INFECTION WITH UT1 BACTERIOPHAGE RESULTS IN ROBUST BIOFILM FORMATION BY Pseudomonas aeruginosa WITH DNA RELEASE AND THE EMERGENCE OF SMALL COLONY VARIANTS

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INFECTION WITH UT1 BACTERIOPHAGE RESULTS IN ROBUST BIOFILM FORMATION BY Pseudomonas aeruginosa WITH DNA RELEASE AND THE EMERGENCE OF SMALL COLONY VARIANTS

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Treatment of infections caused by Pseudomonas aeruginosa, a gram-negative and opportunistic pathogenic bacterium, in immunocompromised human hosts has become a challenge because of the emergence of antibiotic resistant strains. The ability of P. aeruginosa to form biofilms is known to heighten antibiotic resistance. An alternate approach to the treatment of these infections is that of using bacteriophage. We sought to determine the effect of various bacteriophage on the formation of P. aeruginosa biofilms which are a hydrated matrix consisting of DNA, proteins, polysaccharides and other cell products. Initial studies in our laboratory showed that exposure of P. aeruginosa PAO1 cultures to various phage (UT1, D3 and D3112) led to an increase in biofilm formation. In this study, we explored how the Pqs quorum sensing system, polysaccharides and DNA contribute to this increase in biofilm formation upon infection with UT1, a phage specific to P. aeruginosa. The mechanism and timing of DNA release in biofilms was also measured. Our results indicate that the Pqs system and those genes involved in alginate and Psl polysaccharide synthesis are not required for the observed phage
induction of biofilms. UT1 phage infection induced greater DNA release by killing a subpopulation of bacterial cells one to four hours post infection. Additionally we observed the emergence of Small Colony Variants (SCV) from infected biofilms when attached cells were plated on LB agar or minimal salts medium supplemented with glucose (MSG). SCV also arose in uninfected biofilms but were only observed when the cells were plated on MSG agar. The SCV phenotype was stable as these colonies were found to breed true upon subculture. Isolated SCV from infected biofilms were tested in biofilm assays to determine their phenotype. The SCV isolates formed more robust biofilms without the addition of phage, equivalent to naïve P. aeruginosa cells upon infection with UT1. This study is the first to show that exogenous phage can lead to enhanced biofilm formation under certain growth conditions with release of DNA and the emergence of stable SCV.
DEDICATION

Dedicated to my parents, Mr. SubbaRayudu Chaganaboyana and Dr. Jayaprada Malleboyana; and my brother, Dr. Ashesh Chaganaboyana
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INTRODUCTION

*Pseudomonas aeruginosa* is a rod shaped, gram negative bacterium measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm. It belongs to the bacterial family *Pseudomonadaceae*. It is a ubiquitous organism and is commonly found in soil, water and on vegetation. The typical *Pseudomonas* bacterium in nature might be found growing in a biofilm, attached to a surface, or in a planktonic form, as a unicellular organism, actively swimming by means of its flagellum.

*P. aeruginosa* is classified as an opportunistic pathogen and is responsible for a high percentage of nosocomial infection (Emori and Gaynes 1993). It has become increasingly recognized as an opportunistic pathogen of clinical relevance. However, *P. aeruginosa* is also an ecologically significant bacterium and its importance in nitrogen cycle especially during denitrification and assimilation has been recognized (Zumft and Mortenson 1975). Further, it also plays a role in bioremediation by degrading specific contaminants such as chlorinated pesticides and crude oil.

As an opportunistic pathogen in immunocompromised patients, *P. aeruginosa* exploits the host defenses to initiate infection and has been implicated in urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bone and joint infections, gastrointestinal infections, ear and eye infections and a variety of systemic infections. *P. aeruginosa* produces exotoxins and numerous virulence factors which
disrupt the host immune system by impairing the function of phagocytic cells and also cause extensive tissue damage (Lynzak et al. 2002).

Immunocompromised patients, such as individuals suffering from cancer or HIV, as well as those with severe burns, are highly susceptible to *P. aeruginosa*. This organism is responsible for the majority of mortality in cystic fibrosis patients. In these patients, it forms highly recalcitrant biofilms within the thick, hypoxic mucus lining the airways. *P. aeruginosa* is also known to form biofilms on medical devices such as catheters, ventilators, contact lenses, sutures, AV valves, etc. (Costerton et al. 1999). In addition to humans, *P. aeruginosa* is a pathogen of a wide range of animal and plant species, including mice, fruit flies, nematode worms, and mustard plants (Hahn 1997; Rahme et al. 2000; Tan and Ausubel 2000; D'Argenio et al. 2002).

*Pseudomonas aeruginosa* is distinguished by its ability to attach to both abiotic (e.g. medical devices) and biotic (e.g. airways) surfaces. Following attachment to a surface, cells have the potential to colonize and form mature biofilms which are hydrated matrices surrounding colonies of bacteria. Biofilms are advantageous to pathogenic bacteria in that this type of life style provides the bacteria with protection from host immune factors and also antibiotics (Costerton 2001).

