BACTERIOPHAGE MULTIPLICATION IN *ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA* MIXED BIOFILM COMMUNITIES

THESIS

Presented to the Graduate Council of
Texas State University–San Marcos
in Partial Fulfillment
of the Requirements

for the Degree

Master of SCIENCE

by

Matthew K. Kay, B.S.

San Marcos, Texas
March 2010

ACKNOWLEDGEMENTS

This project was funded through the Homer Prince Microbiology Endowment to GMA and a grant from the Norman Hackerman Advanced Research Program of the Texas Higher Education Coordinating Board to RJCM (003615-0037-2007). Dr. Aron comes first on the list for his guidance, patience, and assistance during the course of the research project. I would also like to express my appreciation for the following contributors: Dr. Robert McLean, Dr. Nihal Dharmasiri, Thomas Erwin, Mary Weber, J. D. McCracken, and Lindsey Wiggins, for their helpful advice, discussions and support. My gratitude goes to my friends Dr. Robert Harpold and Dr. Chris Moore, for suggesting I should go get my Master's while I've got the chance. You guys were right, those who don't use their brains when they've got them are letting everyone else down. Thanks for the support, y'all.

I would especially like to thank my mother, librarian Chris Kay, for all her love and encouragement during this time in my life. I would also like to thank her side of the family for all the support and goodwill through these years at the university. Without all their help and patronage this degree would not have been possible.

This manuscript was submitted on 3/26/10.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

ABSTRACT

BACTERIOPHAGE MULTIPLICATION IN *ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA* MIXED BIOFILM COMMUNITIES

by

Matthew K. Kay, B.S.

Texas State University – San Marcos

March 2010

SUPERVISING PROFESSOR: GARY M. ARON

The use of single species bacteriophage infection to control bacterial populations in mixed planktonic cultures has met limited success. Since many chronic infections occur as mixed species biofilms we investigated the effect of double species phage

infection in both mixed species planktonic culture and biofilms. This study reports the effect of *Escherichia coli* bacteriophage λW60 and *Pseudomonas aeruginosa* bacteriophage PB-1 infection on the viability of mixed species biofilm and planktonic cultures. In both mixed species planktonic and biofilm communities, *E. coli* and *P. aeruginosa* maintained stable cell populations in the presence of their phage. Both *E. coli* and *P. aeruginosa* developed phage resistance in planktonic culture, however, reduced resistance was observed in biofilm communities. Increased phage titers and reduced resistance in biofilms suggests phage are capable of replicating on susceptible cells in biofilms. Electron Microscopy of biofilm supernatants revealed bacteriophage particles enmeshed in extracellular polymeric substance (EPS). Treatment of biofilm supernatants with Tween-20 resulted in an increase in infectivity which suggests biofilms' EPS can act as a natural reservoir for phage and affords protection from phage infection. The results of this study demonstrate that the use of double species phage infection did not result in an additive bacteriocidal effect in either mixed planktonic or biofilm communities, but may be effective in preventing spread from established biofilms.

INTRODUCTION

Since the early 1900's bacteriophage have been used in the treatment of bacterial infections (4, 53). Phage host specificity, lack or reduction of immunogenicity, and antibiotic resistance has led to a renewed interest in the use of phage as therapeutic antibacterial agents (4, 34, 53). The recent introduction of bacteriophage in clinical trials has produced encouraging results (63, 27, 47). Phage therapy has been shown to be effective in controlling chronic infectious *Staphylococcus epidermidis* biofilms with catheter hydrogels (14). Recently, a study using bacteriophage preparations targeting antibiotic-resistant *Pseudomonas aeruginosa* otitis infections lowered bacterial counts significantly (66). Phage therapy has also been effective in controlling dysentery with oral phage administration, and with direct application of bacteriophage lysates in UTIs, skin diseases, sinusitis, and respiratory infections (63). Since many chronic infections occur as mixed culture biofilms, the investigation of phage on mixed culture biofilms is of importance. The objectives of this research was to determine the effect of mixed species phage infection on the viability of mixed bacterial biofilm communities and determine the effect mixed species biofilm growth has on phage multiplication.

1.0 BACTERIA

Escherichia coli is a Gram-negative bacillus native to the enteric tracts of most mammals. It is an opportunist responsible for large outbreaks of food contamination-borne diarrhea leading to hemolytic uremic syndrome (6). The bacterium is 2μm - 6μm in length, with colonies on nutrient agar appearing smooth, low, convex, moist, and grey with a shiny surface and entire edge (6). They are facultative anaerobes with both respiratory and fermentative modes of metabolism (6). *E. coli* is capable of producing enterotoxins and possesses many fimbriae for attachment to mucosal surfaces (6). Cell surface structures, including fimbriae, have been shown to be implicated in the formation of biofilms in uropathogenic *E. coli* Dr+ strains (67). *E. coli* MG1655 EPS contains proteins related to amino acid metabolism, carbohydrate metabolism, cell walls, membranes, cellular processes and translation. As *E. coli* switches from exponential to stationary phase in LB media, the carbohydrate/protein ratio increased over time for bound EPS, and decreased for free EPS (16). The increase in protein content for free EPS in stationary phase may be due to bacteria shedding their outer membrane or periplasmic proteins during the growth phase, which may influence phage replication machinery (16). A large increase in the amount of secreted outer membrane porin proteins (including OmpA, OmpX, OmpW, and OmpC) may contribute to the increased antibiotic resistance of *E. coli* biofilms, as many antibiotics target these sites for entry (16).

Pseudomonas aeruginosa is a ubiquitous Gram-negative soil bacterium. Infection occurs in injured, burned, and immune-deficient patients and is the primary causative organism in cystic fibrosis (35). It is responsible for a large portion of nosocomial infections (22). It is a bacillus, 1.5μm - 3μm in length, slightly curved, and motile with a single polar flagella (6). Colonies on nutrient agar are smooth, shiny, round, and exhibit a greenish pigment (6). *P. aeruginosa* is aerobic and has only one metabolic pathway with oxygen as the terminal electron acceptor (6). Phenazines produced by this organism play a number of roles, including antibacterial activity (ex. pyocyanin), signaling and community development, and redox activity (59). A recent study by Wang et al. has shown that phenazine-facilitated electron transfer promotes anaerobic survival in both planktonic and biofilm cultures (59). *P. aeruginosa* can grow a biofilm of considerable thickness (>200 μm) and can secrete large amounts of alginate polysaccharide for its EPS (22). When grown in a biofilm, *P. aeruginosa* may exhibit up to 100 times higher resistance to antibiotics than corresponding planktonic cells (22). *P. aeruginosa* biofilm grown cells were shown to change regulation of motility, alginate production, and quorum sensing (49). Protein analysis indicated increased levels of proteins involved in metabolism, phospholipid and LPS-biosynthesis, membrane transport and secretion, and adaptive and protective mechanisms (51). Stationary phase planktonic studies have shown planktonically-grown *P. aeruginosa* is morphologically similar to cells in established biofilms (59).

The interactions between *E. coli* and *P. aeruginosa* in planktonic culture and in biofilms vary greatly depending on the environment. For example, high concentrations of pyocyanin produced by *P. aeruginosa* in a mixed culture with *E. coli* could be toxic to

E. coli (24). Planktonic *E. coli* growth was inhibited in glucose-minimal media at a concentration of 1 x 10^{-4} per ml pyocyanin (24). The oxygen reactive species produced by the various phenazines of *P. aeruginosa* are suspected to interfere with the aerobic metabolism of *E. coli*, which forces it to produce catalase. This prevents accumulation of intracellular O_2^-, and eventually becomes toxic at high concentrations (59). In biofilms, the metabolic pathway of *E. coli* would be primarily anaerobic and not as susceptible to pyocyanin as in planktonic culture (51, 61, 16).

2.0 BACTERIOPHAGE

λW60 (family *Siphoviridae*) is lytic for *E. coli* MG1655. It has a head 60nm in diameter and a long, non-contractile tail 170nm in length (46). Its genome is linear double-stranded DNA ~48,000bp with single stranded 12-base 5' sticky ends (31). The capsid consists of 8 proteins, and the tail and tail fiber are composed of 12 gene products (31). A mutation in the CI repressor disables the lysogenic phase of the virus ensuring a lytic pathway and prevents survival of phage-infected bacteria as lysogens (39). As a result, the phage is highly lytic with clear circular plaques approximately 0.5-1 mm in diameter. Bacteriophage lambda initiates infection by activating the J protein located on the tip of its tail fiber at a *lamB* porin protein site in *E. coli*'s outer membrane (31). The *lamB* maltose transport porin may be used in conjunction with the OmpC outer membrane protein to infect *E. coli* as a single phage receptor binding site (57). A mutation in λW60 capsid protein E allows λW60 to avoid the reticuloendothelium system and survive for long periods in the circulatory systems of *E. coli* CRM1 infected mice

(39). Purified λW60 phage stock has been used to treat lethal injections of *E. coli* CRM1 induced bacteremia in BALB/c mice with a 100% survival rate (39).

Phage PB-1 (family *Myoviridae*) is lytic for *P. aeruginosa* strain PA01. It possesses an octahedral head 64nm in diameter and a non-flexible, contractile tail 150nm in length (46, 5, 10). It has a bulls-eye ("halo") plaque morphology approximately 1 mm in diameter. Its genome consists of linear, non-permuted double stranded DNA ~65,000bp in length and encodes ~90 different proteins (10). The acid-resistant virion particles are composed of at least 22 different proteins (10). PB-1 characteristics include capsomers that appear as cup-like depressions on the phage heads and a conspicuous criss-cross pattern on the tail (10). PB-1 phage and its relatives use receptors in the bacterial lipopolysaccharide (LPS) layer (10, 44). *P. aeruginosa* phage E79 binds to smooth LPS in suspension and is inactivated in the presence of sonicated LPS (28-29). Multiple LPS binding sites for PB-1 may be a factor in infection since multiple LPS receptors composed of alanine and galactosamine residues are targets for E79 phage (28-29). Several hypothetical proteins in PB-1 phage code functions which may include glycanate polysaccharide depolymerase (possibly gp66) as well as viral outer coat proteins and may act as receptors for binding bacterial-sourced depolymerase (10, 22). This would help infectious virus penetrate the exo-polysaccharide layer in biofilms (10). Phages belonging to this group have been used in human phage therapy trials. Therapeutic application of *P. aeruginosa* phage 14/1 on burn patients with *P. aeruginosa* infections was 37% successful (35, 38).

3.0 PLANKTONIC INFECTION AND BACTERIOPHAGE RESISTANCE

Planktonic phage interactions are important in that they play a vital role in recycling nutrients in nature, regulating microbial ecosystems and facilitating gene transfers among bacterial populations (20, 11, 7, 52, 54). The large numbers of phage found present in ocean water ($>10^6$ PFUs ml^{-1}) indicate that phage are ubiquitous in nature, however only a small fraction of phage are infectious to their host at any given time (20). Since mixed bacterial cultures are to be expected in native environments, mixed phage infections are expected to be part of the environment as well (26, 11). Planktonic mixed bacterial-phage interactions studies suggest that phage can expand on the fastest growing host in a given ecological setting, a "kill-the-winner" hypothesis (9, 48). Harcombe et al. demonstrated planktonic extinction events can occur in mixed cultures with a single host-specific phage present (23). For mixed phage infection (phage cocktails), the focus has been on a single host for the mixed phage, for example, *Pseudomonas aeruginosa* phage cocktails in hydrogels applied to indwelling catheters (19).

Planktonic phage infection produces the most bacteria-phage interaction, with cell lysis and dissemination of progeny phage occurring most rapidly in logarithmically growing cultures (7). A planktonic phage infection typically undergoes a series of rapid lytic cycles where phage titers reach a maximum population density shortly after infection, with alternating cycles of resistant bacteria and newly infectious phage particles able to infect resistant cells (7). Usually a balance of infected cells and infectious phage is maintained over time, but the population of susceptible cells in

planktonic cultures becomes replaced by resistant populations (43, 9). With co-treatment using antibiotics to remove resistant cell populations (which may be more susceptible due to changes in cell membranes to remove phage receptors), phage therapy may be successful in reducing or eliminating bacterial infections (4, 39, 52). However, the therapeutic use of phage to treat persistent bacterial infections can be ineffective, due to both the development of bacterial resistance and the presence of a biofilm (17, 15).

4.0 BIOFILMS

Biofilms consist of structured, enclosed communities generally attached to a solid substratum and comprise the majority of bacteria in natural ecosystems (51). Various biofilm-related structures can occur, including streamers, water channels, stacks and fronds, all of which depend on environmental conditions (27). These biofilm communities are matrix-enclosed micro-colonies separated by water channels which are the main entryway for nutrients and wastes to cycle through the biofilm (51). The sticky matrix enclosing these communities is called extracellular polymeric substance (EPS) (14). EPS generally refers to the complex heterogeneous substances outside the bacteria cells, consisting of polysaccharides, proteins, nucleic acid, and other biopolymers. EPS can be characterized by its proximity to the cell surface, i.e., EPS tightly linked to the cell surface is known as capsular EPS, whereas EPS not directly attached to the cell surface is known as slime or free EPS (16). Free EPS can be secreted by micro-colonies to considerable thickness (>200 μm) by some species of bacteria, including *P. aeruginosa*, for added protection (22). EPS is also mainly responsible for the structural integrity of

cellular aggregates in biofilms, providing an attractive force to hold the micro-community together (16).

