RAMIFICATIONS OF RECEPTOR UTILIZATION FOR TWO BACTERIOPHAGES OF *Pseudomonas aeruginosa*

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CHAPTER I

INTRODUCTION

Gene transfer between bacteria holds gross consequences for species diversity and evolution. The ability of bacteria to transfer genetic material is made more important with the advent of using Genetically Engineered Microorganisms (GEMs) in such tasks as bioremediation, wastewater treatment, and mineral processing (Ripp, 1996). While these are noble efforts, it is possible that not enough effort has been devoted to understanding entire ecosystems of microorganisms. This includes both natural cell-based life forms and viruses that utilize them as hosts.

Genetic exchange happens primarily via three well-studied mechanisms. Transformation, the ability of a cell to take up naked DNA, was first documented by Avery, MacLeod, and McCarty in 1944 (Avery et. al., 1944). This has been studied most extensively in *Escherichia coli* whereby one strand of linear double-stranded DNA is degraded in order to provide the needed energy for injection of the other. The DNA then undergoes a single-stranded aggression event in which it undergoes a RecA mediated homologous recombination event. While said mechanisms have been examined in the *E. coli* system, the same mechanisms do not necessarily hold true for other gram-negative

bacteria such as those in the genus *Pseudomonas* (R.V. Miller, personal communication). Conjugation, a second type of gene transfer, occurs when one cell bearing a conjugal pilus ("male") attaches to a non-pilus-possessing ("female") cell and passes DNA into the recipient. The exact mechanisms by which this takes place are not fully understood. Transduction of prokaryotic DNA is transfer that is mediated by bacteriophages.

Studies have implicated transduction as the major manner in which gene transfer is arbitrated *in situ* (Miller et. al., 1977). Bergh and his colleagues discovered in 1989 that phage titers in marine waters were actually seven times greater than had been previously assumed (Bergh et. al., 1989), and this may provide a venue by which frequent transduction events come to pass. Transduction may not seem to be a common candidate for genetic exchange because the DNA recipient may in turn be killed in the process. However, a little-studied process known as pseudolysogeny may be responsible for the bulk of the transfer (Ripp and Miller, 1997).

During pseudolysogeny, a susceptible host cell exists in a viable but non-culturable state, as may be the case for many microorganisms as they exist in the environment. This cell becomes infected by a phage which is capable of either vegetative or reductive (lysogenic) growth as we know it in the laboratory. The virus, once inside the starving cell, must maintain itself in a harsh environment devoid of nutrients.

If this virus holds genetic material from the prior host cell from which it came, this DNA is now introduced into the new host (transduced). It may now recombine into the host chromosome or into a plasmid. Either way, this new gene may introduce the phenotype of the previously infected cell. In this way, transduction of GEMs into the natural flora of an

ecosystem can give rise to a "Genetically Engineered Environment." Experimental application of phage DNA probes to the environment has shown that such transfer is observable (Ogunseitan et. al., 1992).

Pseudomonas aeruginosa is ubiquitous in the environment and can easily be isolated from freshwater lakes (Ripp, 1996). The entire genome of Pseudomonas aeruginosa PAO has been sequenced at the University of Washington, though this sequence information has not yet been published. An omnipresent bacteriophage, UT1, was isolated from a freshwater source in 1988 (Ogunseitan, 1988). Another phage, UNL-1, was propagated from a spontaneously arising plaque at the University of Nebraska-Lincoln (Shaffer et. al., 1999). UNL-1 does not replicate in starved cells, and it does not mediate generalized transduction.

This study examines properties of UT1 and UNL-1. Specifically, this work aims to show that the receptor for these two phages lies within the lipopolysaccharide layer and that this confers identical host ranges. Molecular properties of the bacteriophages are also considered.

CHAPTER II

LITERATURE REVIEW

Bacteriophage Distribution in the Environment. It is well known that bacteriophages can undergo both reductive and vegetative life cycles, but methods presently in use for detecting phages rely on their lytic activity when infecting a specific host. Should this lytic behavior be the norm in the environment, it stands to reason that there would be little to no host bacteria living in the habitat. Furthermore, there would be no dynamic interaction between phages and their hosts. This notion was contradicted by Ogunseitan (Ogunseitan et. al., 1990). Bacterial viruses have a finite half-life in the environment (Roszak and Colwell, 1987).

One reason why UT1 was chosen in previous studies was that it was actually isolated from a lake sample and not induced from a laboratory lysogen (Ogunseitan et. al, 1990). In order to mimic actual environmental microbiology, it is paramount to make all attempts possible to employ actual environmental isolates of both bacteriophages and their hosts.

Pseudolysogenic Relationships. Pseudolysogeny was first described by Twort (Twort, 1915), but this phenomenon wasn't fully recognized until 1961 (Romig and

Brodetsky, 1961) when it was used to describe the stasis between various soil bacilli and the viruses that infect them.

It was hypothesized that pseudolysogeny happens in response to low levels of nutrients. To test this hypothesis, a chemostat run with chemically defined minimal media was used to control growth of *Pseudomonas* hosts which had been inoculated with UT1 at an MOI of approximately one. Detection of phage was then accomplished by infective center assays taken at intervals during the run. Indeed, a constant low level of phage production was seen at times when few nutrients were available. Spiking the medium with richer nutrients (yeast extract) induced the production of viable phage particles. This increased the phage:bacterium ratio (Ripp and Miller, 1997). Phages from a freshwater environment were also probed with labeled UT1 DNA and shown to be positive (Ripp and Miller, 1997). This confirmed that UT1 was an omnipresent phage in that environment or that UT1-like phages were ubiquitous. In a separate study, UT1 DNA was shown to be present in hosts that had been infected and then starved for five years (Schrader et. al., 1999). UT1 DNA can thus seemingly exist in a pseudoprophage state in the lab or *in situ*.

A study by Ogunseitan et. al. (1990) described the importance of lakewater transduction events. Several phages [UT1, D3, DS1, F116 (temperate), E79 (virulent), and M1 (a mixed population)] were utilized in the study (Ogunseitan et. al., 1992). Phage DNA probes were constructed via nick translation using radioactive nucleotides, and these probes hybridized to genomic DNA isloated from wild bacteria in a lake to test for phage lysogenization. Many of the probes did indeed reveal a strong hybridization signal.

Bacteriophage Receptors. While much attention has been given to the molecular biology of bacteriophages and the events that regulate their life cycles, relatively little effort has been geared toward understanding the initial event in virus infection, attachment to the host cell.

Animal cells have no cell walls, and animal viruses thus tend to adsorb to protein or carbohydrate moities on the outside of the host cell. This explains their high tissue/organ specificity, as different types of differentiated cells within a multicelluar organism display different surface proteins (often to aid in a given function).

Bacteriophages, on the other hand, utilize host cells that do have cell walls that are often quite complicated in nature. Furthermore, the cell wall is often the outermost structure of the host. This is certainly the case with *P. aeruginosa* (Meadow, 1975). Aside from phospholipids, the major component of the cell wall is lipopolysaccharide (Meadow, 1975). Bacteriophages may use this abundant moiety as an attachment site.

Lipopolysaccharide (LPS) has been shown to be the receptor for many bacteriophages. *Pseudomonas* phages 68, PB1 (Bradley and Pitt, 1974), B3, D3, G101, and E79 have all been shown to be specific for the cell wall (Jacoby, 1974). The LPS has been identified as the receptor for T3, T4, and T7 in *Escherichia coli* (Weidel, 1958), T2 and T4 in *Shigella dysenteriae* (Goldham et. al., 1975), for P22 in *Salmonella typhimurium* (Lindberg, 1973), and for phage 1P in *Rhizobium trifolii* (Zajac et. al, 1975). More recently, LPS has been shown to be the receptor for *P. aeruginosa* cytotoxin-converting phage ΦCTX (Yokota et. al, 1994) It is of interest to note that these are primarily bacteriophages in the *Myoviridae* genus.

Of utmost importance is the discovery of the receptor for *Pseudomonas* phage E79 (Jarrell and Kropinski, 1977). In the Jarrell study, E79-resistant mutants were generated by introducing a culture of *P. aeruginosa* PAO 307 to a high concentration of E79. Such mutants were shown to have a rough colony morphology, which is characteristic of bacteria that are deficient in LPS. LPS was then purified from *P. aeruginosa* PAO 307. The LPS was incubated with phage, and the resulting mixture was titered by a plaque assay, using PAO 307 as a host. The fractions containing the most LPS yielded a lower phage titer (efficiency of plating). The investigators concluded that exogenous LPS competed for binding sites on the phage particles. It was not alluded to, an LPS binding event may have induced a needed conformational change in phage proteins that elicited a DNA expulsion event into the medium. This would have yielded "ghost" phage particles that were neither able to bind cells nor to generate a successful infection. In any case, incubation of phage with LPS caused the decreased efficiency of plating that occurred. This indicated that LPS is likely the receptor for E79.

Pili have been shown to be receptors for C22, M6, PE69, and C5 (Bradley and Pitt, 1975), as well as the widely-used generalized transducing phage F116 (Pemberton, 1973). *P. aeruginosa* PAO possesses four to five of these phage-attaching pili/cell (Pemberton, 1973). These pili are constitutively expressed, and they can be observed using any standard negative staining technique coupled with visualization in the transmission electron microscope.

The Cell Wall in *Pseudomonas aeruginosa*. Lipopolysaccharide is a common component of gram negative bacteria, but the lipopolysaccharide is often quite different

between species and even strains of the same species. This is true for members of the *Pseudomonas* genus (Meadow, 1975) between differing species and strains.

The outer membrane and wall of the *P. aeruginosa* cell is characterized by 9 distinct layers visible in a thin section electron micrograph of a cell. These have been designated L_1 through L_9 (Lickfield et al., 1972). Of these, L_1, L_3, L_5, L_7, and L_9 are electron dense, and L_1 is the outermost layer. The even numbered layers are transparent when viewed in the electron microscope.

For purposes of nomenclature, the cell wall (16 nm) consists of layers L_1 through L_6. L_5 (3 nm) is the mucopeptide layer that is within the periplasmic space. In *E. coli*, the mucopeptide (peptidoglycan) is linked to the outer layers of the wall via a lipoprotein which can be removed by trypsin (Braun and Sieglin, 1970). There is much less lipoprotein in the *P. aeruginosa* mucopeptide-outer membrane junction, but there still may exist some sort of lipoprotein-mediated joining of the peptidoglycan and outer membrane. (Meadow, 1975).

The outer membrane (7 nm) is L_1 through L_3 (Gilleland et. al., 1973). This constitutes the exterior layers of L_1-L_6 referred to above. The cytoplasmic membrane (7 nm) is L_7 through L_9. The lipid A portion of the lipopolysaccharide has been shown to be located within L_1-L_3, while the polysaccharide portion is extrudes outward from the cell (Gilleland et. al., 1973). There is likely a common basic lipopolysaccharide molecule which is modified in different strains (Meadow, 1975). This means that a phage which can use *P. aeruginosa* PAO lipopolysaccharide as a receptor might not be able to infect a different strain such as *Pseudomonas aeruginosa* FRD.

The LPS of *P. aeruginosa* can be cleaved into its lipid A and polysaccharide segments by mild acid hydrolysis, though this also cleaves some glycoside linkages (Fensom and Meadow, 1970). The lipid A lacks β-hydroxyl myristic acid, which is the major component of the LPS of most Gram negative bacteria (Fensom and Gray, 1969). The major *P. aeruginosa* LPS lipid is 3-hydroxy myristic acid (12:0). Also present are 3-hydroxy 10:0 and 2-hydroxy 12:0 fatty acids (Chester et. al., 1973). The lipid A moiety is synthesized from a β-1,6-linked disaccharide of glucosamine. The glucosamine hydroxyls are esterified with fatty acids while 3-OH myristic acid (12:0) is amide linked to the glucosamine amino group. The remaining hydroxyls can be substituted with palmitic acid (16:0) and/or 2-hydroxy myristic acid (12:0) (Drewry et. al., 1973). All three of the hydroxy acids of the lipid A can be synthesized from the coenzyme A derivatives or the derivatives of the corresponding saturated fatty acid. Hydroxylation takes place before incorporation into lipid A, and the adding of these acids to the glucosamine backbone is one of the final stages in the assembly of the outer layers of the wall (Humphreys et. al., 1972).

Polysaccharide fractions of the LPS contain glucose, rhamnose, galactosamine, 2-keto-3-deoxyoctonic acid, and alanine. Other amino sugars have been discovered in some strains but not all. There is indeed high variability among the *P. aeruginosa* strains with respect to the LPS polysaccharide (Meadow, 1975).

Aside from the cell wall, *P. aeruginosa* has also been shown to possess a cell glycocalyx (envelope) under certain conditions. Specifically, certain strains produce mucoid colonies or excrete large amounts of their glycocalyx when exposed to increased amounts of gluconate and magnesium (Vogel and Bonner, 1956). In fact, this extracellular substance

is important to macrophage evasion and pathogenesis, especially in cystic fibrosis patients (Govan, 1975). Furthermore, this envelope promotes cell-to-cell adhesion of bacteria that may assist in biofilm formation (in nature) or increased resistance of phagocytosis by alveolar macrophages (within a host) (Reynolds et. al., 1975). Electron micrographs of thin sections reveal that the envelope can extend as much as 2 micrometers from the bacterial surface (Costerton et. al., 1979).

Bacteriophages of *Pseudomonas aeruginosa.* *P. aeruginosa* has a number of viruses that researchers have used for genetic studies as well as for basic microbiology. Unlike the coliphages, viruses of *P. aeruginosa* have letter designations to group phages that are immunoreactive to each other (Holloway et. al., 1960). The phages are thus grouped by letter (A, B, C, D, E, etc.). This tradition has been broken in recent years due to the increase in number of phages isolated from natural environments. Furthermore, there has been a shift toward the usage of phages to study transduction and away from the study of immunoreactivity of bacteriophages. A summary of several important *Pseudomonas* bacteriophages is shown in Table 2.1.

