Although the evolutionary distance between phage LLPP5 and E79 remains to be ascertained, the considerable level of DNA sequence similarity that exist between the two phages indicate a recent divergence, probably resulting from a major deletion in the genome of phage E79 or a corresponding insertion into the genome of phage LLPP5. However, the physiological functions (involving host interaction and transduction) of the genetic difference between the two viruses can only be determined by future molecular analyses.

The frequency of transduction of the traits observed here is probably high for what might occur in nature, considering the heterogeneity of both phage, donors, and possible recipients that can be expected to exist in the natural habitat. Conversely, situations may occur, for instance within epilithic and epiphytic populations, when bacterial and specific phage densities are high enough to permit high frequencies of transduction, establishment, and expression of certain traits, particularly when selective pressures are exerted. In conclusion, this investigation supports the possibility of using bacteriophages for the deliberate dissemination of genetic information into a naturally occuring bacterial population.

Summary of Observations.

1. Phage LLPP5 mediated the transduction of mercury and streptomycin resistance determinants to the lakewater host strain, *P. aeruginosa* LLPA10 at frequencies of 1.67 - 3.89 x 10^{-7} and 2.5 x 10^{-8} per plaque forming unit respectively.

2. The heterogenous phage population recovered from Ft. Loudon lakewater mediated the transduction of plasmid encoded carbenicillin resistance to the lakewater host strain, *P. aeruginosa* LLPA10 at a frequency of 1.5 x 10^{-7} per plaque forming unit.

3. The laboratory host strain, *P. aeruginosa* RM273 was not receptive to transduction mediated by phage LLPP5.

4. Phage DS1 transduced streptomycin resistance to the lakewater strain, *P. aeruginosa* LLPA10 at a frequency of 5.06 x 10^{-6} per plaque forming unit.

CHAPTER VI

OVERVIEW

Bacteriophage ecology has been defined as " the interaction of phage with the environment and with their host cells and the effects of such interactions on the composition and productivity of particular habitats" (Primrose *et al.*, 1982). The interaction of phage with the environment may determine an essentially predictable distribution pattern of phage in various ecological milieux. Therefore, the occurrence of phage in water, sediment, soil, or sewage depends largely on the stability of phage particles in that environment to survive long enough till vegetative replication can occur in a susceptible host. However, vegetative growth of phage depends on the abundance of host cells. Hence, the effect of environmental factors on phage ecology intercepts the effect of the second factor, which is the interaction of phage with their host . Depending on the type of phage, and the metabolic

status of the host population, a particular infection may be vegetatively productive, releasing numerous phage progeny, or it may be silent and redundant, with the phage genome residing in the host population. The choice of growth process undertaken by the phage genome is thought to depend even on prevailing environmental parameters such as nutrient availability for the host cells to maintain the various physiological processes necessary for genomic replication. Accordingly, nutrient availability in a particular habitat depends on its composition and productivity. The status of the composition and productivity of an environment can be regarded as either the consequence or the determinant of phage activity in a particular niche. The productivity of an environment depends on the genetic composition of its various biological entities. The occurrence of phage within the bacterial community may directly influence the productivity of that community by their ability to precipitate genetic exchange between bacterial strains. There appears to exist therefore, an *idem per idem* association between the various factors that influence bacteriophage ecology in the environment (Fgure 6.1). The present effort has attempted to characterize the various aspects of phage ecology for an important group of bacteria, namely *Pseudomonas aeruginosa*, starting with an investigation of the general distribution pattern of phage in their various forms of existence, and the peculiarities of habitats that may influence the observed patterns (Chapter II). In Chapter III, specific

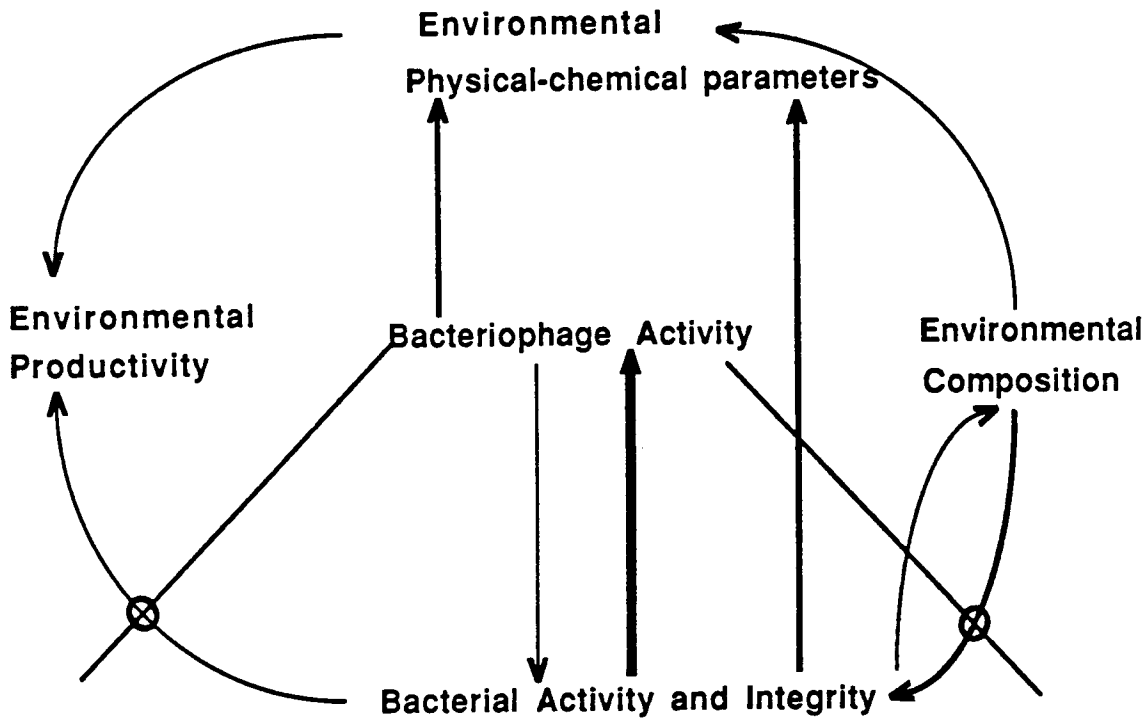


Figure 6.1. Interdependence of bacteriophage ecology on the productivity of microbial communities. Intensity and direction of arrows emphasize influence.

prototypes of phage and host were isolated and characterized to an extent that would facilitate an understanding of the molecular basis of interactions that occur between the coupled system *in situ*. In Chapter IV, the dynamic nature of such interactions was investigated, within the considerations of environmental and biological factors that may affect the persistence, and stability of the interactions. Chapter V dealt with a major aspect the of possible consequences of the interactive behavior of phage and bacteria, that being genetic modification and exchange by transduction. The cycle of the determinants, activities, and repercussions of phage occurrence in Lake Loudon may exemplify the process as it occurs for most other phage-bacterial systems in freshwater. However, it may be that only the periphery of this cycle was outlined in this study. Understanding the assembly rules by which bacteriophage-and-host coupled systems are governed in freshwater habitats remains a formidable and fascinating challenge.

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