Gene expression in mixed bacterial cultures differs significantly than that expressed in single culture (8, 11, 51). Bacteria, especially in mixed biofilm communities, are known to use a complex system of cooperation, coordination, and synchronization (61). For example, mixed species in biofilm communities use quorum sensing to establish gene expression which is regulated by population densities (40). N-acyl homoserine lactones (AHLs) act as signaling molecules by *Chromobacterium violaceum* which leads to production of violacein (37). These molecules have anti-microbial properties and have a function similar to *Pseudomonas* phenazines (ex: pyocyanin), which are also produced in response to population density and can eliminate bacterial competitors (24, 59). Pyocyanin contributes to anaerobic metabolism through redox reactions and electron shuttling. This would be beneficial in a dense biofilm environment in which access to oxidants is limited (59). Phenazines may also benefit the biofilm community as a whole by inhibiting nearby planktonic growth and serving as signaling molecules to attract beneficial bacterial co-operators compatible with the community (61).

The exo-polysaccharide matrix is a major form of protection from extracellular substances, including antimicrobials and bacteriophage (54). The composition of *E. coli* EPS includes membrane lipids, proteins, nucleic acids, and various polysaccharides, and also the polysaccharide adhesion molecule, β-1,6-N-acetyl-δ-glucosamine (16, 58). Genes for adhesins and fimbriae attachment, outer membrane proteins, lipid A biosynthesis, and outer membrane lipoproteins were found overexpressed in early *E. coli*

biofilm synthesis (50). The composition of *P. aeruginosa* EPS is polyanionic alginate, consisting of a block copolymer of mannuronic and guluronic acids (22). In another study by Wozniak et al. showed alginate production in *P. aeruginosa* strains PA14 and PAO1 was significantly lower than that observed in clinical isolates, and this alginate loss could be compensated by an increase in LPS production for scaffolding (65). In all cases, however, use of bacteriophages to treat biofilm-related infections may be ineffective due to the presence of the EPS as a glycocalyx diffusional barrier to the virus (22).

The subsequent co-integration of a biofilm with its various bacterial hosts can lead to structural changes and phenotypes different from their planktonic state, which can influence the effectiveness of phage and antibiotics as therapeutic agents (8, 51). Antibiotic resistance in clinical biofilm-related infections remains a continuing problem (13, 15). Depending on the types of cells present in the biofilm and the variable composition of the EPS, the lytic success of phage may be lowered against a biofilm community (45). These changes may affect the ability of host-specific phage to penetrate and infect mixed species biofilms.

5.0 BIOFILM INFECTION

Cocktails containing phage which utilize different strategies for infection may be effective in preventing mixed biofilm formation or slowing their spread and dissemination (2). The use of phage cocktails to prevent new biofilm formation has been successful in catheter hydrogels and bacteriophage-containing antibacterial preparations (13-14, 15, 19). Phage therapy, however, was of limited use against established *P.*

aeruginosa biofilms (22). Corbin et al. demonstrated phage T4 was able to concentrate an order of magnitude higher within *E. coli* K-12 biofilms, although phage did not reduce bacterial counts within the biofilm (12). The ability of phage to elute from biofilms over a longer period of time compared to planktonic infection was observed in *Staphylococcus aureus* biofilms (47). Phage release from infected biofilms over long periods of time may effectively inhibit spread of the infection by disseminating bacteria. Prolonged treatment with high titer λ and P22 phage was required to control bacteremia in *E.coli* and *Salmonella typhimurium* infected mice (39). Continuous release of infectious phage in the surrounding environment from phage infected biofilms may prevent spread of the bacterial infection.

Phage-borne polysaccharide depolymerase has the ability to degrade bacterial polysaccharides (2). The phage glycanase is associated with the spikes attached to the baseplate (33). The phage degrades the EPS polymer to reach the cell surface and binds to the outer-membrane receptor. Phage polysaccharide depolymerase, is polysaccharide specific, and are associated with specific types and strains of phage (27). Phage which have enzymes for degrading gram negative EPS are typified by haloes ("bulls-eye") surrounding plaques. The haloes represent cleared areas in which excess soluble enzyme is released upon cell lysis (54). *P. aeruginosa* phage are capable of diffusing through alginate gels and able to reduce viscosity by 40% and cell numbers by 99% in 20-day-old *P. aeruginosa* biofilms (22). However, EPS degrading capability was present in purified whole-cell suspension, which indicates the alginate depolymerase is present in the host bacterium itself, and the phage may attach it to coat protein (22). Mixed culture biofilms which contain one species that produces EPS may afford protection from phage infection

to both species present. Thus, infection with one phage that possesses or induces a polysaccharide depolymerase may result in the loss of EPS protection in mixed culture to other bacterial species from their respective phages (27). Use of phage with exo-polysaccharide glycanase enzyme activity may allow a co-infecting non-glycanase phage into an established mixed biofilm community.

6.0 BACTERIOPHAGE RESISTANCE IN BIOFILMS

Bacteria have evolved mechanisms to protect themselves from phage infection, including genomic recombination, deletion and growth in biofilms (62, 55, 34, 15). The protection afforded by EPS may prevent phage infection leading to a passive phage resistance where the host biofilm population is still susceptible. Exo-polysaccharide matrices appear to be a first line of passive defense in excluding phage from cells residing within biofilms. Other strategies to protect against phage infection include persister cell subpopulations which are specialized survivor cells with altered dormancy phenotypes (47, 43, 8, 30). Lowered or altered gene expression of the host or decreased ability of the phage to penetrate biofilms may contribute to the reduced efficiency of specific phage therapy (43, 47).

Persister cell phenotypes may also play a role in preventing phage replication inside biofilms (30). Keren et al. suggested that biofilm survival may in fact be due to increased levels of persister cells within biofilms, rather than expression of biofilm-specific resistance mechanisms (30). Promoters for a lytic switch in lysogenic or delayed

lytic-phase phage may not be activated due to poor or lowered gene expression by the host, which is essentially in a deep dormancy state (30,43). Dormant cells are still susceptible to latent phage infections even though their lytic promoters have been deactivated inside the biofilm (43, 42). Dormant cell phenotypes may become phage susceptible once released into the planktonic environment (43).

Programmed cell death in biofilm communities, in addition to being a source of recycled nutrients, may also affect the rate in which bacteriophage are released into the environment (3, 54). Lysis of *P. aeruginosa* within biofilms may be a defense mechanism against phage infection within the biofilm micro-colony structure (3, 60). Mixed culture biofilms also appear to have a greater effect protecting their co-cultured species than single species biofilms, indicating cooperation in mixed culture biofilms (8). Biofilm biomass increased by >167% in a four species marine bacteria co-culture, along with increased resistance to hydrogen peroxide and tetracycline (8). Mixed species biofilm communities also offer increased protection and resistance to environmental stressors that single culture biofilms cannot employ (51, 8). These additive biofilm strategies may prevent phage infection in mixed biofilms, even from phage strains carrying polysaccharide depolymerase.

The varying cost of resistance to different bacteriophage could influence the effect a specific bacteriophage infection could have on mixed cultures. Thus, development of phage resistance based on cell membrane receptors may be determined by how many different receptors a specific phage can utilize for infection. In planktonic mixed cultures, the presence of specific phage as an exogenous stressor can lead to the extinction of a bacterial species. This was shown with *E. coli* and *Salmonella enterica*

mixed cultures infected with *E. coli* phage T7 (23). A combined phage infection, along with interspecies bacterial competition in biofilms, may be effective in lowering certain populations in mixed biofilms. The use of multiple phage infection in mixed planktonic and biofilm cultures would provide knowledge of either phage competition or cooperation in natural settings. Specific bacterial co-factors, such as phenazine concentrations, metabolic byproducts, and different growth rates of the organisms could all affect the population sizes of phage over time. Reduced metabolic pathways available to infected bacteria, as well as a reduction in actively dividing hosts (persister cells) could also affect phage populations in biofilms (51).

This study investigated the effect of mixed species phage infection on the viability of mixed bacterial biofilm communities and the effect of mixed species culture on phage multiplication. Both single and mixed phage infection failed to eliminate the phage host in either planktonic or biofilm communities. Both *E. coli and P. aeruginosa* mixed planktonic and biofilm communities developed phage resistance, which indicates phage are able to penetrate biofilms and carry out cell infection. This phage resistance, however, may be limited to infections at the surface of the biofilm. Although the proposed activity of PB-1 glycanase may have helped λW60 penetrate a mixed biofilm, mixed phage infection had no significant advantage in reducing cell populations compared to the single infection. However, biofilm infections were incomplete since high levels of phage resistance were not observed, as was the case with planktonic infection. High phage titers along with the presence of susceptible cells in biofilm communities revealed phage are capable of maintaining or increasing titers in biofilms over long periods of time. Electron microscopy of infected mixed biofilms reveals phage trapped

within EPS. The results indicate that biofilms can provide protection from and act as a natural reservoir for λW60 and PB-1 phage populations. However, phage may be capable of replicating on released susceptible cells from the biofilm community and reducing or preventing transmission from existing biofilms, leading to a successful self-replicating antibacterial agent.

MATERIALS AND METHODS

Bacteria and bacteriophage. *Escherichia coli* MG1655 (ATCC 700926) and *Pseudomonas aeruginosa* PAO1 (ATCC 47053) were grown in Luria Bertani (LB) broth (Accumedia Manufacturers, Inc., Lansing, Michigan) at 37°C in a continuous rotating shaker water bath (Thermoscientific Max Q 2000, Waltham, Massachusetts). High titer bacteriophage stocks of λW60 (ATCC 97537) and PB-1 (ATCC 15692-B3) were prepared using the agar overlay technique described by Adams (1). Lysed overlays were scraped into 10ml LB broth and phage eluted at 4°C for 4h. Cell debris was pelleted at 3220g (Eppendorf Centrifuge 5810R V3.5, Eppendorf Internation, Westbury, New York), the supernatant filtered (0.22μm) (Fisherbrand 25mm Syringe Filter, FisherScientific Inc., Dublin, Ireland), and stored at 4°C.

Planktonic growth. *E. coli* and *P. aeruginosa* were grown 18h in LB broth at 37°C in a rotating shaker bath (Thermoscientific Max Q 2000, Waltham, MA). For monoculture, 10ml LB broth in 25ml Erlenmeyer flasks was inoculated with 100μl of either *E. coli* or *P. aeruginosa* at a final cell density of 10^6 cells per ml. For mixed culture, 10ml LB broth was inoculated with 100μl each of *E. coli* and *P. aeruginosa.* For phage infection, mono and mixed cultures were infected at a MOI of 10 pfu ml^{-1} with λW60 and PB-1.

Cultures were incubated in a 37°C horizontal shaking water bath at 60rpm (FormaScientific Model 2564 Shaker Bath, FormaScientific Inc., Marietta, Ohio). At 24h intervals, cultures were assayed for both colony forming units (cfu) and plaque forming units (pfu). *E. coli* and *P. aeruginosa* were differentiated in mixed cultures by assay on LB media which contained either 20μg ml^{-1} cefsulodin or 100μg ml^{-1} ampicillin. Both mono and mixed cultures were diluted 1:100 into 10ml fresh LB broth in new 25ml Erlenmeyer flasks daily.

Biofilm growth. Silicone rubber disks, 7 x 1 mm (Dapro Rubber Inc., Tulsa, Oklamhoma) were placed in 250ml beakers which contained 50ml LB broth and inoculated with overnight cultures of *E. coli* and *P. aeruginosa* at a final cell density of 10^6 cells per ml. Mono and mixed cultures were incubated in a 37°C horizontal shaking bath at 60rpm for 2d at 37°C, with media replacement at 24h intervals. Disks which contained biofilm growth were rinsed with 20ml 0.01M PBS in sterile petri dishes for 1min to remove planktonic debris, and placed in individual 28 x 61mm 20ml scintillation vials (VW74504-20, Kimble Chase Life Science, Vineland, New Jersey) which contained 10ml LB broth and returned to incubation at 37°C at 60rpm in a horizontal shaking water bath. For phage infection, λW60, PB-1, or a mixture of both phage were added at a biofilm density mm^2 MOI 10 to broth in vials. Infected and uninfected biofilm disks were selected at 24h intervals, sonicated (FS 20 Fisher Scientific Mechanical Ultrasonic Cleaner, Fisher Scientific, Pittsburg, Pennslyvania) for 5min, vortexed for 30s and assayed for cfu and pfu. At 24h intervals, 9ml spent media was removed and 9ml fresh

media was added to the remaining biofilm growth vials. Cfu and pfu values were converted to biofilm density mm^2 (21).

Bacteriophage resistance and bacteriophage selection. Planktonic and biofilm mono and mixed cultures were grown and infected as previously described. Four bacterial isolates were chosen from each mono and mixed culture following 5d incubation. Each bacterial isolate was grown and used as indicator cells to assay the original infecting phage population. To determine if bacteriophage were selected to infect a resistant cell population, supernatant from planktonic and biofilm 5d cultures was assayed for infectivity using both the initial bacterial stock and 5d bacterial isolates as indicator cells.