Characterization of Bacteriophage UT1. Oladele Ogunseitan first isolated this bacteriophage (Ogunseitan, 1988) and named it LLPP5 for "Lake Loudon *Pseudomonas* Phage, plaque 5." Lake Loudon is a freshwater lake near the University of Tennessee. For simplicity, the name was changed to UT1 in the literature. While many of the transducing properties of the virus were characterized and published (Ogunseitan et. al, 1990), properties

Table 2.1 Bacteriophages of *Pseudomonas aeruginosa*

Bacteriophage	Family	Receptor	Life Cycle	Reference
	"Classical" Phages			
B86	*Siphoviridae*	**	Temperate	Kilbane et. al., 1988
D3	*Siphoviridae*	(Cell Wall)	Temperate	Miller et. al., 1974
E79	*Myoviridae*	LPS	Lytic	Jarrell et. a., 1977
F116	*Podoviridae*	Pilus	Temperate	Miller et. al., 1974
G101	*Siphoviridae*	**	Temperate	Miller et. al., 1974
	Recently Discovered Phages			
CTX	*Myoviridae*	LPS	Temperate	Yokota et. al., 1994
ACQ	**	**	Temperate	Schrader et. al., 1997
BLB	**	**	Temperate	Schrader et. al., 1997
UNL-1	*Myoviridae*	LPS	Temperate	Shaffer et. al., 1999
UT1	*Myoviridae*	LPS	Temperate	Ripp et. al., 1994

**Not studied or undetermined

of the virus itself were not. This phage was preliminarily classified by its virion morphology, namely that the head is octahedral with a 67.5 nm diameter about its horizontal axis, its collar is 10 nm, and it has a contractile sheath that measures 12 nm thick. There is also a 125 nm non-flexible tail core (Ogunseitan, 1988).

Phage morphology is important for at least two reasons. If UT1 is indeed mostly lytic, pseudolysogeny may be a crucial alternative to lytic replication inside an environment devoid of nutrients. Second, the size of the phage head (which contains the DNA) determines the amount of DNA that can be transduced. The electron microscopy results prompted a classification in the ICTV-approved phage family *Myoviridae* (Ogunseitan, 1988).

Phages with inflexible tail cores are predominantly members of the *Myoviridae* family (Murphy et. al., 1996), and this is a family populated by bacteriophages that undergo preponderantly lytic life cycles. It seems logical that this phage may prefer a lytic cycle.

The primary physical property of UT1 described in the literature is its likeness to *Pseudomonas* phage E79, a highly lytic bacteriophage similar to coliphage T4. Evidence of E79 and UT1 similarity was similar virion morphology and similar restriction patterns. E79 DNA does hybridize with labeled UT1 DNA (Ogunseitan et. al, 1990).

UT1 Host Range and Receptor. A limited host range for bacteriophage UT1 has been alluded to in the thesis originally reporting discovery of the virus (Ogunseitan, 1988), but nothing further on the matter has appeared in the peer-reviewed literature. The lone attribute used to determine host susceptibility of UT1 was the ability to form plaques

(Ogunseitan, 1988). Furthermore, only a few *Pseudomonas aeruginosa* strains were used in that study.

The mechanism that UT1 uses for cell entry has not been elucidated, though Ogunseitan did mention that "absorption of phage (UT1) to host cells appears to be similar to those of previously characterized, contractile tail phages" (Ogunseitan, 1988). This generalized mechanism was first described in 1967 (Bradley, 1967).

Recently Characterized *Pseudomonas* Bacteriophages. Because viruses are so ubiquitous in the environment, phages should be easily identifiable in many freshwater lakes. This has been demonstrated multiple times and in multiple environments (Ripp, 1996; Ogunseitan, 1988). Indeed, bacteriophages ACQ and BLB were isolated from Antelope Creek in Lincoln, Nebraska. ACQ is a nonUV-inducible lysogenic phage of *P. aeruginosa* with plaque morphology and immunity properties identical to the specialized transducing *P. aeruginosa* phage D3. BLB is a UV-inducible lysogenic generalized transducing phage of *P. aeruginosa* (Schrader et. al., 1997).

Another *Pseudomonas* virus, UNL-1, was isolated from a spontaneously arising plaque at the University of Nebraska-Lincoln. It was partially characterized by Shaffer et. al. (1999). Upon viewing UNL-1 particles in a transmission electron microscope following a negative stain with phosphotungstate, the phage was classified to be a member of the Myoviridae family. The head is approximately 80 nm in diameter, and its contractile tail measures 200 nm in length (Shaffer et. al., 1999).

The G/C content of the UNL-1 genome was determined to be 68% when examined spectroscopically. As was the case with UT1, DNA restriction fragment lengths were added in an effort to determine the length of the entire viral genome. It was estimated to be 48 kb long (Shaffer et. al., 1999), a length that is much shorter than UT1 and E79. Also unlike UT1, UNL-1 was shown to not be capable of mediating generalized transduction (Shaffer et. al., 1999). Despite this difference, however, UNL-1 has genome sequence similarity to UT1 of at least 86%.

Research on *Pseudomonas* bacteriophages is not limited to freshwater ecosystems. Researchers in Japan uncovered a bacteriophage, ΦCTX, that carries a *P. aeruginosa* cytotoxin. ΦCTX was isolated from a clinical strain of *P. aeruginosa* that had been purified from an individual with a *P. aeruginosa* infection, and it was determined that ΦCTX lysogenizes many clinically-important *P. aeruginosa* strains. It was shown that *P. aeruginosa* strains lysogenized with this virus were capable of producing the cytotoxin (Hyashi et. al., 1990). For this bacteriophage, the receptor was demonstrated to be in the LPS core region (Yokota et. al., 1994). The complete genome of ΦCTX was also sequenced, but this bacteriophage has not been compared to viruses isolated from the environment such as E79, UT1, or UNL-1 (Nakayama et. al., 1999).

CHAPTER III

IDENTIFICATION OF LPS AS THE HOST RECEPTOR FOR BACTERIOPHAGES UT1 AND UNL-1

Introduction

It is of paramount interest to understand how attachment of the bacteriophage to the host occurs. When the nature of this interaction is known, one can more clearly begin to learn more about when and where phages can exhibit dynamic interactions with their host bacteria. The consequences of studying phage-host symbioses will be addressed in later chapters.

Variations in cell surface structures exist across genera, species, and strains of bacteria. In order for a bacteriophage to have a limited host range, it must attach to a fairly specific receptor; and the receptor would have to be conserved over those hosts which it could infect. The only other alternative to using a common receptor would be to use different receptors for different hosts, but this would require that the phage would likely have to use different tail fibers for different hosts. This phenomenon has not been reported for any bacteriophages of *P. aeruginosa.* The purpose of this study is to elucidate the receptor for bacteriophages UT1 and UNL-1.

Materials and Methods

Bacterial Strains and Bacteriophages. *P. aeruginosa* PAO 303 is an *argB21* mutant of *P. aeruginosa* PAO1, the prototype member of the species. Other strains used are listed in Table 3.1. *Pseudomonas* phage UT1 was provided by R. V. Miller, and phage UNL-1 by T. Kokjohn.

Bacteriophage Plaque Assays. The method of Silhavy et. al. (1984) was employed. Host bacteria were grown to late logarithmic phase. Phage dilution (500 µl) was added to 4.4 ml of soft agar (1% BBL Trypticase, 0.5% sodium chloride, 0.65% agar, 48°C), and 100 µl of host bacteria were then added to the mixture and mixed by rolling for 10 seconds. The soft agar was then poured onto a Luria-Bertani (LB) plate (1.3% agar), allowed to cool, and stored at the appropriate temperature for host bacterial growth. Plaques were observed for morphology and counted the following day.

UT1 Propagation on Various Strains of *P. aeruginosa* PAO. UT1 (10^3 pfu) was inoculated into a 10 ml culture of bacteria at mid-log phase. After 10 hours, the cells were pelleted by centrifugation, and the resulting lysate was titered using the above assay.

Preparation of *P. aeruginosa* RM 4500. The method of Jarrell and Kropinski (1977) was utilized. *Pseudomonas aeruginosa* PAO303 were grown to mid-logarithmic phase and inoculated into 4.5 ml low-melting point phage titration agar (1% BBL Trypticase, 0.5% sodium chloride, 0.65% agar, 48°C). The mixture was gently mixed and poured onto

Table 3.1 UT1 Titer Propagated on Various *P. aeruginosa* Strains

PAO67	*his*$^-$	2 X 10^{10} pfu/ml
PAO886	*his*$^-$ *leu*$^-$ *pro*$^-$ *ade*$^-$ *ura*$^-$ *B1*$^-$	1 X 10^{10} pfu/ml
PAO860	*his*$^-$ *met*$^-$ *ade*$^-$ *ura*$^-$ *B1*$^-$	1 X 10^{8} pfu/ml
PAO8	*ilv*$^-$ *met*$^-$ Sm^R	1 X 10^{7} pfu/ml*
PAO844	*his*$^-$ *arg*$^-$ *ade*$^-$	5 X 10^{8} pfu/ml
PAO859	*his*$^-$ *met*$^-$ *ade*$^-$ *ura*$^-$	1 X 10^{7} pfu/ml*
PAO664	*pro*$^-$ *ade*$^-$	7 X 10^{9} pfu/ml
PAO871	*his*$^-$ *lys*$^-$ *ade*$^-$ *ura*$^-$ *B1*$^-$	2 X 10^{10} pfu/ml
PAO1	prototroph	8 X 10^{9} pfu/ml

*plaques formed in the acceptable range of 30-300 were available on 1 plate only.

an LB nutrient agar plate and allowed to cool. After 5 min., 20 µl of a high-titer E79 phage stock was administered to the center of the plate. The plate was allowed to dry and then placed at 37° C overnight. The following day, a rough colony (*Pseudomonas aeruginosa* PAO E^r) had grown up near the center of the cleared area of bacteria. This colony was restreaked and designated *P. aeruginosa* RM 4500.

Preparation of LPS by Phenol-Water Extraction. The method of Westphal (1969) was used to purify LPS. Twenty g (dry weight) of bacteria were suspended in 350 ml of water at 65° C. An equal volume of 65° C phenol was added and left to stir in a 65° C water bath for 15 min.. The mixture was then cooled to 10° C in an ice bath, and the emulsion centrifuged at 1000 x g for 40 min. This separated the mixture into a water layer, a phenol layer, and an insoluble residue formed at the phase interface. The water layer was saved, and the remaining phenol/residue was re-extracted with another 350 ml of preheated water. The resulting water extracts were dialyzed 4 days against nanopure water and treated with Proteinase K (20 µg/ml) after dialysis.

Preparation of LPS by Triton X-100 Treatment and Salt Precipitation. The method of Uchida and Mizushima (1987) was used. A 50 ml culture of late logarithmic-phase bacteria was spun down at speed of 12,000 x g for 20 min. and resuspended in 2.2 ml distilled water. To the mixture were added successively 0.4 ml of 100 m*M* Tris-HCl (pH 8.0), 0.4 ml of 0.5 *M* $MgCl_2$ and 1.0 ml of 8% Triton X-100. The vessel was tightly capped and heated in boiling water for 10 min.. After cooling, the mixture was centrifuged at

15,000 x g for 15 min. The resulting pellet was washed once with 4.0 ml of 10 m*M* Tris-HCl (pH 8.0)-10m*M* $MgCl_2$. The resulting pellet was resuspended in 4.0 ml of 10 m*M* Tris-HCl (pH 8.0)-10 m*M* $MgCl_2$ as well as 1.0 ml each of distilled water, 0.2 *M* EDTA (pH 8.0), 2 *M* NaCl, and 8% Triton X-100, and incubated at 37° C for 60 min. (while shaking). The mixture was then centrifuged at 15,000 x g for 15 min. The supernatant (~8 ml) was recovered, 0.8 ml of 1 *M* $MgCl_2$ added, stirred, and incubated again at 37° C for 60 min. The resulting cloudy solution was centrifuged at 100,000 x g (33,100 rpm in a Beckman 70.1 Ti rotor) for 90 min. at 4° C. The LPS (the pellet) was then resuspended in 4.0 ml 10 m*M* Tris-HCl (pH 8.0), treated with Proteinase K (20 μg/ml) at 37° C overnight, washed once with 4.0 ml of 10 m*M* Tris-HCl (pH 8.0)-10 m*M* $MgCl_2$, and re-centrifuged. The LPS pellet was dried and reconstituted in water.

Results and Discussion

Propagation of UT1 on Various Strains of *P. aeruginosa*. In an effort to identify membrane-bound proteins as possible viral targets, many independent mutants of *P. aeruginosa* were analyzed for their ability to propagate phage UT1. The exact nature of most of the mutations in these strains had not been studied, but it was anticipated that some of the autotrophic properties of these mutants could have stemmed from mutations in transporters and/or pathway-dependent enzymes. All mutant strains were capable of phage production, but the efficiency of UT1 growth on different strains varied. One explanation of the variable UT1 titers produced could have been an increased phage need for the nutrients for which the host was autotrophic. We did note that the mutant status of the

organisms caused markedly higher generation times. Because they grew at such disparate rates, they were thus likely less able to produce phage at comparable titers.

Bacteriophages UT1 and UNL-1 Bind Uniformily Over the Host Cell Wall. To examine the distribution of phage binding receptors on the bacterial host, electron microscopy was performed. Electron micrographs (Figures 3.1-3.4) show that bacteriophage particles of UT1 and UNL-1 are capable of binding to multiple sites along the *P. aeruginosa* PAO303 cell wall. No phages were observed binding to pili. Indeed, Figure 3.5 reveals the tail fibers of a UT1 phage particle juxtaposed over a pilus. Two distinct bacteriophage particles can in fact be seen attaching to both sides to the L_1-L_3 layer (the outer cell wall) of *P. aeruginosa* PAO303 in Figure 3.6, the layer that contains the lipid A portion of its lipopolysaccharide. These results suggest that UT1 does not bind to pili but rather to some molecule distributed over the surface of the bacteria.