Infectious centers, free, and trapped virions. Planktonic and biofilm mono and mixed cultures were grown and infected as previously described. Planktonic and biofilm medium from 5d cultures were assayed before and following filtration (0.22 μm) to determine infectivity due to infectious phage particles and infectious centers, respectively. Biofilm supernatants were mixed with either 0.5ml choroform per ml supernatant to lyse infectious centers or 0.1ml 0.5% Tween-20 (Sigma Aldrich Inc., Saint Louis, Missouri) per ml supernatant to release EPS trapped phage. Treated supernatants were assayed for infectivity as previously described.

Electron microscopy. Medium (10µl) containing phage were placed on 400µm formvar/carbon coated copper grids (SPI Supplies, West Chester, PA) and allowed to dry for 1 min. Excess liquid was removed with No. 1 Whatman filter paper (Whatman, Inc., Florham Park, NJ) and phage negatively stained with either 2% PTA (phosphotungstic acid) or 2% UA (Uranyl acetate) for 5min. Excess stain was removed using Whatman filter paper and grids were air dried and stored in sterile petri plates prior to viewing. All grids were examined in a JEOL 1200 EXII TEM (JEOL, Skandinaviska AB).

RESULTS

Without phage (planktonic). *Escherichia coli* and *Pseudomonas aeruginosa* were grown in both mixed and monoculture to determine if each could grow as well in the presence of one another as in the absence. In monoculture, *E. coli* and *P. aeruginosa* maintained cell densities of approximately $1x10^9$ to $1x10^{10}$ cells per ml, respectively, for 5d (Fig. 1A). The mean $\log_{10}$ density of *E. coli* and *P. aeruginosa* was 9.4 ± 0.09 and 9.8 ± 0.24 cfu per ml, respectively. In mixed culture, *E. coli* reached a density of $1x10^8$ (Fig. 1B). The mean $\log_{10}$ density for *E. coli* and *P. aeruginosa* was 8.7 ± 0.31 and 9.6 ± 0.14 cfu per ml, respectively. The data indicates both bacterial populations were able to maintain steady state growth under conditions of daily spent medium replacement with fresh medium. *E. coli* did not attain as high a cell density in the presence of *P. aeruginosa* compared to growth in the absence of *P. aeruginosa.* The data suggests in mixed culture *P. aeruginosa* is slightly inhibitory to the growth of *E. coli.*

E. coli with λW60 (planktonic). To determine if *E. coli* could establish a stable population in the presence of λW60, *E. coli* mono and mixed cultures were infected with λW60 and cell populations were determined at 24h intervals for 5d. In monoculture, *E. coli* in the presence of phage λW60 maintained a cell density of $1x10^9$ cells per ml, which was similar to uninfected cultures (Figs. 2A and 1A). The titer of phage λW60 decreased

99% in both monoculture and mixed culture (Figs. 2A and 2B). The mean $\log_{10}$ density of *E. coli* and phage λW60 was 9.3 ± 0.12 cfu per ml, and 6.5 ± 1.27 pfu per ml, respectively. In mixed culture, *E. coli* maintained steady state growth at approximately $1x10^7$ cells per ml. The mean $\log_{10}$ density of *E. coli* and *P. aeruginosa* in mixed culture was 8.2 ± 0.51 and 9.4 ± 0.22 cfu per ml, respectively. Phage λW60 mean $\log_{10}$ density was 6.0 ± 2.13 pfu per ml. The data suggests *E. coli* was able to develop a phage resistant population in both mixed and monoculture. *E. coli* did not attain as high a cell density in mixed culture compared to its growth in monoculture, which suggests *P. aeruginosa* is slightly inhibitory to the growth of *E. coli.*

P. aeruginosa with PB-1 (planktonic). To determine if *P. aeruginosa* could establish a stable population in the presence of PB-1, *P. aeruginosa* mono and mixed cultures were infected with PB-1 and cell populations were determined at 24h intervals for 5d. In monoculture, the density of *P. aeruginosa* in the presence of phage PB-1 maintained a steady state population of approximately $1x10^8$ to $1x10^9$ cells per ml (Fig. 3A). The titer of phage PB-1 increased from $1x10^8$ to $1x10^{10}$ pfu per ml. The mean $\log_{10}$ cell density of *P. aeruginosa* was 8.7 ± 0.44 cfu per ml and the mean $\log_{10}$ density of phage PB-1 was 9.7 ± 1.11 pfu per ml. In mixed culture, the density of *P. aeruginosa* and phage PB-1 was similar to that seen in monoculture (Figs. 3B and 3A). The $\log_{10}$ cell density of *P. aeruginosa* and *E. coli* was 8.5 ± 0.38 and 8.6 ± 0.2 cfu per ml, respectively, in mixed culture, and the mean $\log_{10}$ density of phage PB-1 was 9.9 ± 0.9 pfu per ml. The data suggests *P. aeruginosa* developed a phage resistant population in both mixed and

monoculture. Furthermore, the growth of *P. aeruginosa* was unaffected by the presence of *E. coli*.

Mixed culture with both phage (planktonic). To determine if *P. aruginosa* and *E. coli* could establish a stable population in the presence of both phage, mixed cultures were infected with λW60 and PB-1. Cell and phage populations were determined at 24h intervals for 5d. Both *E. coli* and *P. aeruginosa* maintained cell densities of $1x10^8$ and $1x10^{10}$ cells per ml, respectively, in the presence of both phage (Figs. 4A and 4B). Phage λW60 titer dropped approximately 99% whereas PB-1 titers remained constant at $1x10^{10}$ pfu per ml. The mean $\log_{10}$ cell density of *E. coli* and *P. aeruginosa* was 8.6 ± 0.2 and 8.8 ± 0.43 cfu per ml, respectively, and the mean $\log_{10}$ density of phages λW60 and PB-1 was 6.5 ± 1.3 and 10.2 ± 0.81 pfu per ml, respectively. The infection of a mixed culture with both phage did not have any additive effect on the growth of the bacteria compared to single infection. The results also indicate both *E. coli* and *P. aeruginosa* develop phage resistant populations in mixed culture.

Without phage (biofilm). *E. coli* and *P. aeruginosa* were grown in both mixed and monoculture biofilms to determine if each could grow as well in the presence of one another. In monoculture, *P. aeruginosa* and *E. coli* maintained densities of $1x10^4$ and $1x10^5$ cells per mm^2, respectively (Fig. 5A). *E. coli* and *P. aeruginosa* had a mean biofilm $\log_{10}$ density of 5.6 ± 0.05 and 4.27 ± 0.17 cells per mm^2. In a mixed biofilm community, both *P. aeruginosa* and *E. coli* maintained cell densities similar to single

species biofilms (Fig. 5B). *E. coli* and *P. aeruginosa* in mixed biofilms had mean biofilm $\log_{10}$ densities of 5.3 ± 0.2 and 4.47 ± 0.15 cells per mm^2. The results indicate *P. aeruginosa* does not inhibit the growth of *E. coli* in biofilms as in planktonic growth.

E. coli with λW60 (biofilm). To determine if *E. coli* could establish a stable biofilm in the presence of λW60, *E. coli* mono and mixed cultures were infected with λW60 and cell populations were determined at 24h intervals for 5d. In monoculture, *E. coli* maintained a density of $1x10^6$ cells per mm^2 (Fig. 6A). λW60 titer decreased >99.99% by 24h following infection and was followed by a 99% increase in titer. The mean biofilm $\log_{10}$ density of *E. coli* and λW60 was 5.6 ± 0.19 cells per mm^2 and 2.2 ± 0.62 pfu per mm^2, respectively. In mixed biofilms with phage λW60, *E. coli* grew as well in the presence of phage as in the absence (Figs. 6B and 5A). *E. coli* and *P. aeruginosa* in mixed biofilms had mean biofilm $\log_{10}$ densities of 5.3 ± 0.23 and 5.0 ± 0.13 cfu per mm^2, respectively. The mean biofilm $\log_{10}$ density for λW60 was 1.4 ± 0.58 pfu per mm^2. The results suggest phage λW60 is not able to replicate as well in biofilms as in planktonic culture and biofilm growth affords *E. coli* protection from phage infection.

P. aeruginosa with PB-1 (biofilm). To determine if *P. aeruginosa* could establish a stable biofilm in the presence of PB-1, *P. aeruginosa* mono and mixed cultures were infected with PB-1 and cell populations were determined at 24h intervals for 5d. In both mixed and monoculture, *P. aeruginosa* grew as well in the presence of phage PB-1 as in the absence of phage (Figs. 7A and 5A). Phage PB-1 maintained a titer of $1x10^6$ pfu per mm^2. In monoculture, the mean biofilm $\log_{10}$ density of *P. aeruginosa* and phage PB-1

was 4.4 ± 0.22 cfu per mm^2 and 5.9 ± 0.58 pfu per mm^2. In mixed culture, the mean biofilm log_{10} densitiy of *P. aeruginosa* and *E. coli* was 4.4 ± 0.2 and 5.2 ± 0.29 cfu per mm^2, respectively (Fig. 7B). Phage PB-1 mean biofilm log_{10} density was 5.3 ± 0.25 pfu per mm^2. The results suggest biofilm growth does not afford *P. aeruginosa* protection from phage infection and PB-1 is able to replicate in both mono and mixed biofilm culture.

Mixed culture with both phage (biofilm). Mixed cultures were infected with both phage to determine if infection with both had a synergistic effect on the viability of the biofilm and phage replication. The results demonstrate infection of mixed biofilms with both phage had no effect on biofilm growth (Figs. 8A and 8B). The growth of mixed biofilms with both phage was similar to the single infection of mixed biofilms and biofilm monocultures. The mean biofilm log_{10} density of *E. coli* and *P. aeruginosa* was 5.4 ± 0.11 and 4.5 ± 0.14 cfu per mm^2, and λW60 and PB-1 phage mean biofilm log_{10} density was 1.6 ± 0.74 and 5.3 ± 0.4 pfu per ml. The results suggest mixed phage infection did not have a synergistic effect on the viability of the biofilm. In addition, the infection of *P. aeruginosa* with its phage did not have a significant effect on the replication of phage λW60.

Bacteriophage resistance. To determine bacteriophage resistance, individual colonies were isolated from planktonic and biofilm cultures which were exposed to phage for 5d, and t_0 phage were assayed using 5d cells as indicators. The results demonstrate both *E. coli* and *P. aeruginosa* in planktonic growth developed resistance to phage, whereas,

biofilm cultures developed partial or no phage resistance (Tables 1 and 2). Overall, in both mixed and monoculture biofilms, *E. coli* developed less resistance compared to *P. aeruginosa*. The development of increased phage resistance by *P. aeruginosa* suggests *P. aeruginosa* is more susceptible to phage infection than is *E. coli* in biofilms.

Phage selection. To determine if phage were selected to infect resistant cells, phage were isolated from planktonic and biofilm cultures which were exposed to bacteriophage for 5d and t_5 phage were assayed using 5d cells as indicators. The results show t_5 phage from planktonic cultures were able to infect t_0 cells but not t_5 cells (Table 3). However, t_5 phage from biofilm cultures were able to infect not only t_0 cells but also t_5 cells. The results of planktonic growth indicate phage were not selected to infect a population of resistant cells (Table 3). In biofilms, however, phage populations were selected which infect resistant cells (Table 4). *P. aeruginosa* biofilm grown cells show no significant difference in resistance to the t_5 phage population as compared to t_0 phage (Table 1). *E. coli* biofilm grown cells showed reduced resistance, indicating either susceptibility to new t_5 phage populations or the lack of development of phage resistance.

Infectivity. To determine if phage infectivity was present as either virions or infectious centers, planktonic and biofilm cultures were assayed for infectivity on t_0 bacteria, both before and after filtration (0.22μm) to remove infectious centers. The results show a majority of plaque forming units in planktonic culture are present as infectious centers and not infectious virions (Fig. 9A). However, biofilm infectivity decreased to $<1 \times 10^0$

pfu per ml after filtration. The results suggest that infectivity is present in biofilms as infectious centers and not virions (Fig. 9B).

Effect of chloroform and Tween-20 on infectivity. To determine if infectivity in biofilms is indeed infectious centers or infectious virus trapped in EPS, biofilm supernatants were treated with either chloroform to lyse infectious centers, or a 0.5% Tween-20 solution to dissolve EPS, filtered (0.22μm), and assayed for infectivity. Results show that treatment with chloroform did not result in an increase in infectivity (Fig. 10). Treatment with 0.5% Tween-20 resulted in an increase in infectivity similar to that seen in planktonic infected cells. The data indicates λW60 and PB-1 infectious phage are present trapped in biofilm EPS.

Electron microscopy. To determine if phage particles could be visualized in infected biofilms, filtered (0.22μm) and Tween-20 treated biofilm supernatants were negatively stained with 2% UA (Uranyl acetate) or 2% PTA (Phosphotungstic acid) using transmission electron microscopy (JEOL 1200 EXII) at 25-30,000X (Figs. 11-13). Results reveal λW60 and PB-1 phage trapped in filtered biofilm EPS (Fig. 12). Tween-20 treated biofilm reveals phage particles free of EPS (Fig. 13). Planktonic grown phage are shown as a control (Fig. 11).

DISCUSSION

The use of bacteriophage as therapeutic agents in the treatment of chronic bacterial infections declined in Western countries after the 1940's, primarily due to the advent of sulfa drugs (4, 52, 53). Sporadic research continued in some areas of the world, including Russia and eastern European countries, but in the western hemisphere phage research was discontinued due to difficulties managing phage purification and host specificity (4, 53). Recently, phage have been enjoying a renaissance in the medical field as increased antibiotic resistance in clinical infections have sent scientists scrambling to find alternatives to multi-drug resistant strains (52). Phage cocktails, or mixtures of phage specific for a single bacterial species, have proven effective in reducing bacterial populations (52). Recently, clinical trials in the UK using a phage-containing preparation on antibiotic-resistant *P. aeruginosa* chronic otitis infections proved successful in reducing bacterial populations (66). Phage are now genetically modified or screened for particular strains to increase their effectiveness (13, 34, 39). Examples include engineered M13 phage targeting gene networks to disable *E. coli* EMG2's SOS DNA repair system, and λW60 phage mutants selected to avoid entrapment by the hosts reticuloendothelium system to increase circulation effectiveness against *E. coli* CRM1 septicemias in mice (34, 39). Strain specific phage SF153 polysaccharide depolymerase

glycanase has been shown to reduce the thickness of a *Enterobacter agglomerans* 53b EPS when grown in a biofilm and allows phage SF153b to penetrate and infect cells (27). The observation that phage are capable of penetrating biofilms is more important than ever as The Centers for Disease Control and Prevention estimates that more than 65% of infections are caused by biofilm-growing bacteria (64). The ability of phage to be successfully modified for enhanced effectiveness as therapeutic agents for use in clinical applications will mean phage will be an important component in treating chronic biofilm-related infections.

This study reports the effect of mixed λW60 and PB-1 bacteriophage infection on the growth of mixed *Escherichia coli* and *Pseudomonas aeruginosa* planktonic and biofilm cultures. Bacteriophage λW60 and PB-1 mixed infection had no significant effect on the growth or cell density of either mixed planktonic or biofilm cultures. Although phage infection of planktonic grown cultures resulted in a 90% reduction in cell population within 24h, infected cultures had recovered over time and returned to their initial cell densities. The results of this study suggest that although the therapeutic use of mixed phage infection to treat chronic infection may not clear infection caused by an established biofilm, phage therapy may be an effective means of controlling the growth and spread of cells which originate from biofilms. The maintenance of phage titers, cells susceptible to phage infection, and infectious phage trapped within biofilm EPS indicates biofilms can act as a reservoir for phage replication.

Bacteriophage λW60 and PB-1 populations maintained high titers in planktonic culture and planktonic cells developed complete resistance. Phage resistance may be due to either selection for resistant cell populations or gene transduction to susceptible cells (32). These findings are in agreement with others which show that phage infection does not have a long-term effect on the growth of cell density in planktonic culture (23, 32). In biofilm infected cultures, however, both λW60 and PB-1 were able to replicate although overall infectious phage titers were lower than in planktonic culture. The reduced phage titers may be due to spatial limitations of the biofilm disks. Lower phage resistance was also present in infected biofilm cells. The results of this study correlates with other studies that have shown a reduced selection for resistant populations and reduced resistance gene transduction in biofilms (27, 47). The presence of susceptible cells in biofilms and not in planktonic culture suggest that phage either cannot penetrate the interior of a biofilm or are unable to replicate in metabolically inactive cells within the biofilm. Phage multiplication has been shown to occur at a reduced rate in biofilm cells which are metabolically less active than planktonically grown cells (43, 54, 51, 61). Cells in a biofilm microcolony use alternative gene pathways in addition to switching on anaerobic metabolism (8, 11, 51). *P. aeruginosa* use phenazines as redox alternatives in anaerobic metabolism, but our results indicated that *P. aeruginosa* had no bacteriocidal effect on *E. coli* in mixed culture biofilms, whereas in mixed planktonic culture pyocyanin concentrations can quickly become lethal (59, 24). The persister cell state with alternative metabolism pathways present in biofilms may lead to poor activation of needed promoters for a lytic switch in phage (43). Even after becoming activated, however, programmed cell death in biofilm communities may lower the number of

bacteriophage released into the environment (3, 54). Programmed cell death may also lead to ineffective phage infection of recently activated bacteria which are slated to die (3). Other factors include the physical impossibility of infection due to entrapment of released phage particles in the biofilm EPS matrix (passive resistance).

The extra cellular polymeric substance itself contains an array of various proteins and carbohydrates which may "camouflage" bacteria within the biofilm from antimicrobials and bacteriophage infections (54). In *E. coli* MG1655 EPS, various carbohydrate metabolism proteins as well as outer membrane porin proteins were found (16). *E. coli* typically sheds its outer membrane during biofilm development and undergoes programmed cell death which may lead to a concentration of membrane macromolecules in biofilm EPS (16). This EPS-associated macromolecular concentration may act as a potential deterrent to λW60 phage infection by acting as an inhibiting adsorbent for the λW60 J protein receptor which binds to *lamB* porin. Expression of *lamB*-maltose specific proteins were increased in *E. coli* biofilms including *lamB*, *malG* and *malE* transporter proteins (16, 50). In *P. aeruginosa*, lipopolysaccharide (LPS) serves as an important component of EPS gene function and propagation (36). The *algC* gene product is a factor in three *P. aeruginosa* virulence-associated saccharides: alginate, LPS, and rhamnolipid formation (41). The presence of 2-linked and 3-linked rhamnose sugars in PAO1 biofilms, both of which are important components of EPS, provide support for the presence of LPS in *P. aeruginosa* biofilms (65). Smooth LPS is a specific receptor for bacteriophages E79 and PB-1, and it has been shown that sonicated LPS can inactivate phage particles in suspension (29). These extracellular LPS particles can act as a neutralizing absorbent in the EPS matrix, binding the tail receptors of PB1-

like phages. Particles bound to EPS were infectious upon release with Tween-20 treatment. Therefore, the binding of phage to receptors in biofilm is temporary and does not permanently inactivate virions.

Decreased bacteriophage resistance to λW60 in *E. coli* biofilms compared to planktonic infected cells may be due to an inability of the phage to penetrate the EPS matrix of a biofilm. λW60 phage titers decreased at 24h post-infection to near undetectable levels in both mixed and monoculture biofilms, but increased to 50% of the initial infecting phage titer. The reduced ability of λW60 phage to replicate in biofilms compared to PB-1 may be due to a portion of infecting or progeny phage trapped in EPS and unable to infect susceptible cells. PB-1 phage titers remained constant in biofilm infected cells, which suggests phage PB-1 is able to penetrate and therefore replicate within *P. aeruginosa* biofilms, more easily than λW60 in *E. coli* biofilms. *P. aeruginosa* developed resistance to PB-1 which supports the notion of bacterial susceptibility to infection in biofilms. Studies with *P. aerugionosa* phage F116 and GL1 suggest bacteriophage migration through *P. aeruginosa* biofilms may be facilitated by a reduction in alginate viscosity brought about by enzymatic degradation and that the source of the enzyme may be the bacteria itself. The phage may utilize this enzyme by attaching it to its outer coat proteins to assist penetration of biofilm exopolysaccharide (22). In addition, *P. aeruginosa* utilizes its *own* polysaccharide depolymerase in response to a biofilm phage infection so that its cells are more likely to become infected and selection for phage resistance may occur. *Enterobacter agglomerans* 53b bacteriophage SF153b possesses a polysaccharide depolymerase enzyme specific for the EPS of strain 53b (27).

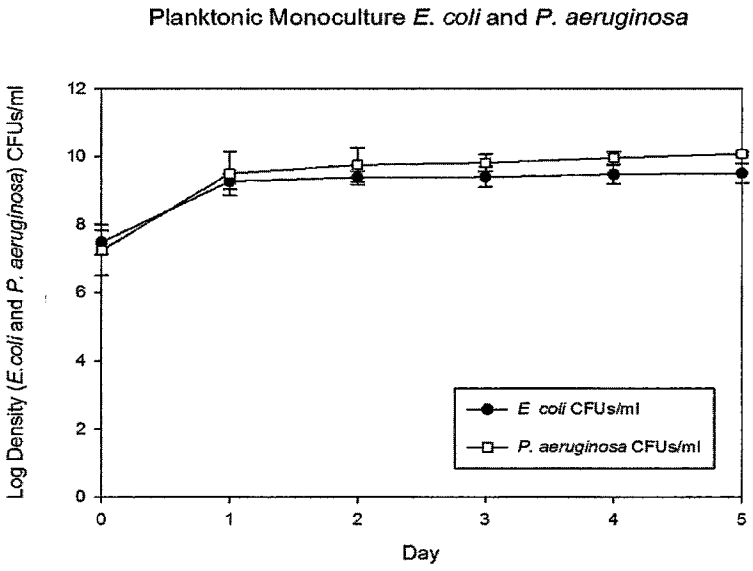
In the same manner, our data suggests phage-utilized depolymerase may be an important component of PB-1 phage activity in biofilms.

Infectivity from biofilms was found to decrease following filtration which indicated infectivity was not due to the presence of free infectious virus but rather infectious centers or possibly virions embedded in EPS. Treatment of biofilms with Tween-20, a surfactant that dissociates biofilm EPS, however, resulted in increased infectivity (18, 56). The observation that treatment with chloroform, which lyses cell membranes, did not cause any increase in infectivity indicated infectious virus was more likely present trapped in extracellular matrix and infectivity was not due to infectious centers (22, 25). Electron microscopy of Tween-20 treated biofilm supernatants revealed both λW60 and PB-1 phage particles eluting from the biofilm matrix. Similar findings were reported by Resch et al. which showed *Staphylococcus aureus* phage SA113 eluting from *S. aureus* biofilms (47). The inability of phage to fully penetrate biofilms by entrapment in EPS may be beneficial by providing a population of susceptible cells. Uninfected biofilm cells can act as a phage host reservoir and lead to new phage replication as susceptible cells are released from the biofilm. This ability of the phage to thrive off susceptible biofilm populations also displays how biofilms can maintain phage counts or even increase them in the surrounding planktonic environment, which might account for high phage titers present in natural environments such as ocean water ($>10^6$ particles ml^{-1}) (7, 20). Several studies have also shown that infected biofilms release progeny phage for longer periods of time than planktonic culture, and phage therapy has the potential to reduce dissemination of bacteria from established biofilms (13, 14, 15, 19). Prophylactic use of *Staphylococcus epidermidis* phage 456 has been effective in

controlling biofilms in catheter hydrogels (14). In the special case of treating chronic biofilm infections, we may have found our first effective self-replicating antimicrobial agent!

Although mixed phage infection did not result in a decrease in bacterial biofilm population we did observe an increase in λW60 bacteriophage resistance in mixed culture with both phage compared to single phage infection (Table 1A and 2A). This suggests a possible increased λW60 infection rate in mixed infection with PB-1. The enzymatic activity of the biofilm penetrating phage PB-1 may also benefit the non-biofilm penetrating phage λW60. In the absence of an actual reduction in biofilm populations, the induction of phage resistance involves the altering or removal of cell wall receptors which decreases overall cell fitness and may enhance the effectiveness of co-administered antibiotics (11, 28). Thus, mixed λW60 and PB-1 phage infection may be more effective in halting biofilm-released planktonic spread or reducing biofilm viscosity in conjunction with antibiotic treatment. With increased research in bacteriophage applications, phage engineered with repressors targeting bacterial promoters for either persister-state dormancy or EPS production may be effective when used in conjunction with a polysaccharide depolymerase glycanase. A study by Lu et al. demonstrated that engineered bacteriophage targeting alternate protein expression pathways could be effective as an antibiotic adjuvant in treating bacterial infections (34). With increased research and development on biofilm and bacteriophage interactions, it seems standardized bacteriophage treatment for biofilm infections will soon become a reality.

A.



B.

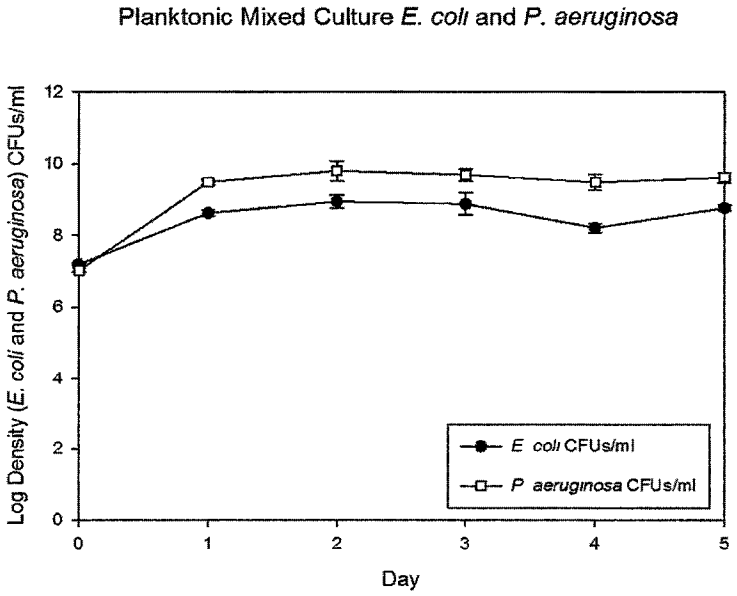
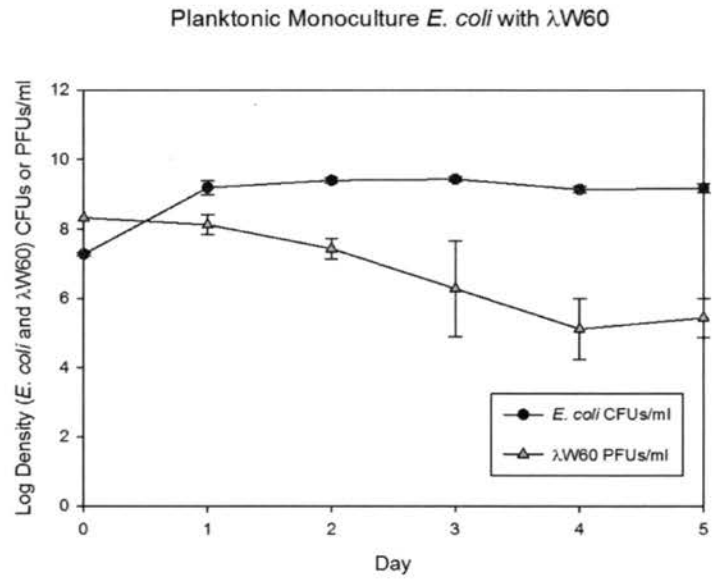


Figure 1. **Mean planktonic bacterial density without bacteriophage**. (A) *E. coli* (●) and *P. aeruginosa* (□) in planktonic monoculture. (B) *E. coli* (●) and *P aeruginosa* (□) in planktonic mixed culture.