Generation of an E79-resistant Organism. UT1 showed very little DNA sequence homology to many previously-characterized *Pseudomonas* bacteriophages (Ogunseitan, 1988). It was demonstrated, however, that UT1 was very similar to Myovirus E79 with respect to DNA sequence (Ogunseitan et. al., 1992). Since LPS has been proven to be the receptor for many Myoviruses including E79 (Jarrell and Kropinski, 1977), lipopolysaccharide was postulated to be a receptor for phages UT1 and UNL-1.

Generating UT1- or UNL-1-resistant organisms using the method of Jarrell and Kropinski (1977) was not useful because both UT1 and UNL-1 have been reported to

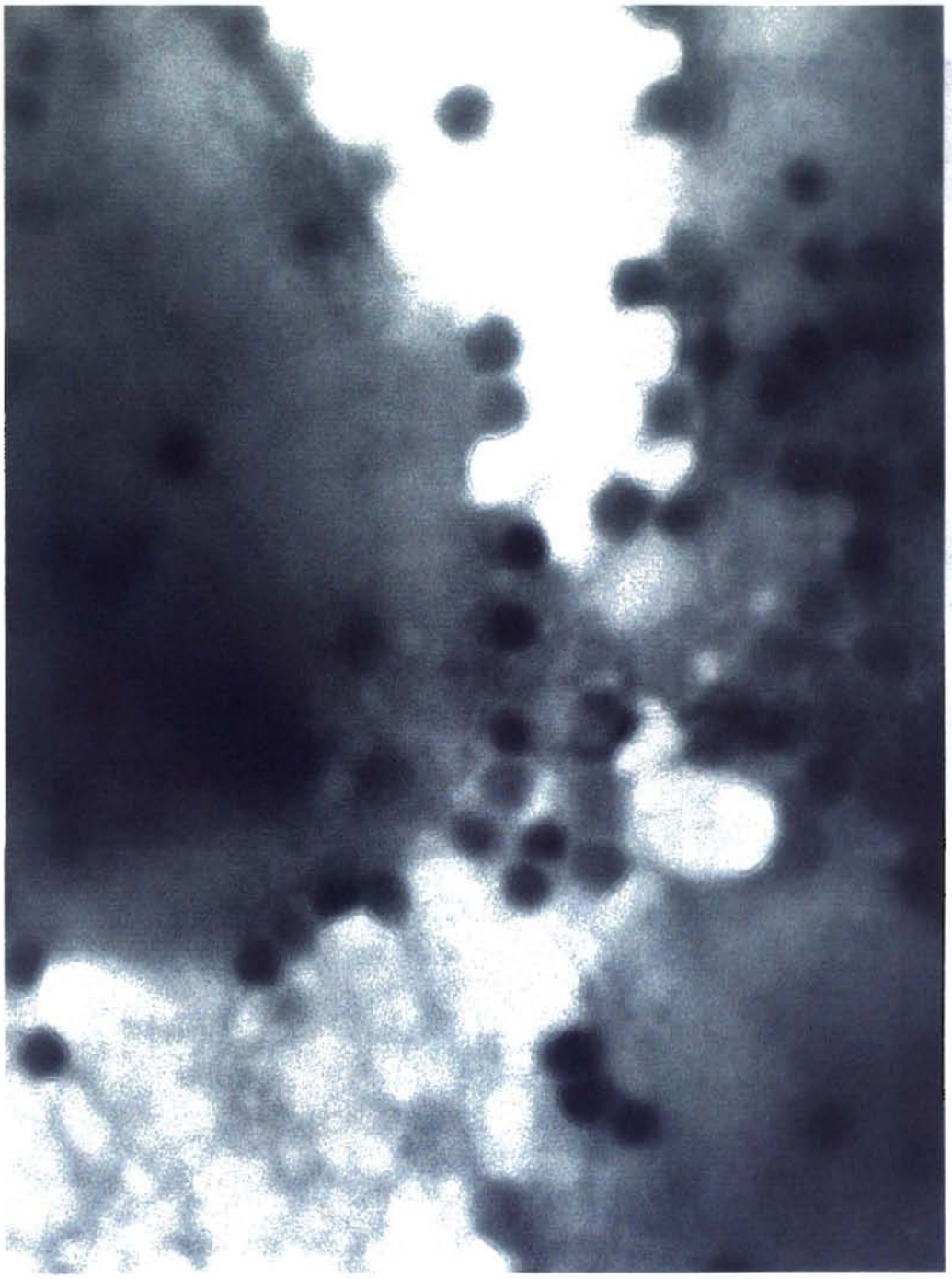

Figure 3.1 UT1 attaching to PAO303 (228,000 X).

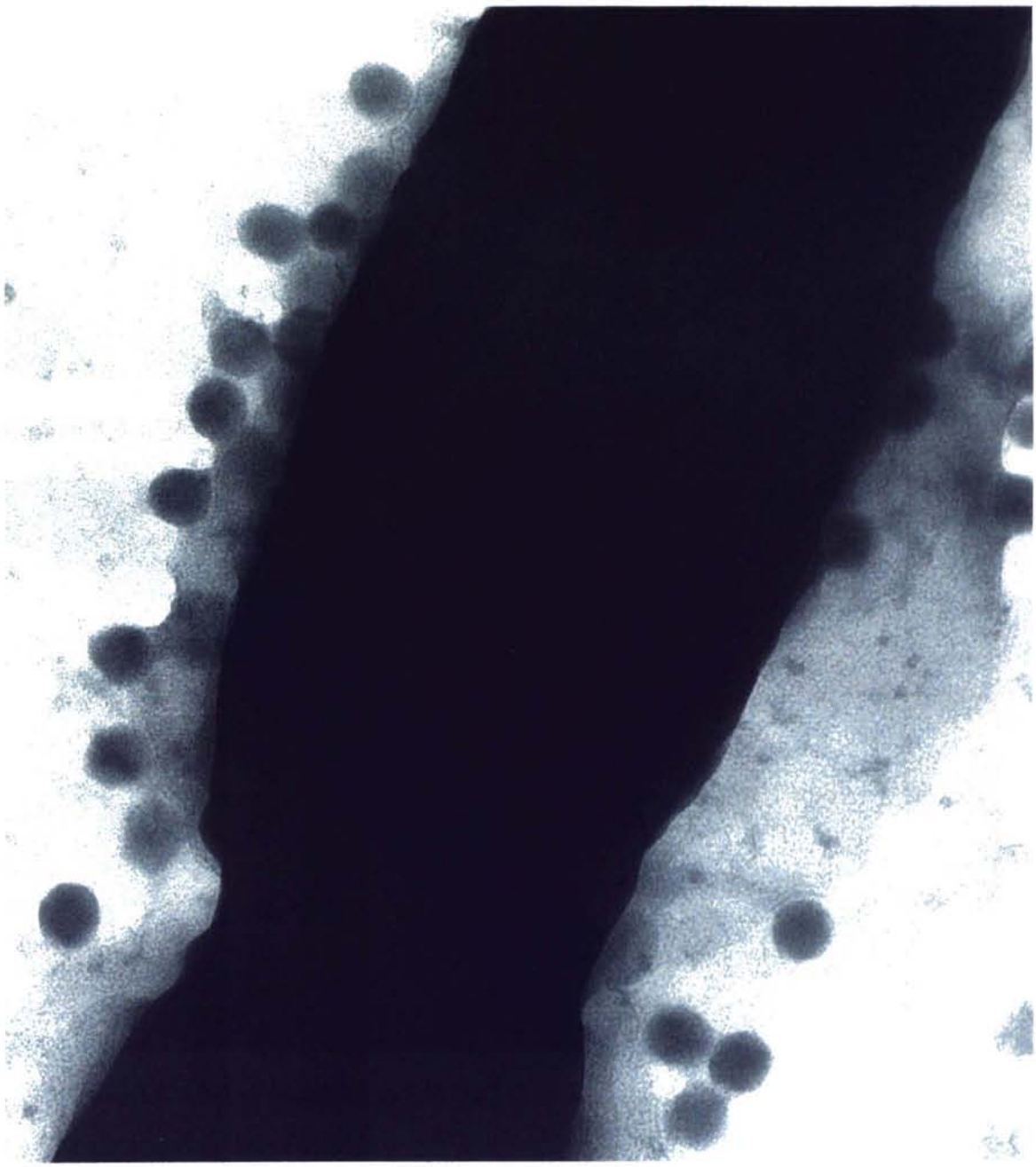

Figure 3.2 UNL-1 attaching to PAO303 (201,600 X).

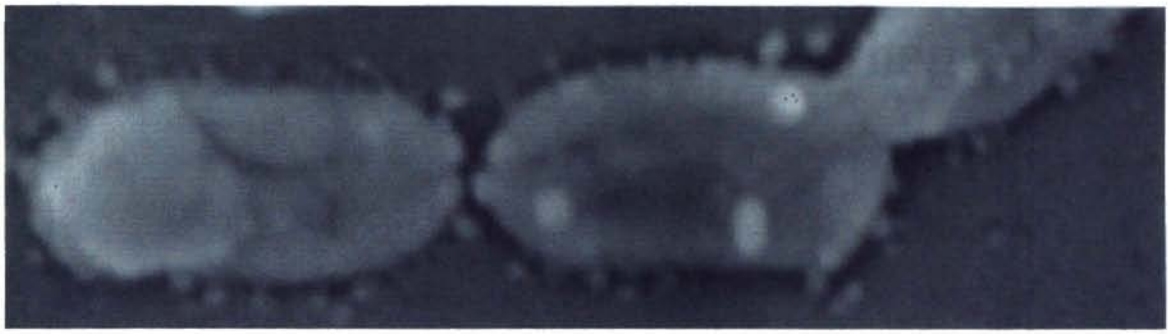

Figure 3.3 SEM of UT1 attaching to PAO1 (31,853 X).

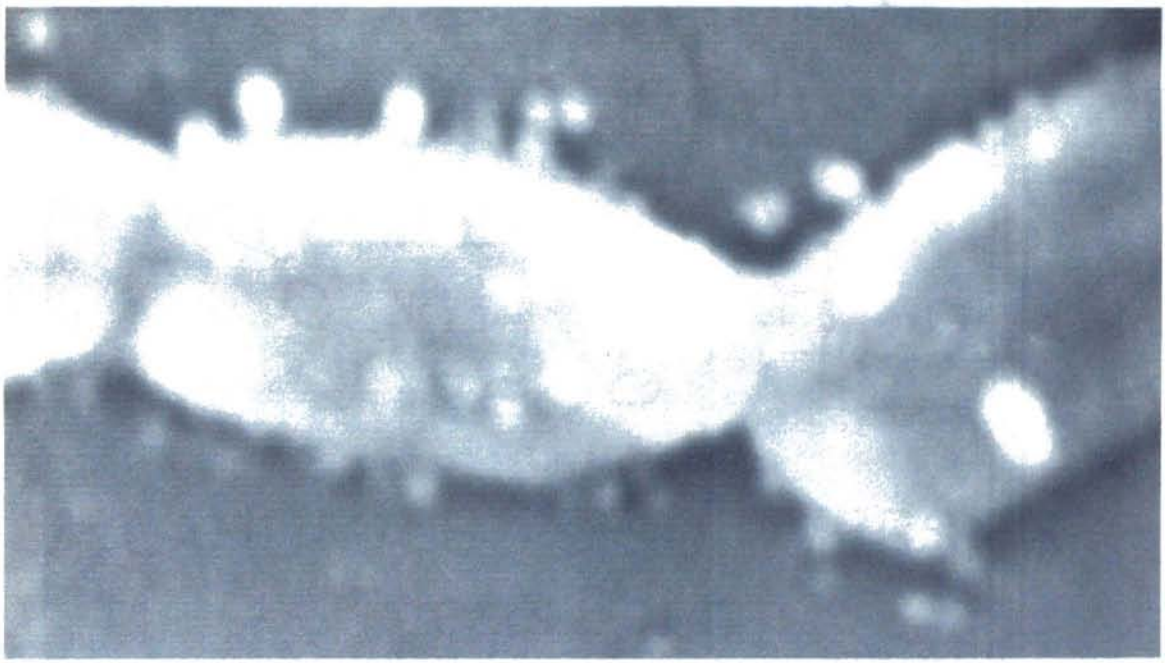

Figure 3.4 SEM of UT1 attaching to PAO1 (56,296 X).