A.



B.

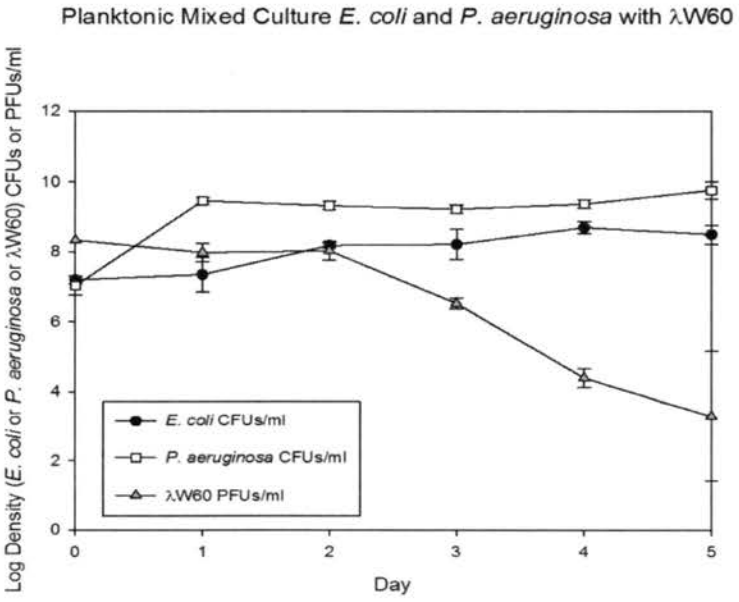
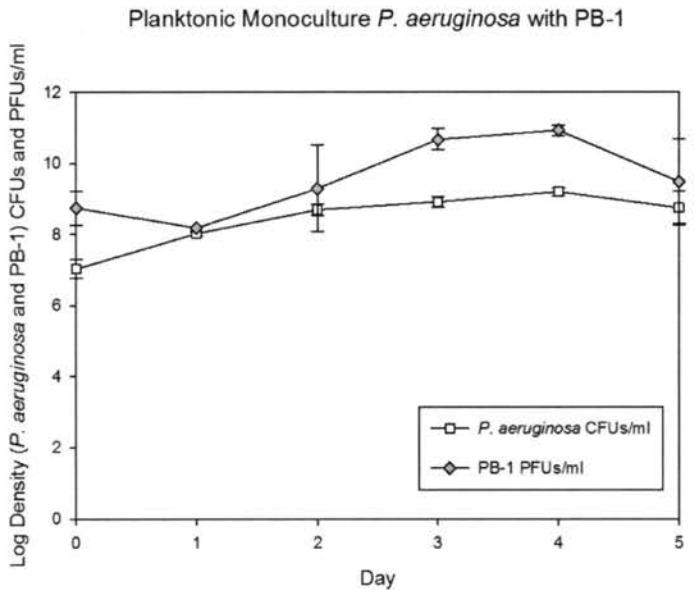


Figure 2. *E. coli* mean planktonic density in the presence of phage λW60. (A) *E. coli* (●) and λW60 (Δ) in planktonic monoculture. (B) *E. coli* (●), *P. aeruginosa* (□), and λW60 (Δ) in planktonic mixed culture.

A.



B.

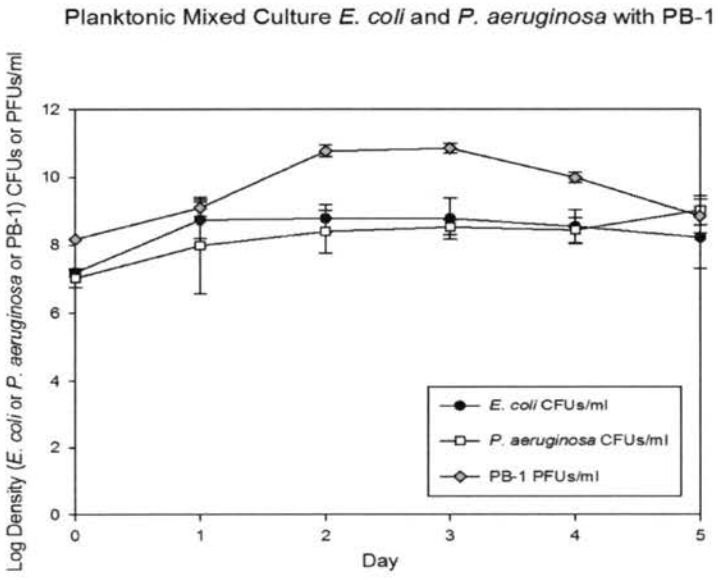
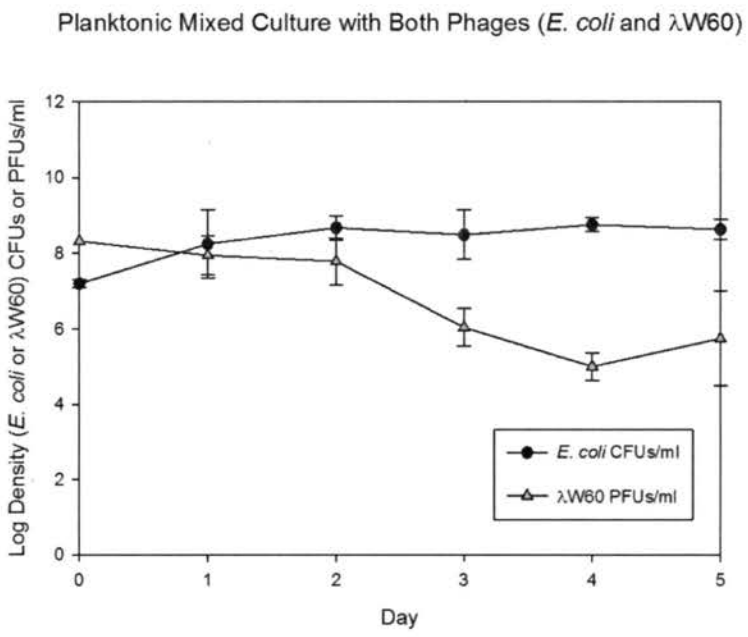


Figure 3. ***P. aeruginosa*** **mean planktonic density in the presence of phage PB-1.** (A) *P. aeruginosa* (□), and PB-1 (◊) in planktonic monoculture. (B) *P. aeruginosa* (□), *E. coli* (●), and PB-1 (◊) in planktonic mixed culture.

A.



B.

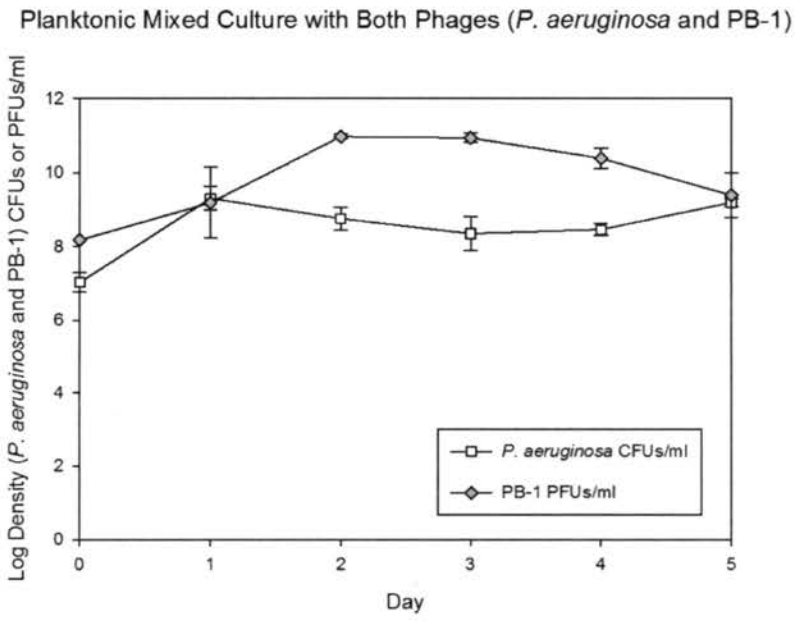
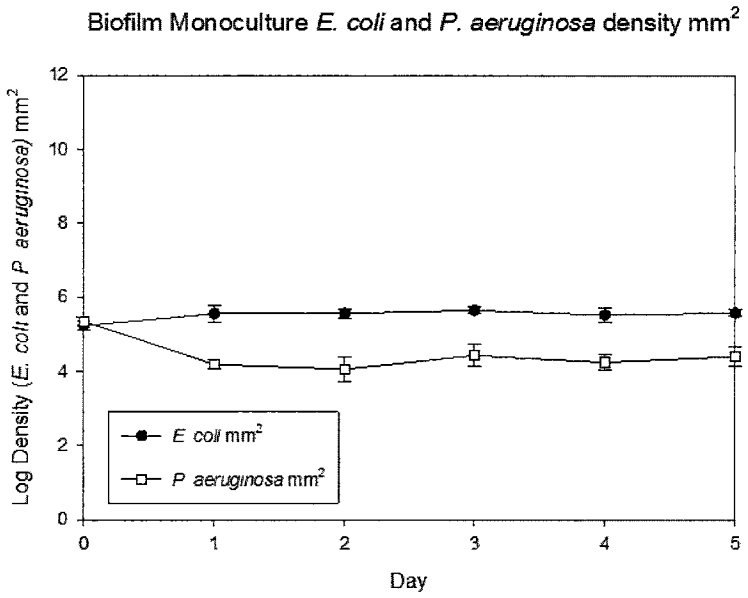


Figure 4. ***E. coli* and *P. aeruginosa* mean planktonic density with phages λW60 and PB-1.** (A) *E. coli* (●) and λW60 (Δ) in planktonic mixed culture with both phage. (B) *P. aeruginosa* (□), and PB-1 (◊) in planktonic mixed culture with both phage.

A.



B.

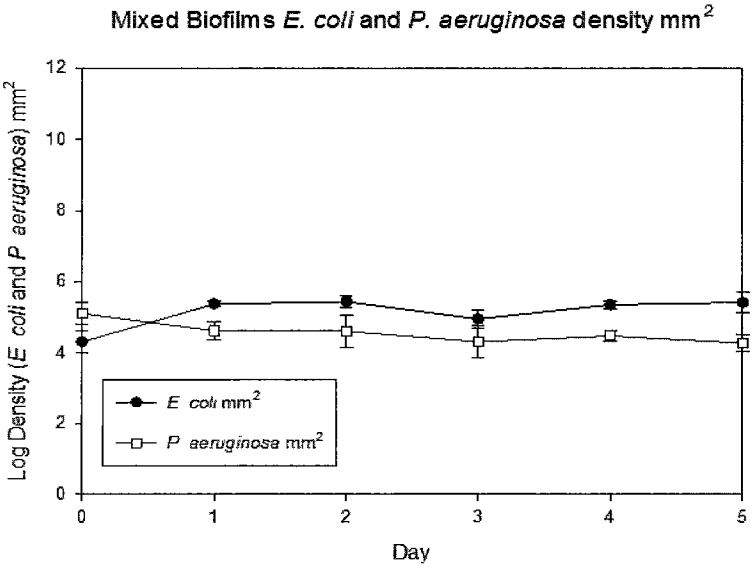
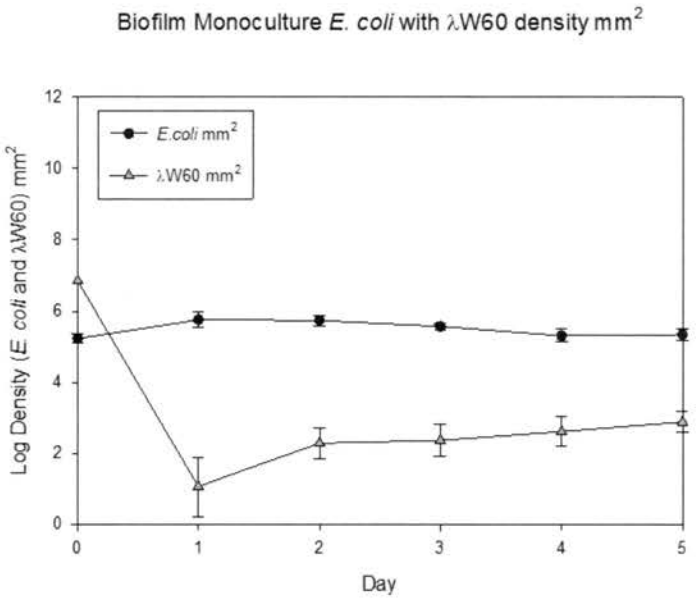


Figure 5. **Mean biofilm bacterial density without bacteriophage.** (A) *E. coli* (●) and *P. aeruginosa* (□) in monoculture biofilm. (B) *E. coli* (●) and *P. aeruginosa* (□) in mixed culture biofilms.