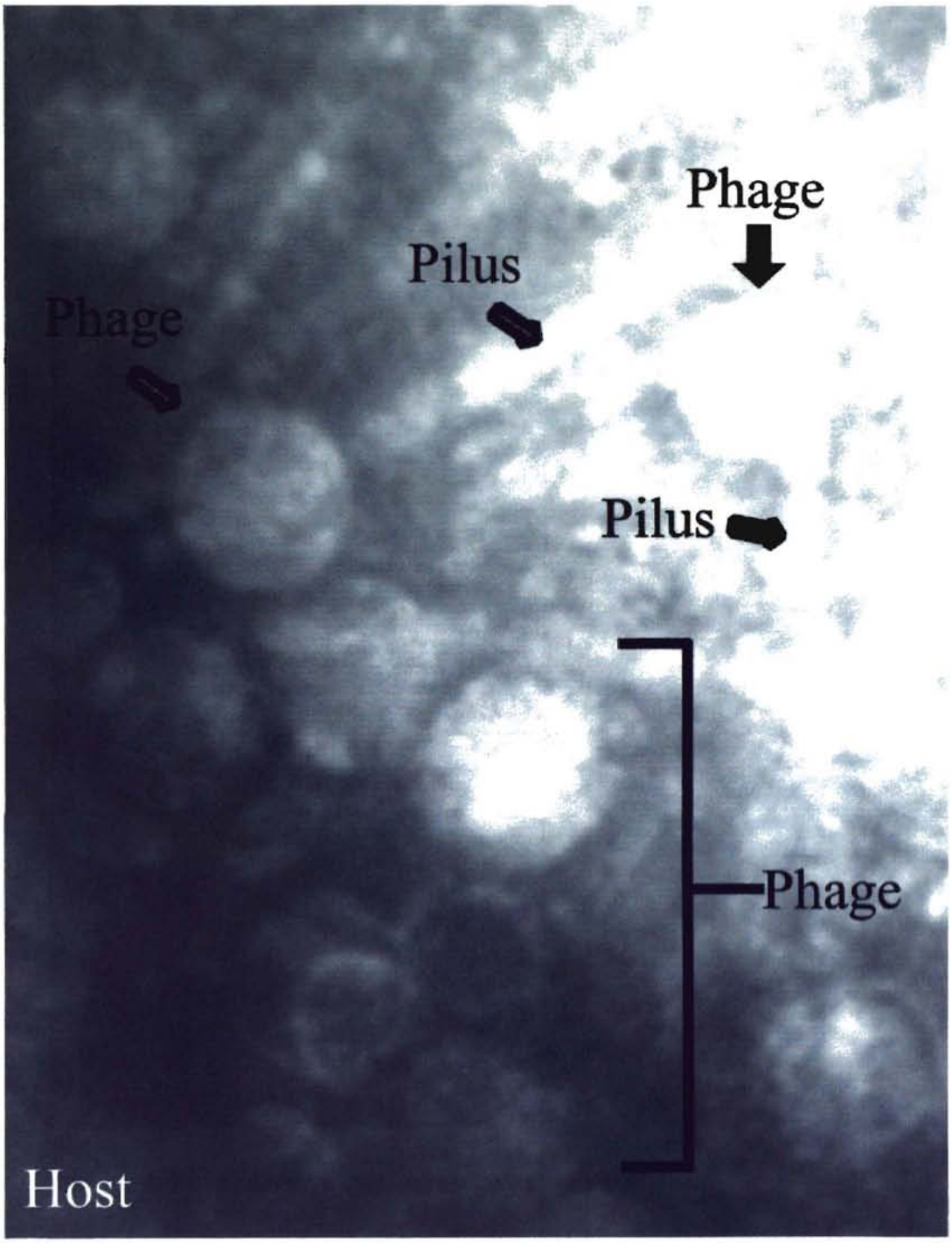

Figure 3.5 UT1 tail fibers juxtaposed over a pilus (360,000 X).

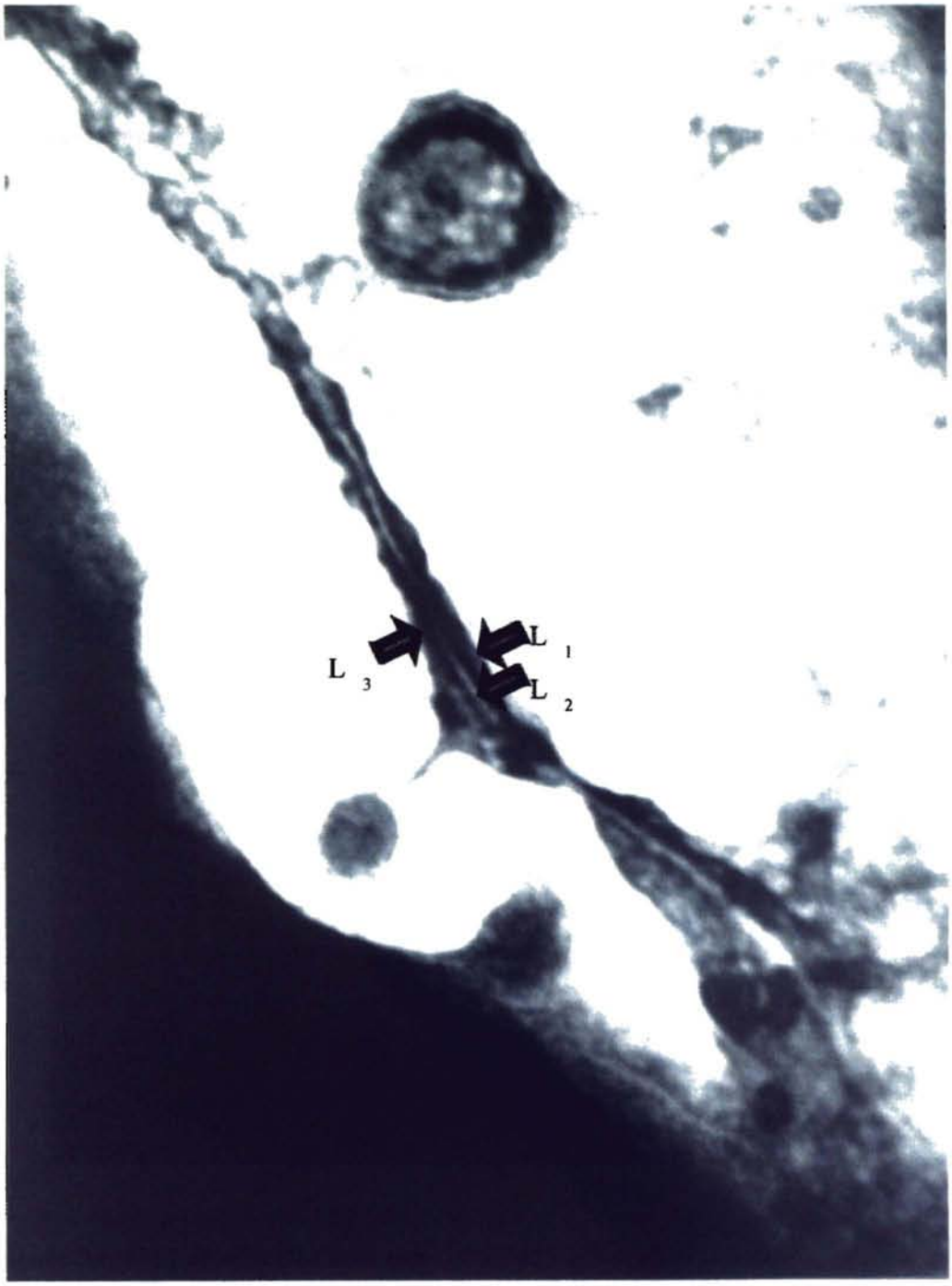

Figure 3.6 UT1 attaching to both sides of a PAO 303 wall (356,364 X).

generate lysogens that might be mistaken for resistant organisms (Ripp, 1996; Shaffer et. al., 1999). This property was confirmed and will be discussed in the next chapter. *Pseudomonas* phage E79, on the other hand, is analogous to coliphage T4 in that it does not lysogenize; E79 forms clear plaques, and it has been demonstrated to use the LPS as its receptor (Jarrell and Kropinski, 1977). When 20 µl of a high titer E79 lysate were inoculated onto a top agar/*P. aeruginosa* PAO303 mixture, a large zone of lysis resulted with several rough colonies emerging within. These colonies were restreaked and named *P. aeruginosa* RM 4500.

Characterization of *P. aeruginosa* RM 4500. *P. aeruginosa* RM 4500 produced rough colonies and was even found to cause rough lawns when spread plated. RM 4500 was shown to be immune to both UNL-1 and UT1 (Chapter 4). RM 4500 did not grow any slower than PAO1 (Figure 3.7), though it apparently produced a suspension of cells that was less optically dense or grew to a lower stationary phase titer. No phage attached to RM 4500 when inoculated with a high titer preparation of UT1 or UNL-1 as visualized by electron microscopy.

Preparation of LPS from PAO303 and RM 4500. The most common method for purifying bacterial lipopolysaccharides is the one developed by Westphal (1969). This method inadequately recovers LPS from rough (LPS deficient) strains (Key et. al., 1970), but it is successful in preparing LPS from a number of gram negative genera. This was certainly the case with *P. aeruginosa* RM 4500, as LPS was unable to be recovered using this hot

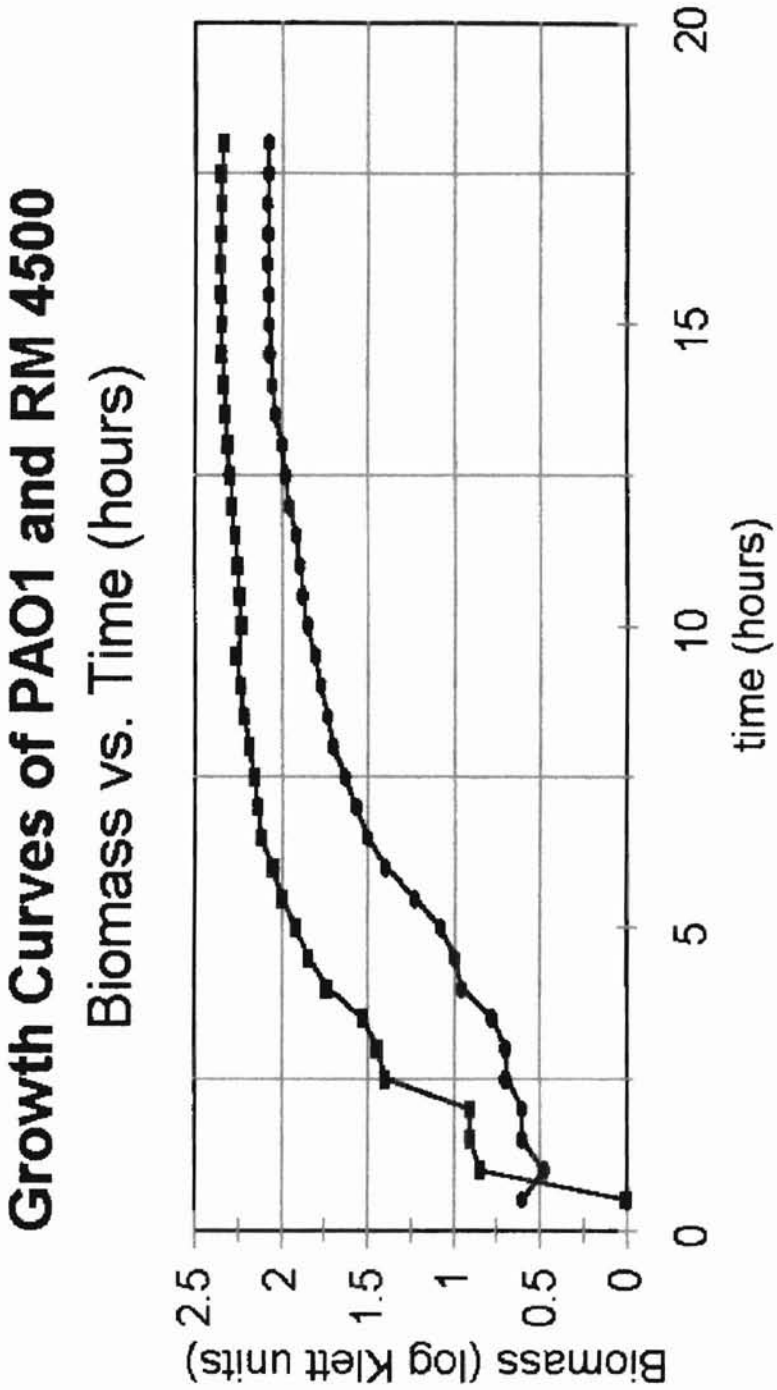

Figure 3.7 Growth curves of PAO1(■) and RM4500 (●).

phenol method. Furthermore, bacterial lawns grown for purposes of plaque assays during a titration assay containing LPS purified by this method grew unusually slowly. Fortunately, the method of Uchida and Mizushima (1987) was capable of extracting LPS from both rough and smooth strains even though it is fairly specific for *P. aeruginosa* LPS. In the final centrifugation step, RM 4500 (the UT1/UNL-1 resistant strain of *P. aeruginosa*) LPS produced a transparent pellet whereas LPS extracted from PAO303 generated a white pellet. Clearly, the lipopolysaccharide molecules of the phage-sensitive and phage-resistant bacteria were dissimilar.

Titration of Bacteriophage with LPS Decreases Efficiency of Plating. If LPS is the receptor for phage UT1 or UNL-1, exogenously provided LPS should decrease phage titers by either blocking the LPS binding sites or causing phage to spontaneously eject their head contents. A reduction in titer following treatment with LPS was observed for both UT1 (Figure 3.8) and UNL-1 (Figure 3.9), but not to the extent observed by Yokota, et. al. (1994) with ΦCTX or by Jarrell and Kropinski (1977) with E79. UT1 and UNL-1 titers were unaffected by incubation with LPS extracted from RM 4500.

Conclusion

The isolation of a UT1/UNL-1 resistant strain of *P. aeruginosa* (RM 4500) was accomplished by generating an organism immune to infection by *Pseudomonas* bacteriophage E79. UT1 and UNL-1 thus likely utilize the same receptor as E79. The rough nature of the colonies created by RM 4500 lends significant support to the fact that RM4500

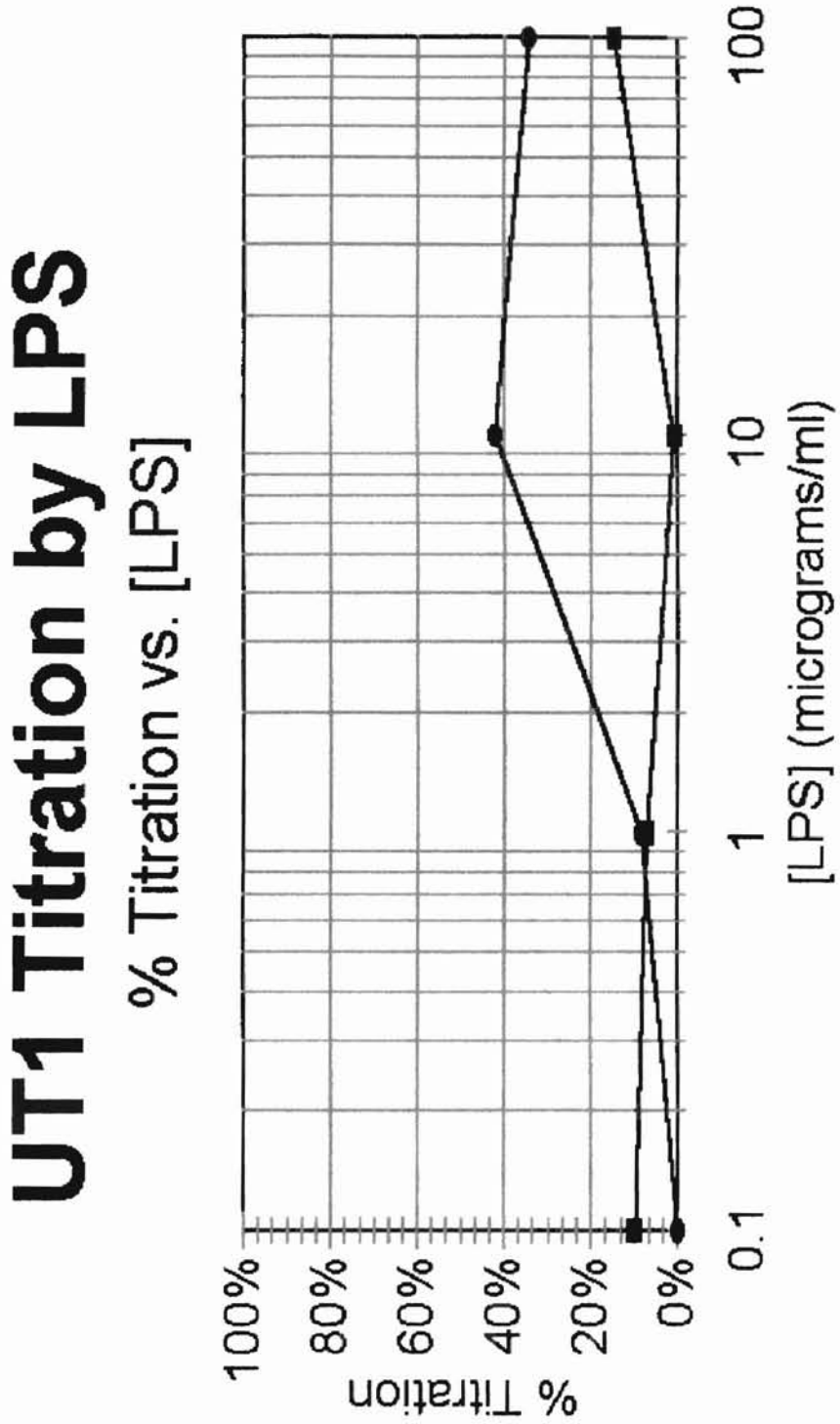

Figure 3.8 LPS effect on virulence of UT1. PAO303 LPS (■) and RM 4500 LPS (●). ANOVA showed significant differences at the LPS concentration of 10 μg/ml.

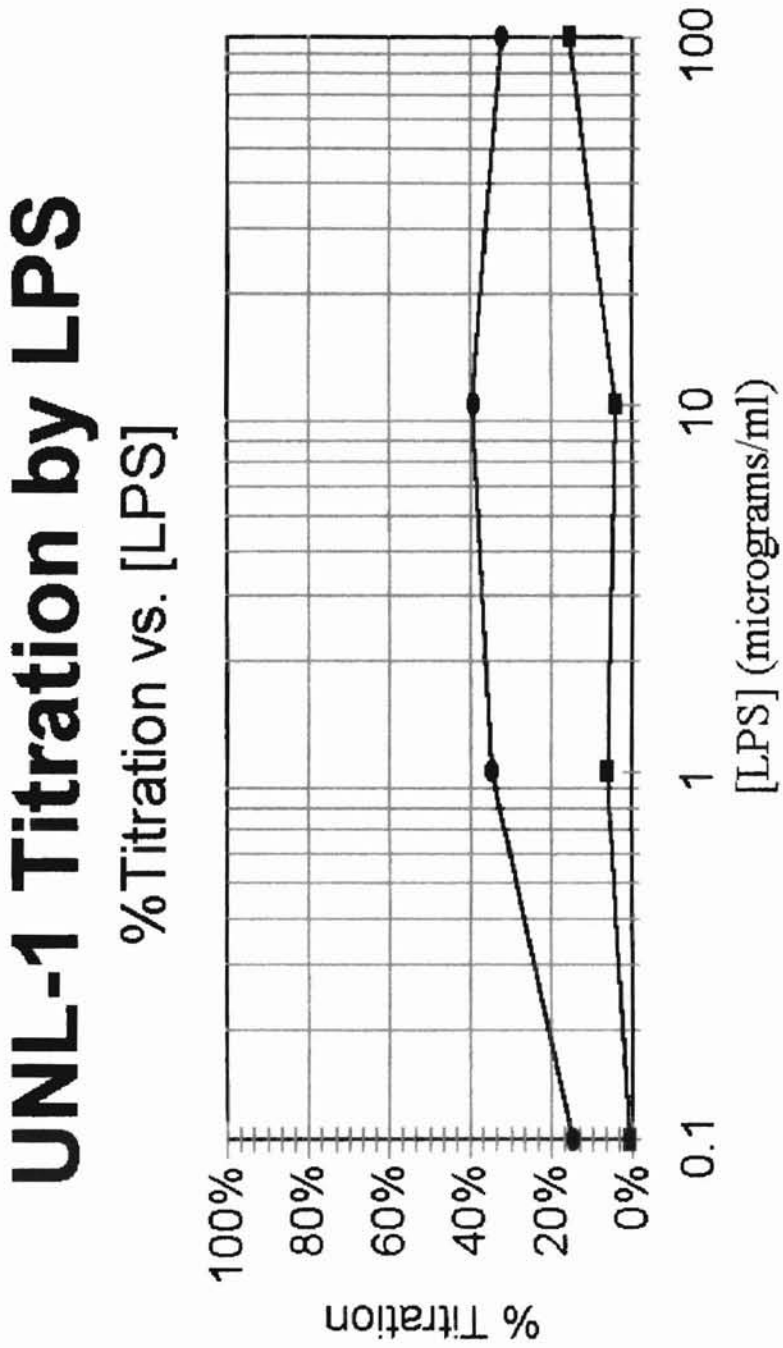

Figure 3.9 LPS effect on virulence of UNL-1. PAO303 LPS (■) and RM 4500 LPS (●). ANOVA showed significant differences for LPS concentrations 1 and 10 µg/ml.

is deficient in its lipopolysaccharide layer, as the lack of one or more portions of the LPS causes irregularities in the way that the cells stack on and around each other. The growth rate of RM 4500 paralleled that of PAO1. As a result, RM 4500 is likely a strain of *P. aeruginosa* that does not have any serious metabolic deficiencies.

Due to the fact that adding free LPS to the phage preparation decreased the infectious titer, it appears that the LPS has some neutralizing ability. In the case of both E79 and ΦCTX, the number of LPS binding sites on phage tails was unascertainable (Jarrell and Kropinski, 1977; Yokota et. al., 1994). Furthermore, the exact nature of lipopolysaccharide binding has yet to be determined for any of the *Pseudomonas* LPS-binding phages. It was postulated by Jarrell and Kropinski (1977) that the phage actually requires both a portion of the polysaccharide and the lipid A in order to get an injection event to occur. Because E79 resistance confers immunity to UT1 and UNL-1, the same might also be true for UNL-1 and UT1. Another possible conclusion is that the LPS may assume a different conformation when it is separated from the cell membrane and that this conformational change affects UT1 and UNL-1binding ability more than it affects E79 or ΦCTX binding ability. The purification method presented here required the use of magnesium chloride to precipitate the LPS. Unfortunately, the added presence of the divalent cation may have chelated the LPS and interfered with phage neutralization.

CHAPTER IV

DEFINING THE HOST RANGES OF BACTERIOPHAGES UT1 AND UNL-1

Introduction

Understanding the host range for a virus is an important step in understanding phage biology. Because all viruses are obligate intracellular parasites, the most important characteristic of a virus is what host it utilizes. In other words, what is its "habitat?" This is indeed an important question for any biological entity.

The host range of UT1 and UNL-1 has not been defined to date. UNL-1 was purified from a spontaneously arising plaque (Shaffer et. al., 1999), and it has only been studied using the original *P. aeruginosa* PAO host. Similarly, UT1 has only been used in the *P. aeruginosa* PAO system. Original work by Ogunseitan (1998) showed that all PAO strains were sensitive to UT1 infection except LPL5, which was a UT1 lysogen. He did not test other strains of *P. aeruginosa*, other *Pseudomonas* species, or any other gram-negative organisms.

It has been shown that UT1 is a generalized transducing phage (Ripp et. al., 1994). If a bacteriophage such as UT1 or UNL-1 could in fact infect other types of bacteria, such a phage could be an important transducing vector. The introduction of genetically

engineered microorganisms into an ecosystem which contains such a wide host range bacteriophage could have significant consequences on the balance of that ecosystem. Moving of genes from a GEM into a natural organism might not yield any significant consequence, as genes transferred might not be active in the new host. If the new gene(s), however, would provide some selective advantage for the new host, some random mutation event (such as that which might occur in a promoter region) might provide the needed force to generate a genetically engineered environment.

Unlike UT1, UNL-1 is not capable of generalized transduction in *P. aeruginosa*. Its high sequence homology to UT1 (Shaffer et. al., 1999) thus leaves us with many interesting questions. No studies to determine the host range of UNL-1 have been published.

In this study, several gram-negatives, *Pseudomonas* species and strains of *P. aeruginosa* were examined. With regard to host range, there are three questions that are pertinent to understanding the bacteriophage:

1. Which organisms can the phage attach to?
2. Which organisms can the phage lyse when introduced in high titer?
3. Which organisms can the phage propagate (produce plaques) on?

Materials and Methods

Preparation of Phage Lysates. *P. aeruginosa* PAO1 or PAO303 (an argB21 mutant) were grown for 8 hours (late mid-logarithmic phase) in Luria-Bertani (LB) broth and then infected with phage at a multiplicity of infection (MOI) of approximately 0.1. At 10 hours post-infection, cells were centrifuged at 4,300 x g or 30 min. in a Sorvall centrifuge.

The supernatant (phage lysate) was then filtered through a 0.45 micron filtering apparatus and stored at 4°C.

Purification of Bacteriophage for Electron Microscopy. Phage lysates were centrifuged through a glycerol gradient at 146,550 x g for 1 hour and fifteen min. at 4°C in a Beckman ultracentrifuge. The supernatant was decanted into a bleach solution. The resulting pellets (purified phage) were suspended in 100 microliters of TE buffer (10 m*M* Tris-HCl, pH 8.6, 10 m*M* EDTA) and infectious phage titrated by plaque assay.

Analysis of Bacteriophage Attachment to Hosts. Purified phage were added to mid-logarithimically growing bacteria at an (MOI) of approximately 100. After an adsorption period of 5 min., a carbon and formvar coated 200 mesh grid was touched to the bacteriophage-host suspension, the suspension allowed to settle for 1 min., and negatively stained with 2.5% uranyl acetate for 1 min. Grids were washed (1 minute) in sterile water, blotted dry, and viewed with a JEOL JEM 100 CX II STEM.

Observation of Lytic Properties. A high titer lysate (50 µl, $>10^{10}$) of phage were added to the center of a lawn of host bacteria on an LB plate and allowed to dry. The plates were allowed to incubate at an optimal growth temperature (30°C for *P. putida, P. fluorescens,* and *B. cepacia*, 37°C for the others) overnight. Bacteria susceptible to lysis by the phage had a large plaque in the precise shape of the phage suspension added to the plate (a spot lysis assay). Bacteria immune to phage were identified by the absence of lysis.

Bacteriophage Plaque Assays. The method described in Chapter 3 was employed. Host bacteria were grown to late logarithmic phase. Phage dilution (500 µl) was added to 4.4 ml of soft agar (1% BBL Trypticase, 0.5% sodium chloride, 0.65% agar, 48°C). Host bacteria (100 µl) were then added to the mixture and mixed by rolling for 10 seconds. The soft agar was then poured onto an LB plate (1.3% agar), allowed to cool, and stored at the appropriate temperature suited for host bacterial growth (30°C for *P. putida*, *P. fluorescens*, and *B. cepacia*, 37°C for the others). Plaques were observed for morphology and counted the following day.

Results and Discussion

Visualization of Phage Attachment to Hosts. A typical example of attachment is provided in Figure 4.1 . Results of phage attachment studies are summarized in Table 4.1. In most cases, few viruses were seen in the background unattached to bacteria. Cases where no viral attachment occurred thus had little or no phage in the field, or the phages were congregated away from the surface of the bacterial cell. A typical instance of non-attachment is provided in Figure 4.2.