A.



B.

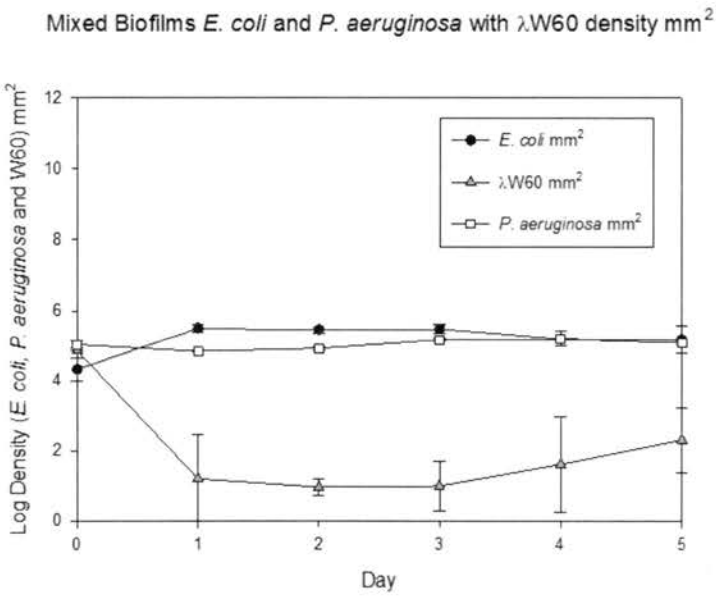
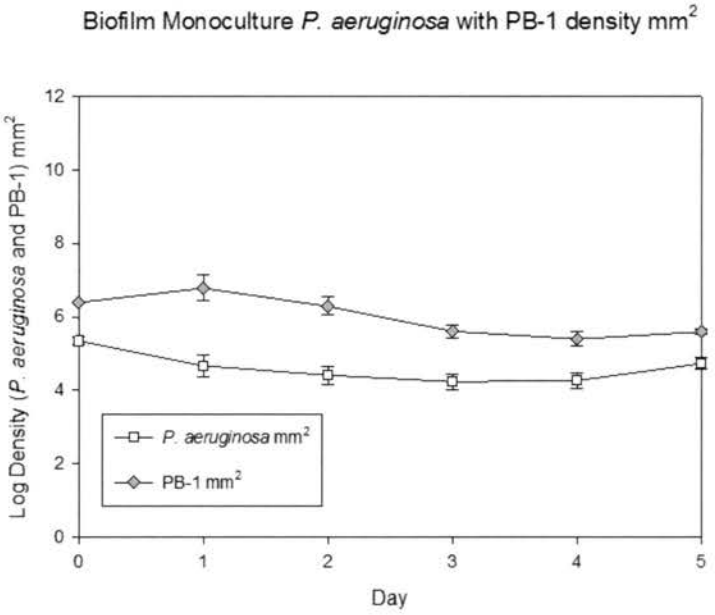


Figure 6. ***E. coli* mean biofilm density in the presence of phage λW60.** (A) *E. coli* (●) and λW60 (Δ) in monoculture biofilm. (B) *E. coli* (●), *P. aeruginosa* (□), and λW60 (Δ) in mixed culture biofilms.

A.



B.

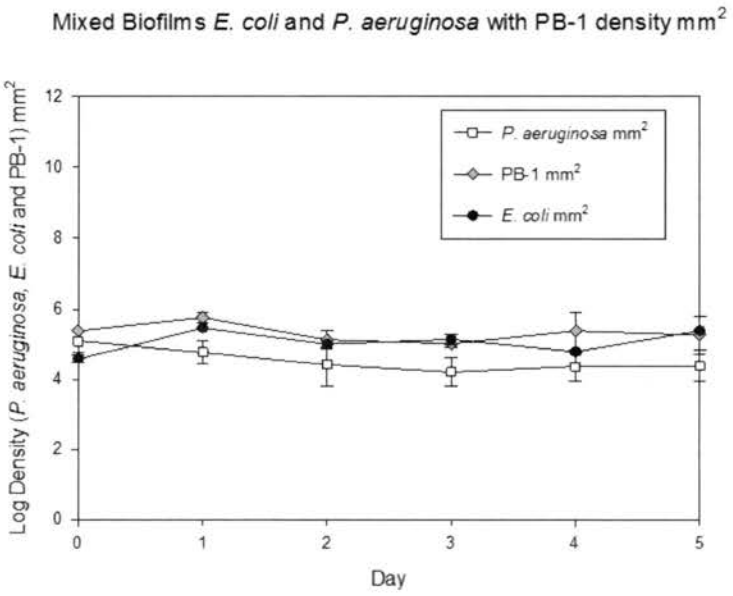
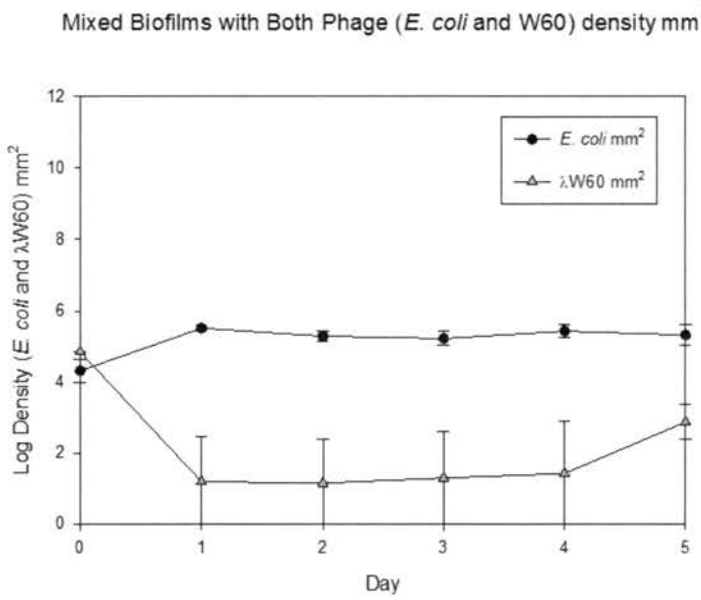


Figure 7. ***P. aeruginosa*** **mean biofilm density in the presence of phage PB-1.** (A) *P. aeruginosa* (□), and PB-1 (◊) in monoculture biofilm. (B) *P. aeruginosa* (□), *E. coli* (●), and PB-1 (◊) in mixed culture biofilms.

A.



B.

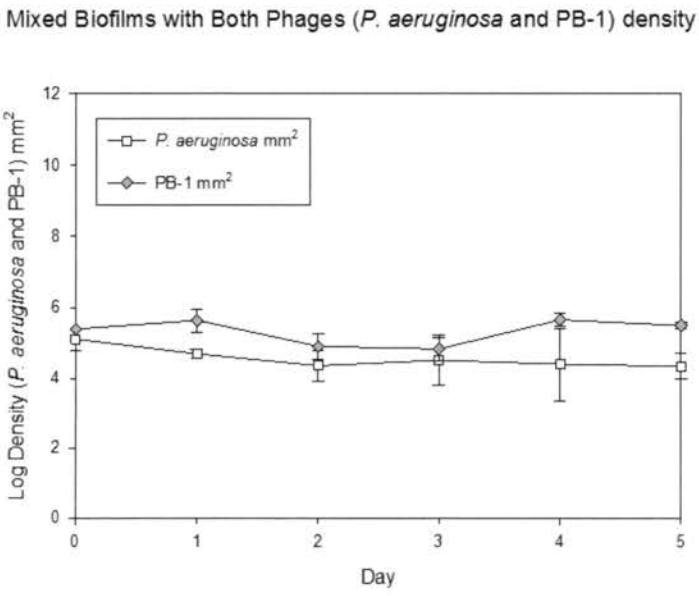


Figure 8. ***E. coli* and *P. aeruginosa* mean biofilm density with phages λW60 and PB-1.** (A) *E. coli* (●) and λW60 (Δ) in biofilm mixed culture with both phage. (B) *P. aeruginosa* (□), and PB-1 (◊) in biofilm mixed culture with both phage.

Table 1. ***E. coli* Resistance to λW60 bacteriophage.**

	E. coli and λW60 Monoculture	Mixed Culture with λW60	Mixed Culture Both Phage (λW60)
Planktonic	100[a]	100	100
Biofilm	0	0	25

[a] percent of colonies isolated found resistant to original t_0 phage; N = 4

Table 2. ***P. aeruginosa* Resistance to PB-1 bacteriophage.**

	P. aeruginosa and PB-1 Monoculture	Mixed Culture with PB-1	Mixed Culture Both Phage (PB-1)
Planktonic	100[a]	100	100
Biofilm	75	50	75

[a] percent of colonies isolated found resistant to original t_0 phage; N = 4

Table 3. **λW60 Phage Selection to Infect Resistant *E. coli*.**

	E. coli and λW60 Monoculture	Mixed Culture with λW60	Mixed Culture Both Phage (λW60)
Planktonic	0[a]	0	0
Biofilm	100	100	100

[a] percent of colonies isolated found susceptible to t_5 phage; N = 4

Table 4. **PB-1 Phage Selection to Infect Resistant *P. aeruginosa*.**

	P. aeruginosa and PB-1 Monoculture	Mixed Culture with PB-1	Mixed Culture Both Phage (PB-1)
Planktonic	0[a]	0	0
Biofilm	33	50	33

[a] percent of colonies isolated found susceptible to t_5 phage; N = 4

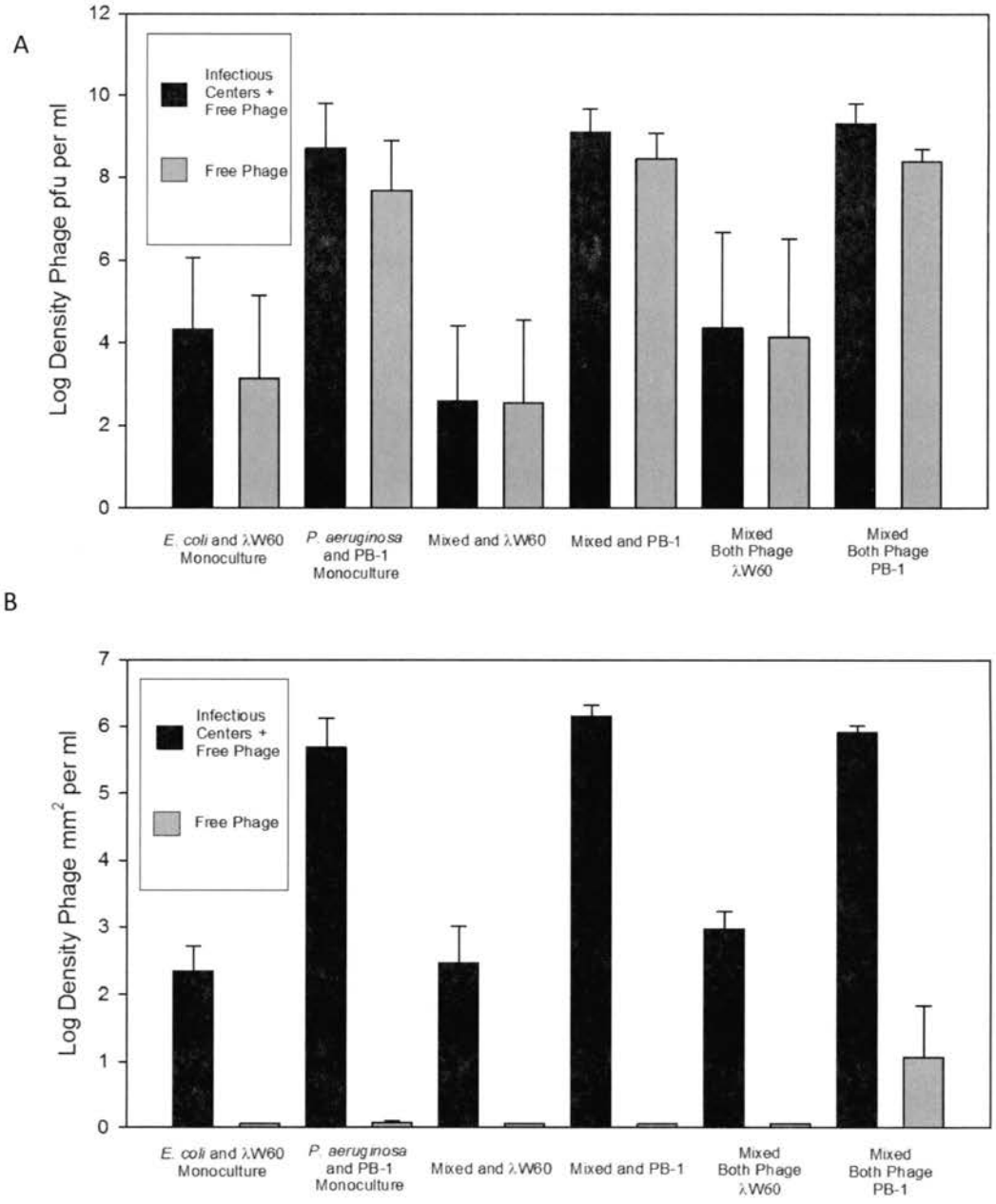


Figure 9. **Infectivity.** (A) Planktonic. Log planktonic unfiltered versus filtered t_5 phage pfu per ml. Infectious centers plus free phage pfu per ml (black); free phage pfu per ml (gray). (B) Biofilm. Log biofilm unfiltered versus filtered t_5 phage mm^2 densities. Infectious centers plus free phage log biofilm mm^2 density (black); free phage log biofilm mm^2 density (gray).

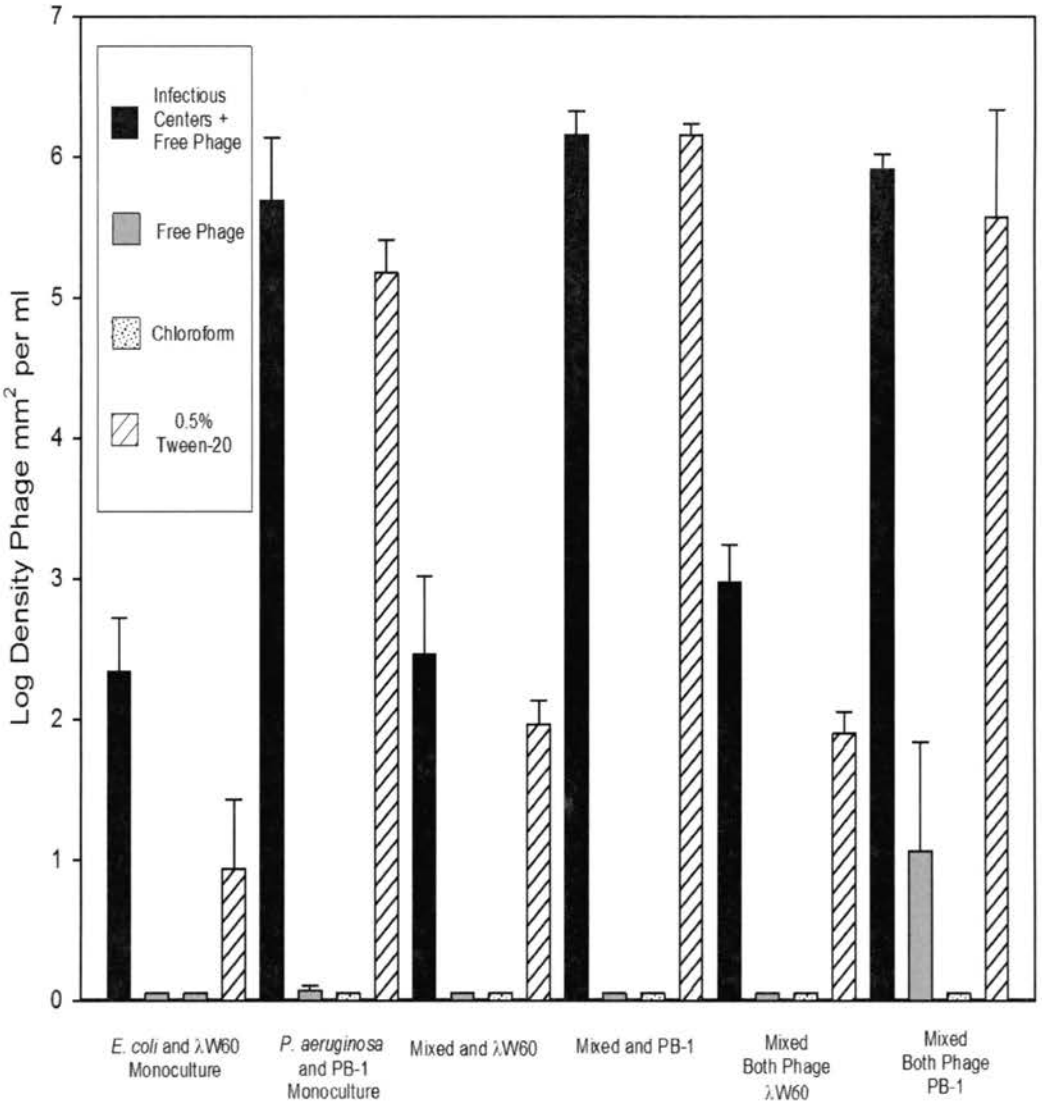


Figure 10. **Effect of chloroform and 0.5% Tween-20 on biofilm infectivity.** Log biofilm mm^2 densities for unfiltered t_5 phage titers versus filtered t_5 phage titers (from Fig. 9), compared to filtered phage titers exposed to chloroform and 0.5% Tween-20 treatment. Infectious centers plus free phage log biofilm mm^2 density (black); free phage log biofilm mm^2 density (gray). Chloroform-treated log biofilm mm^2 density (dots); 0.5% Tween-20-treated log biofilm mm^2 density (stripes).

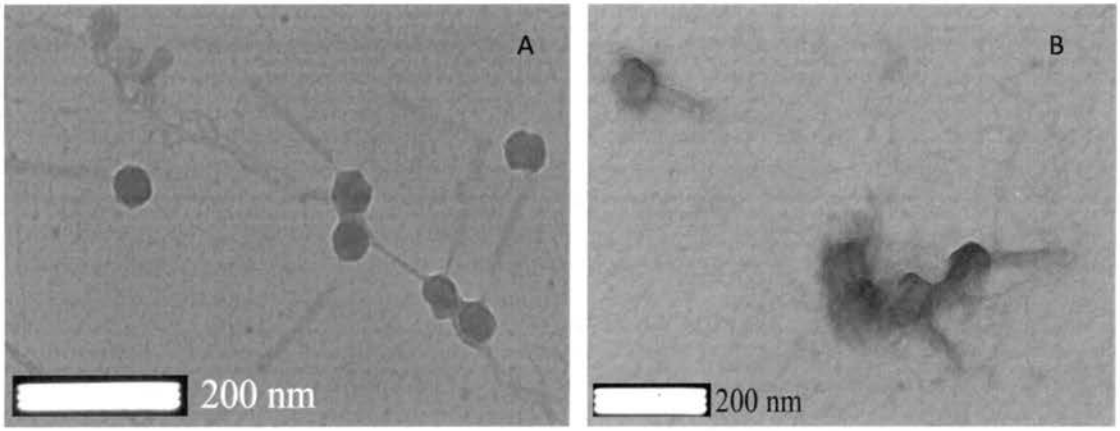


Figure 11. **Electron microscopy.** λW60 phage (A) and PB-1 (B) from planktonically grown cells. Negatively stained with 2% UA using transmission electron microscopy at 25K and 30K magnification.

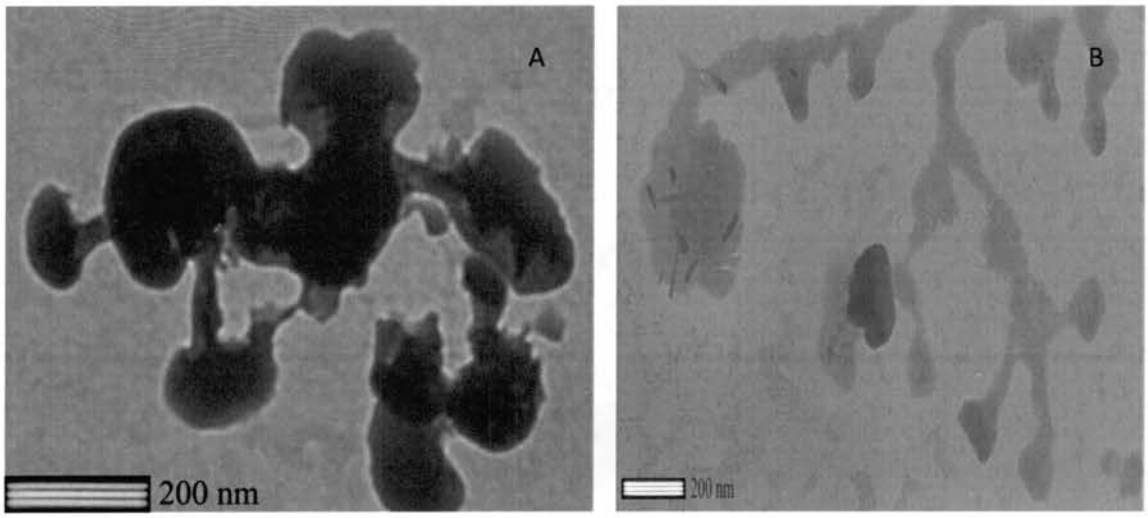


Figure 12. **Electron microscopy.** λW60 (A) and λW60 plus PB-1 (B) from single and mixed biofilm infected cultures. Negative stain with 2% PTA using transmission electron microscopy at 30K magnification.

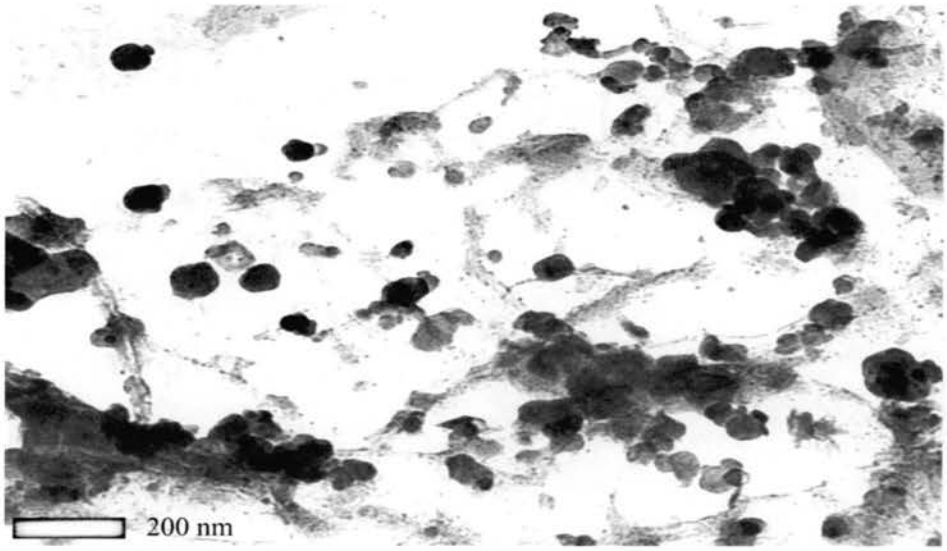


Figure 13. **Electron microscopy.** λW60 and PB-1 from mixed biofilm infected culture following 0.5% Tween-20 treatment. Negative stain with 2% PTA 30K magnification.

REFERENCES

1. **M. H. Adams**. 1956. Bacteriophages. **97-119**.

2. **M. H. Adams, Park, B. H.** 1956. An enzyme produced by a phage-host cell system. II. The properties of the polysaccharide depolymerase. Virology. **2**: 719-736.

3. **K. W. Bayles**. 2007. The biological role of death and lysis in biofilm development. Nat Rev Microbiol. **5** (9): 721-726.

4. **J. Borysowski, Gorski, A.** 2008. Is phage therapy acceptable in the immunocompromised host? Int J Infect Dis. **12** (5): 466-471.

5. **D. E. Bradley, Robertson, D.** 1968. The structure and infective process of a contractile *Psuedomonas aeruginosa* bacteriophage. J Gen Virol. **45** (1): 90-97.

6. **D. J. Brenner, Kreig, N. R., Staley, J. T.** 2005. Bergey's Manual of Systematic Bacteriology, 2nd ed.

7. **C. J. Briggs, Hoopes, M. F.** 2004. Stabilizing effects in spatial parasitoid-host and predator-prey models: a review. Theor Popul Biol. **65**: 299-315.

8. **M. Burmølle, Webb, J. S., Rao, D., Hansen, L. H., Sørensen, S. J., Kjelleberg, S.** 2006. Enhanced Biofilm Formation and Increased Resistance to Antimicrobial Agents and Bacterial Invasion Are Caused by Synergistic Interactions in Multispecies Biofilms. Applied and Environ Microbio. **72** (6): 3916-3923.

9. **B. J. Cairns, Timms, A. R., Jansen, V. A., Connerton, I. F., Payne, R. J. H.** 2009. Quantitative Models of *In Vitro* Bacteriophage-Host Dynamics and Their Application to Phage Therapy.

10. **P. Ceyssens, Miroshnikov, K., Mattheus, W., Krylov, V., Robben, J., Noben, J., Vanderschraeghe, S., Sykilinda, N., Kropinski, A. M., Volckaert, G., Mesyanzhinov, V., Lavigne, R.** 2009. Comparative analysis of the widespread and conserved PB1-like viruses infecting *Pseudomonas aeruginosa*. Environmental Microbiology. **11** (11): 2874-2883.