Phage Lysis of Host Bacteria. Results of bacteriophage lysis studies are summarized in Table 4.3. Photographs of typical positive and negative results are shown in Figure 4.3. In general, zones of lysis were visibly apparent. *P. aeruginosa* PAT gave an unusual densely turbid lytic zone. Others were mostly clear in appearance. It was initially

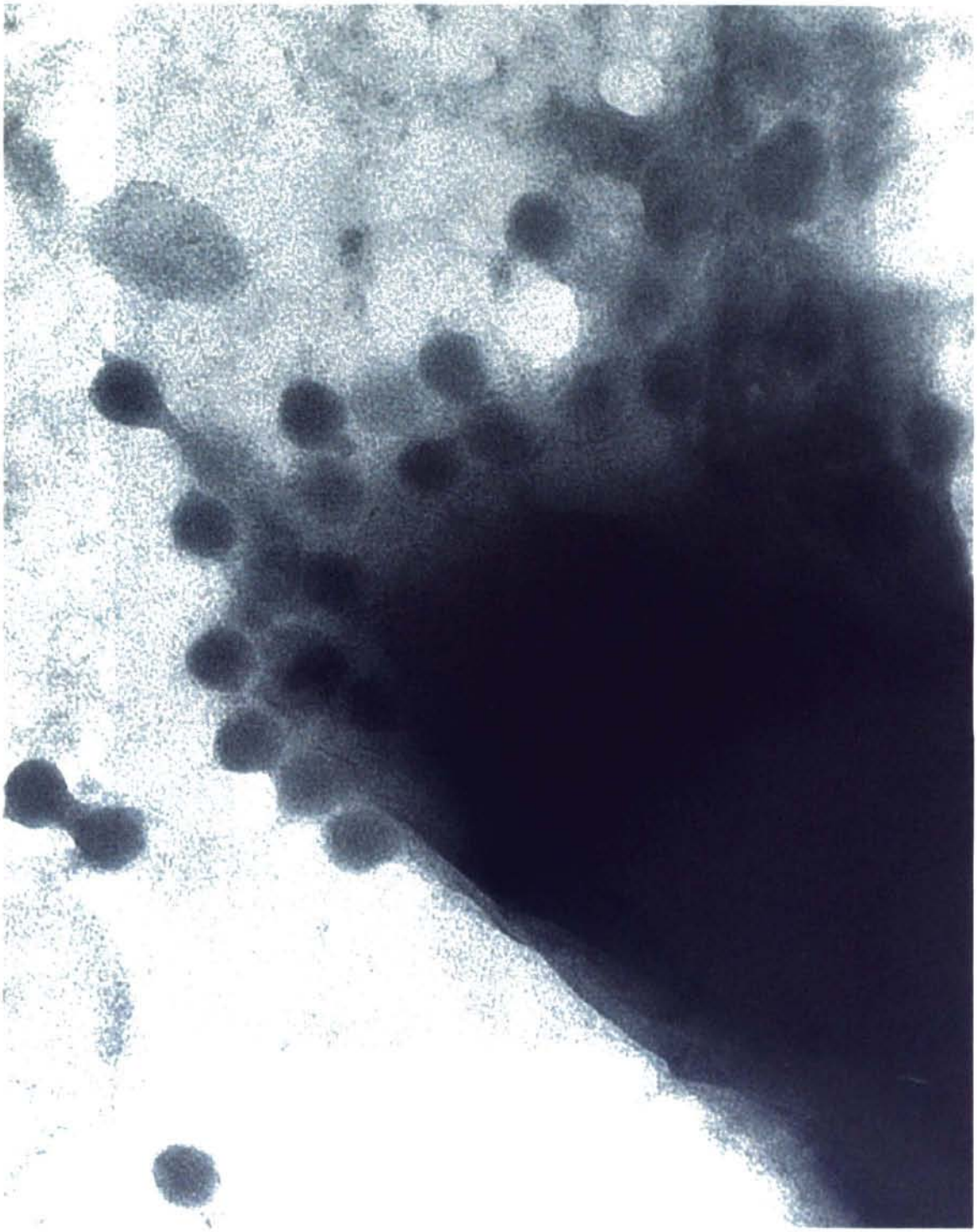

Figure 4.1 UNL-1 binding to PAO303 (235,714 X).

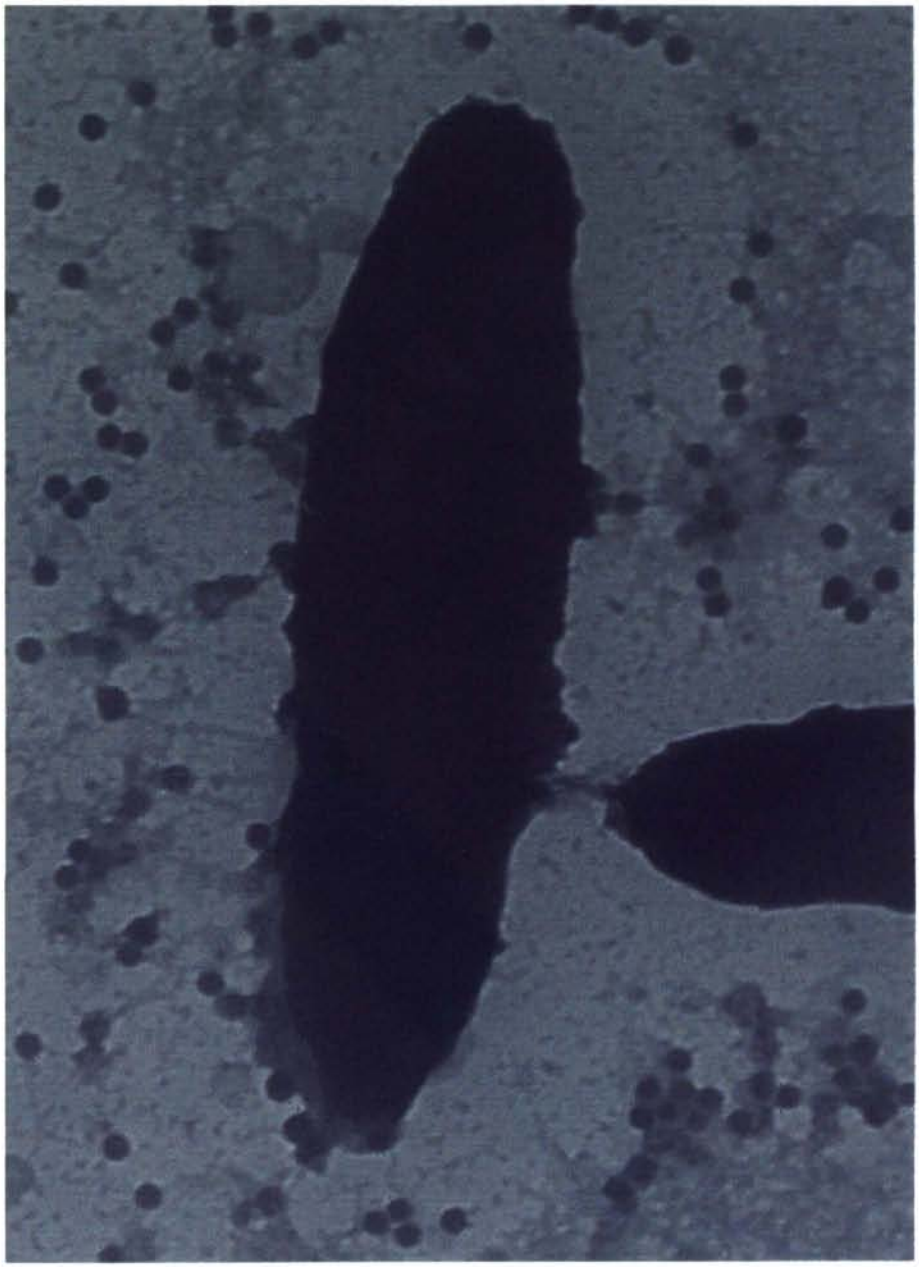

Figure 4.2 RM 4500 exposed to UT1 (65,143 X).

Table 4.1 Phage Attachment to Various Bacterial Strains

Bacterial Strain Tested	UT1	UNL-1
Pseudomonas aeruginosa PAO1	++	++
Pseudomonas aeruginosa PAO303	++	++
Pseudomonas aeruginosa PAT	++	++
Pseudomonas aeruginosa RM 4500	-	-
Pseudomonas aeruginosa LLPA10	++	++
Pseudomonas aeruginosa LPL5	+	+
Pseudomonas aeruginosa RM 2097	++	+
Pseudomonas aeruginosa RM 759	+	++
Pseudomonas aeruginosa FRD	-	-
Burkholderia cepacia	++	+
Pseudomonas putida	-	-
Pseudomonas fluorescens	-	-
Salmonella typhimurium	-	-
Escherichia coli B*	-	-
Seratia marcesans	-	-
Vibrio natriegans	-	-

++ many phage particles were observed bound to the bacterial cells
+ some phage particles were observed bound, though not the majority
- very few phage particles were observed bound if any

Table 4.2 Phage Lysis of Bacterial Lawns

Bacterial Strain Tested	UT1	UNL-1
Pseudomonas aeruginosa PAO1	+	+
Pseudomonas aeruginosa PAO303	+	+
Pseudomonas aeruginosa PAT	+	+
Pseudomonas aeruginosa RM 4500	-	-
Pseudomonas aeruginosa LLPA10	+	+
Pseudomonas aeruginosa LPL5	+	+
Pseudomonas aeruginosa RM 2097	+	+
Pseudomonas aeruginosa RM 759	+	+
Pseudomonas aeruginosa FRD	-	-
Burkholderia cepacia	+	+
Pseudomonas putida	-	-
Pseudomonas fluorescens	-	-
Pseudomonas syringae	-	-
Salmonella typhimurium	-	-
Escherichia coli B*	-	-
Seratia marcesans	-	-
Vibrio natriegans	-	-

+ a zone of lysis was observed
- a bacterial lawn grew unperturbed

Table 4.3 Plaque Production on Various Strains of Bacteria

Bacterial Strain Tested	UT1	UNL-1
Pseudomonas aeruginosa PAO1	+	+
Pseudomonas aeruginosa PAO303	+	+
Pseudomonas aeruginosa PAT	+	+
Pseudomonas aeruginosa RM 4500	-	-
Pseudomonas aeruginosa LLPA10	+	+
Pseudomonas aeruginosa LPL5	+	+
Pseudomonas aeruginosa RM 2097	+	+
Pseudomonas aeruginosa RM 759	+	+
Pseudomonas aeruginosa FRD	-	-
Burkholderia cepacia	+	+
Pseudomonas putida	-	-
Pseudomonas fluorescens	-	-
Salmonella typhimurium	-	-
Escherichia coli B*	-	-
Seratia marcesans	-	-
Vibrio natriegans	-	-

+ plaques were produced
- no plaques were observed

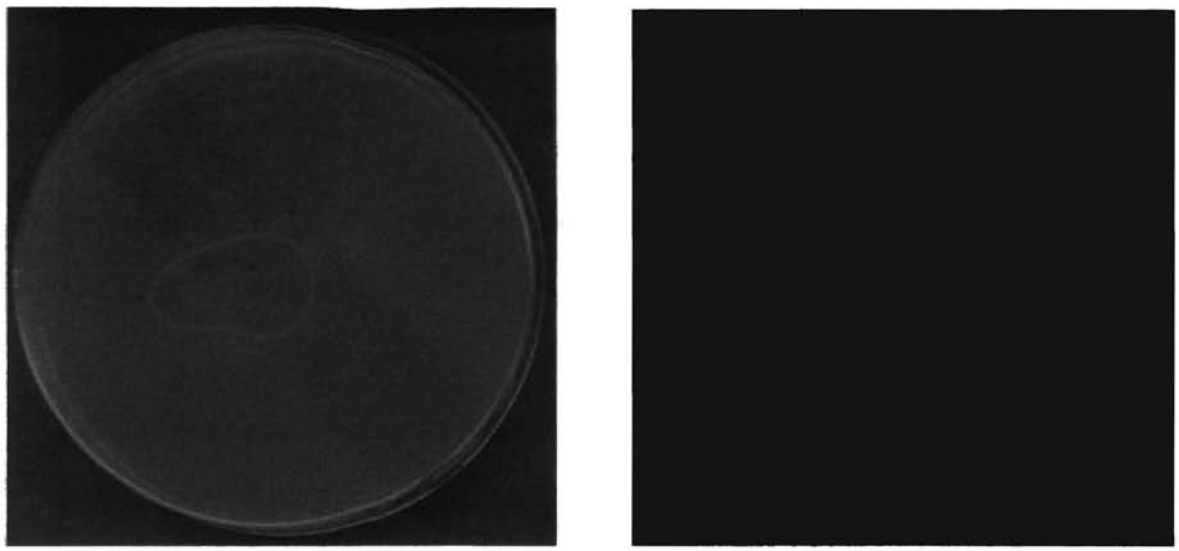

Figure 4.3 Typical results of bacteriophage lysis. Left: UT1 plated onto a PAO303 lawn (note the zone of lysis). Right: UNL-1 plated onto a LPL5 lawn (no lysis observed).

thought that *Escherichia coli* could serve as a host for UT1 and UNL-1, but these results were later shown to be the result of contamination with *P. aeruginosa* PAO303.

Ability of Phage to Form Plaques on Hosts. Results of phage plaque assay were consistent with phage adherence assays. There were no cases of plaque formation in strains that did not experience lysis when dosed with a high titer of phage. The highest degree of turbidity of the plaques was shown with *P. aeruginosa* PAT. This could be due to a high degree of lysogeny or an increased resistance. Interestingly, plates that showed the highest number of plaques also exhibited the largest amount of pyocyanin released into the agar medium except when there was so much phage on the plate as to cause confluent lysis over the surface of the whole petri dish. In all cases where plaques were formed, the titer was not significantly different from the titer of the positive control, *P. aeruginosa* PAO303. Sensitive strains were all of derivatives of PAO1 except for *B. cepacia*. Virus propagated on *P. aeruginosa* PAO303 had the same efficiency of plating when titrating on PAT hosts and PAO hosts. Virus propagated on *P. aeruginosa* PAT had the same efficiency of plating when titrating on PAO303 hosts and PAT hosts. *P. aeruginosa* FRD was immune to infection.

Conclusion

P. aeruginosa phages UT1 and UNL-1 have identical host ranges with respect to the bacterial host strains tested in this study. The only non-PAO strains sensitive to those phage were *Burkholderia* (formerly *Pseudomonas*) *cepacia* and *Pseudomonas aeruginosa* PAT. This shows that UT1 and UNL-1 are highly selective with respect to their choice of receptor.

Interestingly, these phages can both go outside of the genus to infect *Burkholderia cepacia*, but they are quite limited within the species of *P. aeruginosa*.