11. **S. Chibani-Chennoufi, Bruttin, A., Marie-Lise D., Brüssow, H.** 2004. Phage-Host Interaction: an Ecological Perspective. J of Bacteriol. **186** (12): 3677-3686.

12. **B. D. Corbin, McLean, R. J., Aron, G. M.** 2001. Bacteriophage T4 multiplication in a glucose-limited *Escherichia coli* biofilm. Can J Microbiol. **47** (7): 680-684.

13. **J. J. Curtin, Donlan, R. M.** 2006. Using Bacteriophages to Reduce Formation of Catheter-Associated Biofilms by *Staphylococcus epidermidis*. Antimicrob Agent Chemother. **50** (4): 1268-1275.

14. **R. M. Donlan**. 2006. Controlling clinically relevant biofilms using bacteriophages. Biofilm Perspectives. **2**

15. **R. M. Donlan**. 2009. Preventing biofilms of clinically relevant organisms using bacteriophage. Trends in Microbiology. **17** (2): 66-72.

16. **K. E. Eboigbodin, Biggs, C. A.** 2008. Characterization of the extracellular polymeric substances produced by *Escherichia coli* using infrared spectroscopic, proteomic, and aggregation studies. Biomacromolecules. **9**: 686-695.

17. **R. Efrony, Atad, T., Rosenberg, E.** 2009. Phage Therapy of Coral White Plague Disease: Properties of Phage BA3. Curr Microbiol. **58**: 139-145.

18. **P. J. Eginton, Holah, J., Allison, D. G., Handley, P. S., Gilbert, P.** 1998. Changes in the strength of attachment of micro-organisms to surfaces following treatment with disinfectants and cleansing agents. Letters in Applied Microbiol. **27**: 100-105.

19. **W. Fu, Foster, T., Mayer, O., Curtin, J. J., Lehman, S. M., Donlan, R. M.** 2010. Bacteriophage Cocktail for the prevention of biofilm formation by *Pseudomonas aeruginosa* on catheters in an *in vitro* model system. Antimicrob Agent Chemother. **54** (1): 397-404.

20. **J. A. Fuhrman.** 1999. Marine viruses and their biogeochemical and ecological effects. Nature. **399**: 541-548.

21. **M. Hamilton, Heersink, J., Buckingham-Meyer, K., Goeres, D.** 2003. The Biofilm Laboratory. Step-by-step protocols for experimental design, analysis, and data interpretation. **1-71.**

22. **G. W. Hanlon, Denyer, S. P., Olliff, C. J., Ibrahim, L. J.** 2001. Reduction in exopolysaccharide viscosity as an aid to bacteriophage penetration through *Pseudomonas aeruginosa* biofilms. Applied and Environ Microbio. **67** (6): 2746-2753.

23. **W. R. Harcombe, Bull, J. J.** 2005. Impact of Phages on Two-Species Bacterial Communities. Applied and Environ Microbio. **71** (9): 5254-5259.

24. **H. M. Hassan, Fridovich, I.** 1980. Mechanism of the Antibiotic Action of Pyocyanine. J of Bacteriol. **141** (1): 156-163.

25. **R. H. Heineman, Bull, J. J.** 2007. Testing Optimality with Experimental Evoluation: Lysis Time in a Bacteriophage. Evolution. **61** (7): 1695-1709.

26. **R. W. Hendrix, Smith, M. C., Burns, R. N., Ford, M. E., Hatfull, G.** 1999. Evolutionary relationships among diverse bacteriophages and prophages: all the world's a phage. Proc. Natl. Acad. Sci. USA. **96**: 2192-2197.

27. **K. A. Hughes, Sutherland, I. W., Jones, M. V.** 1998. Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. Microbio. **144**: 3039-3047.

28. **K. Jarrell, Kropinski, A.** 1977. Identification of the cell wall receptor for bacteriophage E79 in *Pseudomonas aeruginosa* strain PAO. J Virol. **23** (3): 461-466.

29. **K. Jarrell, Kropinski, A.** 1981. *Pseudomonas aeruginosa* bacteriophage psiPLS27-lipopolysaccharide interactions. J Virol. **40** (2): 411-420.

30. **I. Keren, Kaldula, N., Spoering, A., Wang, Y., Lewis, K.** 2003. Persister cells and tolerance to antimicrobials. FEMS Microbiol Letters. **230**: 13-18.

31. **D. M. Knipe, Howley, P. M.** 2001. Fundamental Virology, 4th edition.

32. **R. Lenski.** 1988. Dynamic interactions between bacteria and virulent bacteriophage. Advances in Microbial Ecology. **10**: 1-44.

33. **A. A. Lindberg.** 1977. Bacterial surface carbohydrates and bacteriophage adsorption.

34. **T. K. Lu, Collins, J. J.** 2009. Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. PNAS. **106** (12): 4629-4634.

35. **J. B. Lyczak, Cannon, C. L., Pier, G. B.** 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes Infectious. **2**: 1051-1060.

36. **M. Matsukawa, Greenberg, E. P.** 2004. Putative exopolysaccharide synthesis genes influence *Pseudomonas aeruginosa* biofilm development. J of Bacteriol. **186** (14): 4449-4456.

37. **K. McLean, Winson, M., Fish, L., Taylor, A., Chabra, S., Camara, M., Daykin, M., Lamb, J., Swift, S., Bycroft, B., Stewart, G., Williams, P.** 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbio. **143**: 3703-3711.

38. **M. Merabishvili, Pirnay, J., Verbeken, G., Chanishvili, N., Tediashvili, M., Lashkhi, N., Glonti, T., Krylov, V., Mast, J., Parys, L. V., Lavigne, R., Volckaert, G., Mattheus, W., Verween, G., De Corte, P., Rose, T., Jennes, S., Zizi, M., De Vos, D., Vaneechoutte, M.** 2009. Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. PLoS ONE. **4** (3): e4944.

39. **C. R. Merril, Biswas, B., Carlton, R., Jensen, N. C., Creed, G. J., Zullo, S., Adhya, S.** 1996. Long-circulating bacteriophage as antibacterial agents. Proc. Natl. Acad. Sci. USA. **93**: 3188-3192.

40. **M. B. Miller, Bassler, B. L.** 2001. Quorum Sensing in Bacteria. Annu Rev Microbiol. **55**: 165-199.

41. **C. Olvera, Goldberg, J. B., Sanchez, R., Soberon-Chavez, G.** 1999. The *Pseudomonas aeruginosa* algC gene product participates in rhamnolipid biosynthesis. FEMS Microbiol Letters. **179** (1): 85-90.

42. **R. E. Osterhout, Figueroa, I. A., Keasling, J. D., Arkin, A. P.** 2007. Global analysis of host response to induction of a latent bacteriophage. BMC Microbiol. **7**: 82.

43. **S. Pearl, Gabay, C., Kishony, R., Oppenheim, A., Balaban, N. Q.** 2008. Nongenetic Individuality in the Host-Phage Interaction. PLoS Biol. **6** (5): 957-964.

44. **E. A. Pleteneva, Shaburova, O. V., Krylov, V. N.** 2009. A formal scheme of adsorptional receptors in *Pseudomonas aeruginosa* and possibilities for its practical implementation. Russian Journal of Genetics. **45** (1): 43-49.

45. **E. A. Pleteneva, Shaburova, O. V., Sukilinda, N. N., Miroshnikov, K. A., Kadykov, V. A., Krylov, S. V., Mesyanzhinov, V. V., Krylov, V. N.** 2008. Study of the Diversity in a Group of Phages of *Pseudomonas aeruginosa* Species PB1 (Myoviridae) and Their Behavior in Adsorption-Resistant Bacterial Mutants. Russian Journal of Genetics. **44** (2): 150-158.

46. **M. H. V. e. a. Regenmortel.** 2000. Virus Taxonomy. **81-88**.

47. **A. Resch, Fehrenbacher, B., Eisele, K., Schaller, M., Götz, F.** 2005. Phage release from biofilm and planktonic *Staphylococcus aureus* cells. FEMS Microbiol Letters. **252**: 89-96.

48. **J. M. Ritchie, D. W. Wagner, W. Acheson, M. K. Waldor**. 2003. Comparison of Shiga toxin production by hemolytic-uremic syndrome-associated and bovine-associated Shiga toxin-producing *Escherichia coli* isolates. Applied and Environ Microbio. **69**: 1059-66.

49. **K. Sauer, Camper, A. K., Ehrlich, G. D., Costerton, J. W., Davies, D. G.** 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J. Bacterio. **184** (4): 1140-1154.

50. **M. A. Schembri, Kjaergaard, K., Klemm, P.** 2003. Global gene expression in *Escherichia coli* biofilms. Molecular Microbiology. **48** (1): 253-267.

51. **P. Stoodley, Sauer, K., Davies, D. G., Costerton, J. W.** 2002. Biofilms as Complex Differentiated Communities. Annu Rev Microbiol. **56**: 187-209.

52. **A. Sulakvelidze, Alavidze, Z., Morris, J. G., Jr.** 2001. Bacteriophage therapy. Antimicrob Agent Chemother. **45** (3): 649-659.

53. **W. C. Summers**. 2001. Bacteriophage Therapy. Annu Rev Microbiol. **55**: 437-451.

54. **I. W. Sutherland, Hughes, K. A., Skillman, L. C., Tait, K.** 2004. The interaction of phage and biofilms. FEMS Microbiol Letters. **232**: 1-6.

55. **Y. Tanji, Hattori, K., Suzuki, K., Miyanaga, K.** 2008. Spontaneous Deletion of a 209-Kilobase-Pair Fragment from the *Escherichia coli* Genome Occurs with Acquisition of Resistance to an Assortment of Infectious Phages. Applied and Environ Microbiol. **74** (14): 4256-4263.

56. **C. M. Toutain-Kidd, Kadivar, S. C.,Bramante, C. T., Bobin, S. A., Zegans, M. E.** 2009. Polysorbate 80 Inhibition of *Pseudomonas aeruginosa* Biofilm Formation and Its Cleavage by the Secreted Lipase LipA. Antimicrob Agent Chemother. **53** (1): 136-145.

57. **J. Wang, Hofnung, M., Charbit, A.** 2000. The C-terminal portion of the tail fiber protein of bacteriophage lambda is responsible for binding to LamB, its receptor at the surface of *Escherichia coli* K-12. J. Bacteriol. **182** (2): 508-512.

58. **X. Wang, Preston III, J. F., Romeo, T.** 2004. The *pgaABCD* locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. J of Bacteriol. **186** (9): 2724-2734.

59. **Y. Wang, Kern, S. E., Newman, D. K.** 2010. Endogenous phenazine antibiotics promote anaerobic survival of *Pseudomonas aeruginosa* via extracellular electron transfer. J of Bacteriol. **192** (1): 365-369.

60. **J. S. Webb, Lau, M., Kjelleberg, S.** 2004. Bacteriophage and Phenotypic Variation in *Pseudomonas seruginosa* Biofilm Development. J of Bacteriol. **186** (23): 8066-8073.

61. **S. A. West, Diggle, S. P., Buckling, A., Gardner, A., Griffin, A. S.** 2007. The Social Lives of Microbes. Annu Rev Ecol Evol Syst. **38**: 53-77.

62. **B. E. Windle, Hays, J. B.** 1986. A phage P1 function that stimulates homologous recombination of the *Escherichia coli* chromosome. Proc. Natl. Acad. Sci. USA. **83**: 3885-3889.

63. **M. Wojciech F., R., Weber-Dąbrowska, B., Górski, A.** 2008. Bacteriophage therapy in children: Facts and prospects. Med Sci Monit. **14** (8): 126-132.

64. **R. D. Wolcott, Ehrlich, G. D.** 2008. Biofilms and Chronic Infections. JAMA. **299** (22): 2682-2684.

65. **D. J. Wozniak, Wyckoff, T. J. O., Starkey, M., Keyser, R., Azadi, P., O'Toole, G. A., Parsek, M. R.** 2003. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. PNAS. **100** (13): 7907-7912.

66. **A. Wright, Hawkins, C. H., Anggard, E. E., Harper, D. R.**. 2009. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa*, a preliminary report of efficacy. Clinical Otolaryngology. **34** (4): 349-357.

67. **B. M. Zalewska-Piatek, Wilkanowicz, S. I., Piatek, R. J., Kur, J. W.** 2009. Biofilm formation as a virulence determinant of uropathogenic *Escherichia coli* Dr+ strains. Pol J of Microbiol. **58** (3): 223-229.

VITA

Matthew Krivacka Kay was born in Anchorage, Alaska, on February 3, 1980, the son of James Robert Kay and Anna Christina Kay. He graduated Leander High School, Leander, Texas, in 1998, and then he began his undergraduate degree at The University of Texas in Austin. He received his B. S. in Microbiology from UT in August 2003. The next few years were spent working as a specimen processor at Clinical Pathology Labs, a refurbishing technician at Dell Computers, and a blood plasma technologist at BIO-MAT USA, in Austin, Texas. At these jobs new hire training was part of the daily regimen in addition to the normal duties. In August 2007 he entered the Graduate College at Texas State University-San Marcos. During his time here at Texas State Matt has joined the American Society for Microbiology and has taught laboratory courses in Immunology, Virology, and Microbiology. He has also spent his spare time dabbling in tenor saxophone, writing, drawing, and 3D animation.

Permanent Address: 2508 Tumbling River Dr.

Leander, TX 78641

This thesis was typed by Matt K. Kay.