Also of interest is the fact that the viruses can grow equally well on PAT and on PAO even after being propagated on the other. This is somewhat riveting because PAT and PAO have restriction/modification systems that restrict each other much like *E. coli* K12 and *E. coli* B. In the case of *E. coli*, Arber's work with coliphage λ was one of the first to show that restriction/modification systems exist between strains of organisms within the same species. In that study, phages first grown on *E. coli* K12 grew poorly on *E. coli* B. Those that did grow on *E. coli* B were very successful at propagating on B in the future, but they then failed to grow well when plated back on K12. If *P. aeruginosa* had been Arber's organism of choice and UT1 or UNL-1 his bacteriophage of choice, we may still be ignorant to the presence of restriction enzymes.

An important characteristic of a phage that has lysogenized is the fact that it confers immunity to superinfection by the same phage. *P. aeruginosa* LPL5 is a bacterial strain that has been shown to be a lysogen of UT1 (Ogunseitan, 1988). LPL5 was immune to both UT1 and UNL-1 when challenged. RM 759 is a lysogen of ΦDS-1 (a relative of Podovirus F116), and RM 2097 is a lysogen of *Pseudomonas* phage D3 (a relative of coliphage λ). Both were sensitive to infection by UT1 and UNL-1.

The host range of phages UT1 and UNL-1 are limited and likely do not extend far beyond the PAO strain. The environmental ramifications of this are such that UT1 or UNL-1 might be a suitable vector for in situ genetic engineering of freshwater ecosystems. UNL-1 could be of particular significance because it does not transduce chromosomal genes to a

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measurable extent, and it doesn't seem to be capable of pseudolysogeny. Its half-life would likely be considerably less as a consequence.

CHAPTER V

RELATEDNESS OF BACTERIOPHAGES UT1 AND UNL-1

Introduction

It has been shown that both UNL-1 and UT1 require the lipopolysaccharide as the receptor for host infection, and the host range of the two bacteriophages are identical. Shaffer et. al.(1999) showed that UNL-1 DNA hybridizes strongly with DNA purified from UT1. The same report also calculated the UNL-1 chromosome at 48 kb, 30 kb less than that of UT1. Many of the UNL-1 DNA restriction fragments were of identical lengths to that of UT1 DNA, but it was demonstrated that UNL-1, unlike UT1, could not mediate generalized transduction (Ripp et. al., 1994). What was strikingly ignored, however, was the fact that UT1 (with a DNA length of 79 kb) possessed a smaller capsid (67.5 nm) than UNL-1 (80 nm) even though the chromosome was larger. This would not agree with the headful rule (the property that phage pack their capsids with as much DNA as possible), but it could be an indicator that UNL-1 may serve as a vector by using the extra head room to transduce foreign DNA.

While many of the ecological consequences and DNA reactivation characteristics of these viruses have been examined, little effort has been placed on trying to discover physical properties of the viruses. The goal of this set of experiments was to look at some

of the physical attributes in an effort to better understand the biology of these phages and to examine the similarities between the viruses.

Materials and Methods

Bacteriophage Purification for Purposes of DNA Analysis. The method of Silhavy et. al. (1984)was used with slight modification. Polyethylene glycol (6000 MW) was added to a phage lysate at a concentration of 10% (w/v) and allowed to stand at 4°C overnight. The phage-PEG pellet was then collected by centrifugation at 6000 x g for 10 min. at 4°C. The pellet was resuspended in 50 m*M* Tris-HCl, pH 7.5, 10 m*M* $MgSO_4$ (TM buffer). Phage was extracted from the solution with an equal volume of chloroform, and the solution was centrifuged at 2000 x g for 10 min. to separate the organic phase. The aqueous phase (4 ml) was then loaded onto a glycerol gradient (2 ml 40% glycerol, 2 ml 5% glycerol) and then centrifuged at 100,000 x g for 60 min. at 4°C. The resulting pellet was resuspended in 1 ml TM buffer and treated with RNase A (final concentration 10 μg/ml) and DNase I (final concentration 1 μg/ml) and allowed to incubate 30 minute at 37°C.

Purification of Bacteriophage DNA. STEP buffer (0.5 ml of 0.5% SDS, 50 m*M* Tris-HCl, pH 7.5, 0.4 *M* EDTA, 1 mg Proteinase K/ml) was added to the above suspension and allowed to incubate overnight. After a 15 min. incubation at 50°C, DNA was extracted successively with Tris-saturated phenol, phenol:chloroform:isopentyl alcohol (25:24:1), and chloroform:isopentyl alcohol (24:1). The DNA was then precipitated with 1/10 volume 3 *M* sodium acetate and 2 volumes cold ethanol. The DNA was then centrifuged 30 min. at

3,000 x g, washed with 70 % ethanol, and then resuspended in 10 m*M* Tris-HCl, pH 7.5, 10 m*M* EDTA. DNA concentration was determined by the ethidium bromide spot method using salmon sperm DNA as a standard.

Restriction Analysis of Bacteriophage DNA. Restriction enzymes were purchased from Promega. Protocols were provided by the supplier. Briefly, 1 µl of 10X restriction buffer, 1 µg DNA, and 1 µl enzyme were diluted to 10 µl with nanopure sterile water and allowed to react at 37°C for two hours.

Agarose Gel Electrophoresis. 1% agarose gels were made with 1X TAE running buffer (0.484 % Tris, 01142 % glacial acetic acid, 0.3722% EDTA). In most cases, the gels were electrophoresed overnight at 25 volts. Lambda DNA was cut with *Hin*dIII and used as a standard.

Visualization of Phage DNA Using Electron Microscopy. Phage DNA (10 µg) was added to a 40 µl staining solution described by Mayer, et. al. (0.2 *M* ammonium acetate, 2 µg/ml cytochrome c, 0.07 *M* formaldehyde, and 0.001 *M* EDTA). This preparation was gently spread over parafilm, and then a drop of it was applied to a formvar and carbon-coated 300-mesh electron microscopy grid. DNA was then stained with 1% uranyl acetate and then viewed in an STEM as described in the previous chapter.

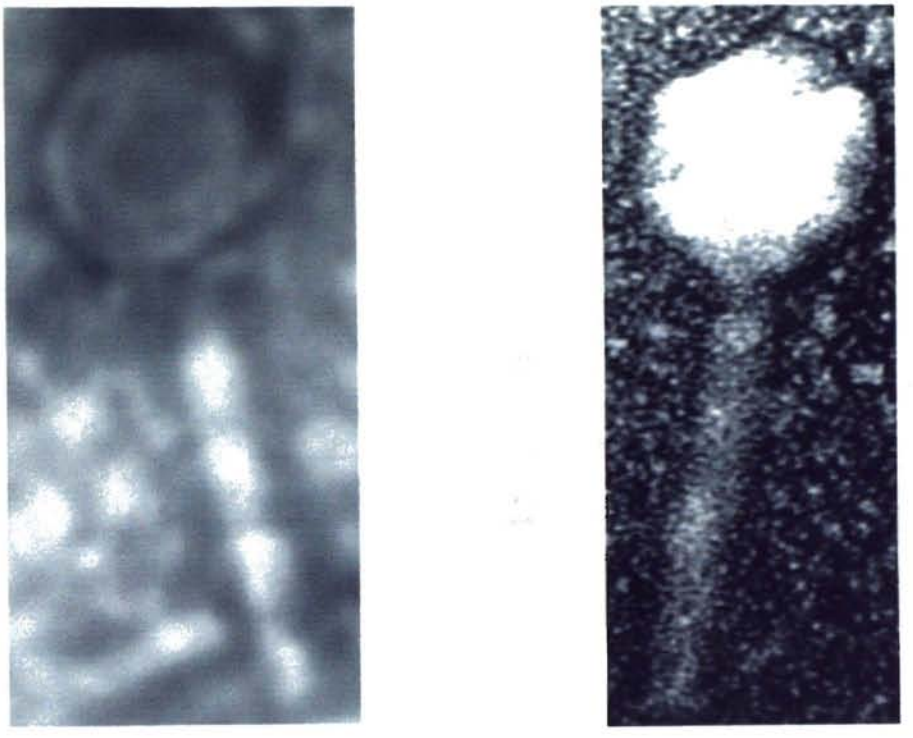

Figure 5.1 Bacteriophages UNL-1 and UT1. Right: UNL-1 (416,000 X), Left: UT1 (420,000 X).

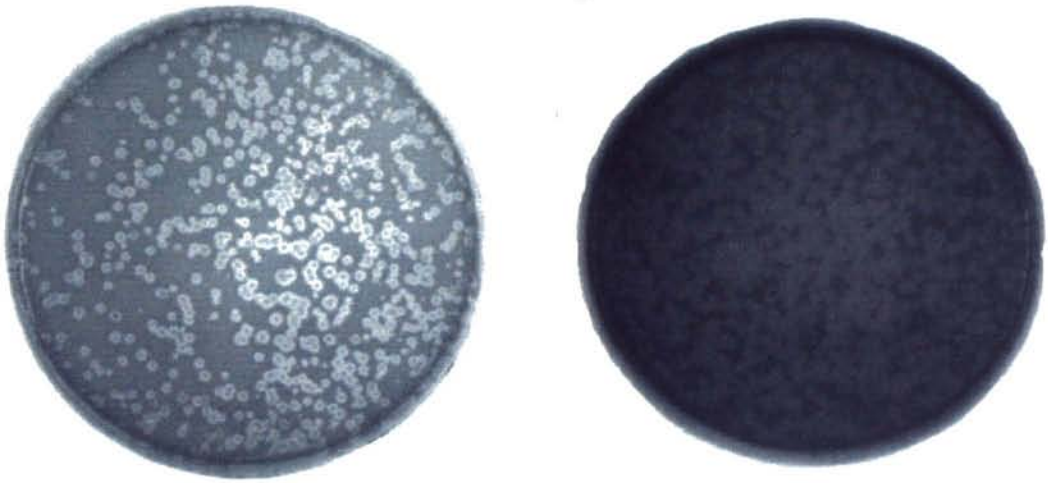

Figure 5.2 Differing plaque morphologies of UT1 and UNL-1. Both phages are capable of both lysogenic plaques (left) and clear, halo-forming plaques (right).

Results and Discussion

Temperate Behavior of UNL-1 and UT1. UNL-1 is classified as a "lytic bacteriophage of *P. aeruginosa*" (Shaffer et. al., 1999), and UT1 is known to be capable of both temperate and vegetative growth. It was determined, however, that UNL-1 cannot grow on *P. aeruginosa* starved for 24 hours (Shaffer et. al., 1999); and it is not capable of generalized transduction. What was not reported, however, was the ability of UNL-1 to produce turbid plaques. Such turbid plaques were observed in this study, and they were particularly apparent when UNL-1 was assayed using *P. aeruginosa* PAT as a host.

The genetic switch between lytic replication and lysogeny in UT1 remains an enigma. For some time in this study, only turbid plaques were observed for UT1 (which were not reported previously for plaque assays using laboratory strains of *P. aeruginosa*). When a spontaneously appearing clear plaque emerged, it was plaque-purified and regrown. Some progeny retained the ability to produce clear plaques. Others reverted back to turbidity.

Analysis of Bacteriophage DNA. The only published values for genome length of UT1 and UNL-1 are based on the addition of the sizes of restriction fragment lengths. It is of importance to note that this is not a reliable measure of DNA length, as lengths of 20 kbp or greater are both difficult to size estimate and unreliable on a standard (non-pulse field) gel. Nonetheless, this experiment was repeated. *Eco*RI was the only enzyme used with UT1 DNA that gave what appeared to be a complete digestion (lanes 1 and 2, Figure 5.3) of DNA fragments into lengths less than 20 kbp. The results are provided in Table 5.1. Sixteen fragments were identified. If *Eco*RI sites occurred completely randomly, one would expect

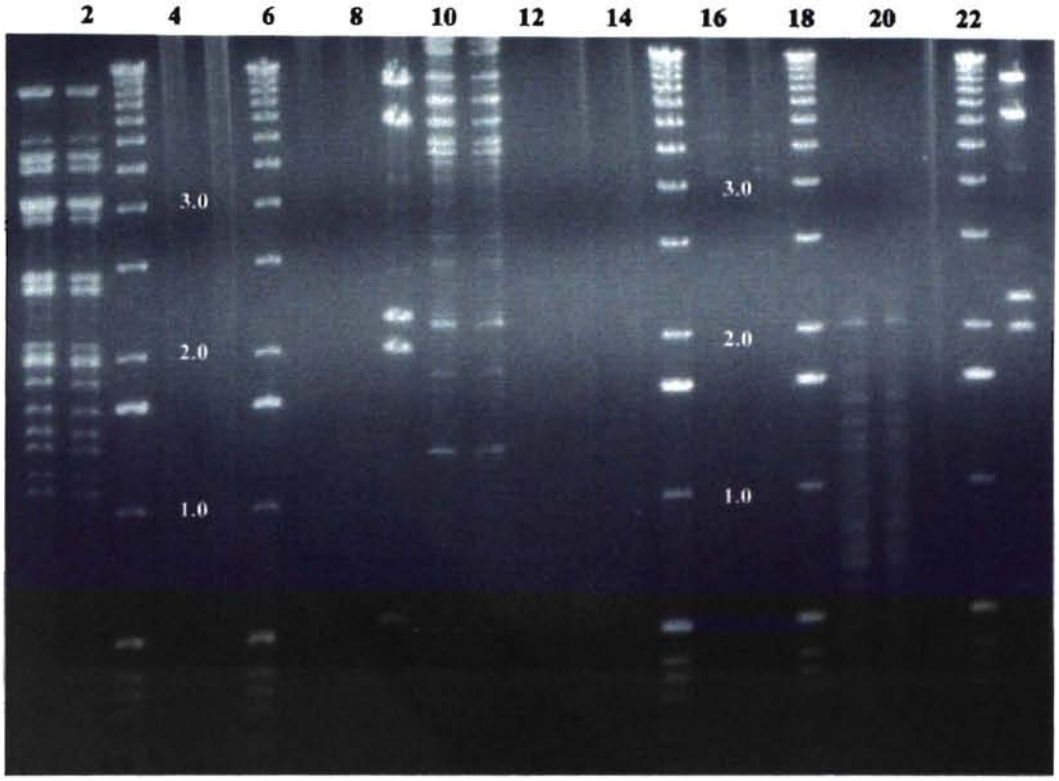

Figure 5.3 Restriction analysis of UT1. Lanes 1 and 2: *Eco*RI; 4 and 5: *Bam*HI; 7 and 8: *Pst*I; 10 and 11: *Hin*dIII; 13 and 14: *Sma*I; 16 and 17: *Sal*I; 19 and 20: *Sau*3A; 3, 6, 15, 18, and 23 : 1 kbp; 9 and 23: λ X *Hin*dIII.

Table 5.1. Approximate Restriction Fragment Lengths of *Eco*RI-digested UT1 DNA

Fragment Number	Length (kbp)
1 (smallest)	1.1
2	1.2
3	1.3
4	1.4
5	1.5
6	1.75
7	2.0 X 2
8	2.1
9	2.4
10	2.45
11	2.95
12	3.05 X 2
13	3.5
14	3.75
15	4.0
16 (largest)	6.0
Total	45.5 kbp

Table 5.2 Approximate Restriction Fragment Lengths of *Hin*dIII-digested UT1 DNA

Fragment Number	Length (kbp)
1 (smallest)	1.25
2	1.75
3	2.2
4	2.3
5	2.5
6	2.7
7	3.0
8	3.25
9	3.5
10	3.75
11	3.8
12	4.4
13	5.8
14	6.0
15	6.4
16	6.6
17	7.5
18	8.2
19 (largest)	9.4
Total	84.3 kbp

it to cut every 4096 base pairs. If UT1 DNA was cut 15 times, it would seem that the DNA would be approximately 61 kb in length. Indeed, adding the *Eco*RI restriction fragment lengths group an estimated genome size of 50.5 kbp.

In contrast to this, *Hin*dIII yielded 19 fragments (statistically, UT1 DNA would thus be nearly 74 kbp with such a random digest). In the case of *Hin*dIII, the fragments added up to 84.3 kbp. This actually fits into the value of 79 +/- 5 kbp noted by Ogunseitan (1988), but one could only speculate what results might be obtained if yet another enzyme was used.

A comparative restriction analysis of phages UT1 and UNL-1 is shown in Figure 5.4. Many common restriction patterns are apparent by looking at the ladders produced. Also of interest is the high sensitivity of the bacteriophages to *Pae*R7, an enzyme purified from *P. aeruginosa*. Based on digests by *Eco*RI, *Cla*I, and *Pae*R7, phage UT1 appears to have one or more bands present not found with UNL-1 DNA. In the *Pae*R7 lanes, a series of 6-9 kbp fragments (possibly four) are present in UT1 that are not in UNL-1 DNA. These fragments approximate the reported differences in DNA lengths of the bacteriophage genomes.

Visualization of the bacteriophage DNA molecules by electron microscopy was possible, but no contour lengths over half a kilobase pair were observed (using the established length of 3.4 Å/bp present in B-form DNA). The longest (straight) molecules seen are provided in Figure 5.5. Many DNA molecules were visualized as large "knots of yarn," but accurate contour lengths could not be calculated from those micrographs. Extensive spreading of the DNA on the parafilm likely caused mechanical breaks in the

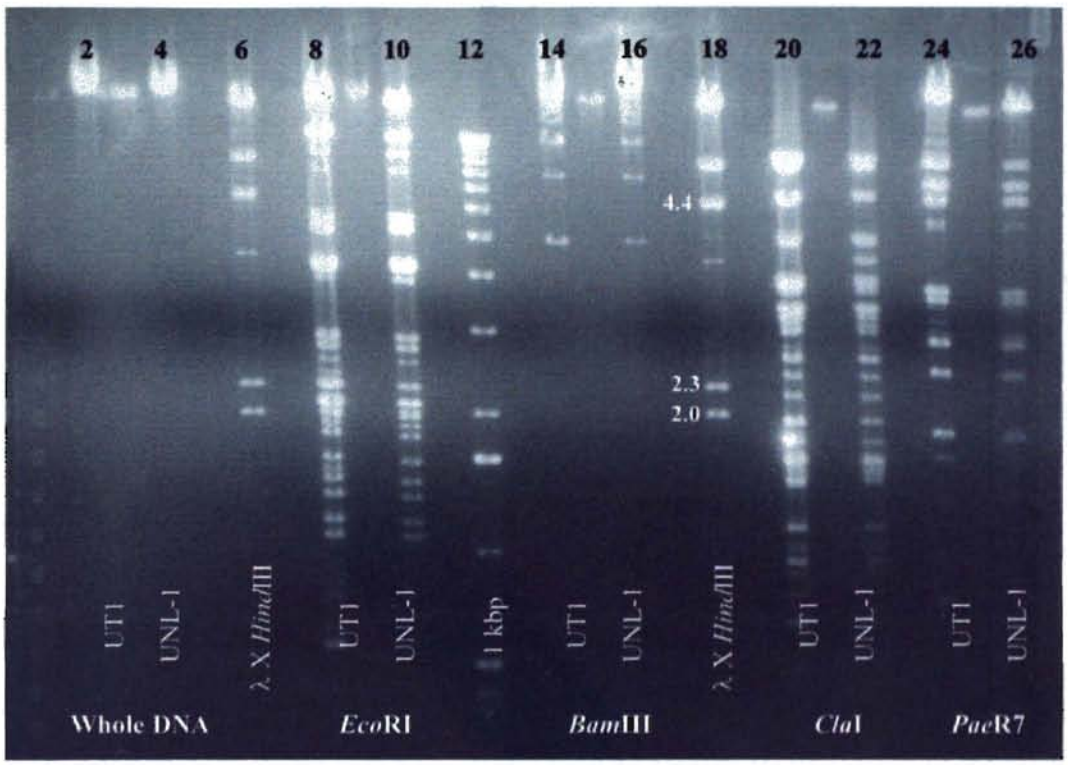

Figure 5.4 Restriction analysis of UT1 and UNL-1 DNA.

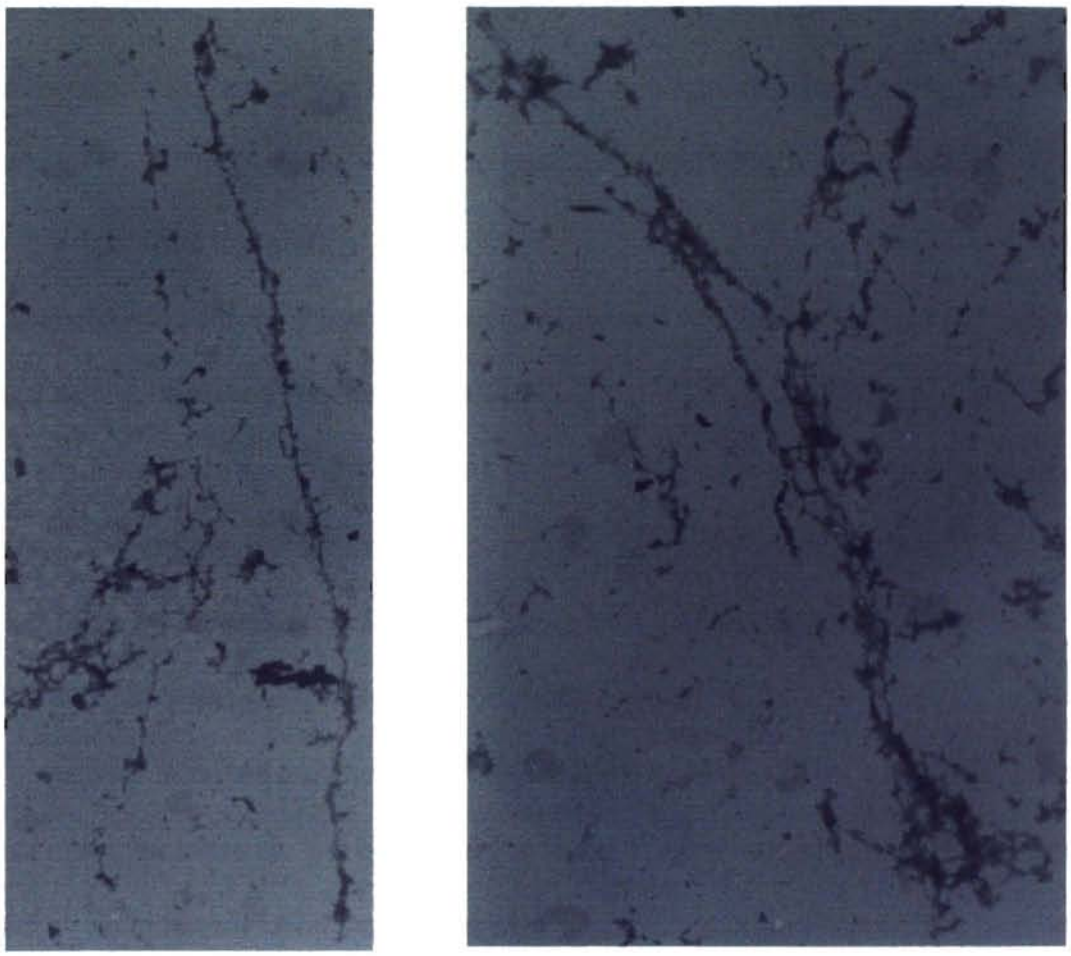

Figure 5.5 Bacteriophage DNA. Left: UT1 (8,640 X), Right: UNL-1 (11,520 X).

molecule, or there may have been a lack of adequate amounts of cytochrome c in the preparation to absorb the electron dense stain.

Conclusion

Bacteriophage UNL-1 may be related to UT1 on an evolutionary level. Previous studies have proven there to be at least 86% identity with respect to DNA sequence between UT1 and UNL-1. This study has shown that there seem to be many restriction fragments that the two phages have in common.

We have also learned that there seems not to be a simplistic answer to the choice of life cycles (temperate or vegetative). Both UNL-1 and UT1 are capable of producing both turbid and clear plaques. This occurred in both the plaque and bacterial lysis assays described in Chapter 4, though the latter was only true when *P. aeruginosa* PAT was used as a host. This could serve as evidence supporting the notion that PAT is really the "natural" host for the virus instead of PAO as was originally thought.

Because it is not truly known where UNL-1 originated (environmental sample, laboratory contaminant, etc.), it is difficult to comment on what real consequences UNL-1 has had or could have on freshwater ecosystems. Evidence in previous studies pointed to UNL-1 as a variant of UT1 in which 30 kbp has been deleted, with the deletion rendering the virus incapable of generalized transduction and unable to engage in pseudolysogenic behavior. However, our data failed to confirm the 30 kbp difference. Such a deletion doesn't seem to explain the increased size of the capsid or what type of role the capsid has

on the "headful rule" for UNL-1, but such measurements based on electron microscopy may not be reliable measurements due to artifacts of the preparation process.

CHAPTER VI

CONCLUSIONS

In a scientific world that has clearly stepped into the age of the gene, formerly advanced techniques such as genetic engineering and bioremediation have become commonplace in today's fast-paced solution-oriented world. It is thus of paramount importance to consider the biology of an ecosystem before attempting to engineer it to our perceived benefit. Because of the widespread occurrence of gene transfer between organisms, we must first understand these mechanisms of transfer so that we do not inadvertently offset the delicate balance that exists in nature.

It is apparent that substantial amounts of genetic transfer occur in the environment as a result of virally-mediated transduction (Ripp et. al., 1997). Transduction has been shown to occur to a significant extent in freshwater systems via freshwater bacteriophages (Ripp et. al., 1996). Specifically, *Pseudomonas* bacteriophage UT1 has probably been a key player in the transduction of many naturally-occurring *Pseudomonas aeruginosa* strains. Until this study was performed, however, little was known about how many different types of organisms this phage could actually infect.

We know now that UT1 is fairly restricted in its ability to infect host bacteria. Specifically, it is really quite limited to *P. aeruginosa* PAO and PAT as well as *Burkholderia cepacia*. Due to its capability to form lysogens rapidly on PAT, it is reasonable to believe that PAT may be its true natural reservoir.

There is significant evidence in this study to support the fact that UT1 gets its ability to infect such a limited host range as a result of its utilization of LPS as a receptor, as LPS is very species- and strain-specific among gram negative bacteria. The implications for such knowledge are widespread. First, one might conclude that introducing new organisms to a community which are not *P. aeruginosa* PAO, PAT, or *B. cepacia* is a relatively safe process with respect to UT1. Of course, this is not to say that some other naturally omnipresent virus would not be able to mediate genetic transfer as viruses are of very high concentration in natural ecosystems. Perhaps a more striking use of this knowledge is to consider the fact that UT1 is so selective that it might actually be used as a vector either in the environment or as a phage therapy. It would have the capability to regulate populations in a very species and strain-specific manner.

Lost in this discussion is the recently-characterized virus UNL-1, which seems to have everything in common with UT1 except its ability to mediate generalized transduction and its penchant for thriving in starved environments. Authors of previously-published UT1 work would almost certainly argue that these were the two characteristics that made UT1 such an appealing virus to work with as an environmental model. However, the lack of an aptitude for pseudolysogeny nearly guarantees a shorter half-life for UNL-1 in a starved environment such as that found in a freshwater community. Because UNL-1 has not been

shown to be a generalized transducing phage and apparently possesses a larger capsid size than UT1, UNL-1 might be the ideal vector for *in situ* genetic engineering.

Even if one is not interested in these phages for their environmental or applied significance, the very biology of these fascinating entities leaves one thirsty with questions. How do these phages "choose" between temperate and vegetative growth? What is the true stimulus that flips the genetic switch? Such bizarre life cycle habits are likely not the norm in the laboratory setting, but they very well may be the rule and not the exception in the environment.

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