Many environmental factors and genes are essential for the formation of stable biofilms. Biofilm formation begins with the attachment of bacterial cells followed by growth and/or migration to form a smooth monolayer of cells. Attachment is followed by microcolony formation then macrocolony formation and finally dispersal of some cells or even clumps of cells (Figure 1). These steps have been recognized as a developmental process (O'Toole et al. 2000). Quorum sensing (QS), a form of cell-to-cell
communication, is necessary for normal biofilm formation by \textit{P. aeruginosa}. In particular the Las QS system is required; this involves the \textit{lasI} (autoinducer production) and \textit{lasR} (transcriptional regulator) genes (Davies \textit{et al.} 1998). These genes regulate, along with \textit{rhlA} and \textit{rpoN}, the transition from microcolony to macrocolony formation. Another signaling molecule is 2-heptyl-3-hydroxy-4-quinolone, known as PQS (Pseudomonas Quinolone Signal). \textit{P. aeruginosa} packages this signaling molecule into membrane vesicles that serve to traffic PQS into the bacterial population (Mashburn and Whiteley 2005).

A variety of polysaccharides are known to increase the stability of biofilms, alginate and psl are among the important ones. Although alginate may not play a significant role in bacterial attachment, biofilm development and formation, it may play an important role in protecting mucoid \textit{P. aeruginosa} biofilm bacteria from the human immune system (Leid \textit{et al.} 2005). Another polysaccharide, psl, enables efficient attachment to surfaces and regulated localized psl operon expression is required for biofilm differentiation (Overhage \textit{et al.} 2005).

Extracellular DNA is one of the major matrix components of \textit{P. aeruginosa} biofilms. It has known to provide more stability to the biofilms by increasing intercellular connections. Extracellular DNA has proven to be similar to whole-genome DNA (Allesen-Holm \textit{et al.} 2006). DNA release in \textit{P. aeruginosa} biofilms is controlled by the \textit{las-rhl} and \textit{pqs} quorum-sensing systems. DNA release in \textit{P. aeruginosa} biofilms is also under iron regulation (Yang \textit{et al.} 2007). It was found that DNase treatment dissolved young \textit{P. aeruginosa} biofilms indicating that DNA is in significant levels in the biofilm. But the treatment of established biofilms with the same did not show the same effect,
implying that there are other compounds which aid in holding the established biofilms together (Whitchurch et al. 2002).

Bacteria within biofilms are highly resistant to traditional antibiotics, and even many planktonic (free-living) infections (bacteremias) are becoming more antibiotic resistant. The emergence of antibiotic resistance had led to an exploration of alternative antibacterial therapies including phage therapy, a therapy involving the use of bacteriophages for treatment of pathogenic bacterial infections. Phage therapy has many potential applications in human medicine, dentistry, veterinary science as well as in agriculture. An important theoretical benefit of phage therapy is that bacteriophages can be much more specific than common drugs. They also have a high therapeutic index that is; phage therapy gives rise to few if any side effects, as opposed to drugs, and does not stress the liver. Also, since phage can replicate in vivo, a very small dosage might be sufficient in the treatment of infections.

It has been shown that pretreatment of catheters with a lytic phage resulted in a 4.5 log reduction in *Staphylococcus epidermis* biofilms compared with untreated catheters (Curtin and Donlan 2006). Some phage produce phage-associated polysaccharases that “eat” the exopolysaccharide matrix of biofilms (Hughes et al. 1998; Hughes et al. 1998).

Since the use of phage therapy is in its infancy and before phage can be used to treat infections in human beings, mechanistic studies are required to determine precisely how *P. aeruginosa* biofilms respond to exogenous phage. We sought to investigate the effect of a bacteriophage, UT1 on biofilm formation by *P. aeruginosa*. UT1 is a filamentous fresh water bacteriophage specific to *P. aeruginosa*. It is lytic phage and is
capable of pseudolysogeny (a state of unstable lysogeny in which the phage genome does not integrate into the bacterial genome) and generalized transduction. It has been proposed that environmentally endemic bacteriophages such as UT1 are formidable transducers of naturally occurring microbial communities (Ripp et al. 1994). Certainly, these phage also play a role in controlling bacterial population in nature, but can they also control population in medically relevant environments? Or will the bacteria respond in a protective manner that would defeat the ability to use them as a treatment modality.

We have previously shown that exposure of *P. aeruginosa* PAO1 cultures to various phage (UT1, D3 and D3112) led to an increase in biofilm formation (Beumer et al., 2006). This effect was observed when cultures were grown on minimal salts medium supplemented with glucose, but not in minimal salts medium supplemented with succinate or Luria broth. Further the induction by phage infection was independent of growth phase and evident at MOIs ranging from 5 to 0.001. Phage that were inactivated by UV light pretreatment did not induce greater biofilm production.

We wanted to know the effect of biofilm relevant polysaccharides on the induction of biofilms by bacteriophage. It is also important to know how QS might influence the formation and stability of biofilms formed by UT1 infected bacterial cells. Experiments comparing wild type PAO1 cells with isogenic mutants in the *las* and *rhl* quorum sensing (QS) systems showed the QS mutants were unaltered in their response to phage (Buemer and Robinson 2006). Hence, screening of a Transposon mutant library to identify all the genes responsible for the induction of biofilm formation by the phage was started. The screening of Tn mutants has, so far, resulted in the identification of one such likely candidate from three 96 welled microtitre plates. This mutant will be sequenced to
identify the gene that is mutated by the transposon. The identification of at least one Tn mutant that exhibits the loss of induction by UT1 phage after screening less than 300 mutants concludes that we should be able to identify all the genes involved in this response.

We must understand the extent of the effect of phage upon biofilm formation. When biofilms form in nature environmental phage are likely to be present and it is logical that bacterial hosts have evolved a response to these phage that will impact biofilm formation. These evolved responses may play out in clinical situations where phage are added in hopes of preventing or remediating biofilms.
INTRODUCTION TO BIOFILMS

A biofilm is a structured community or population of microorganisms encapsulated in a self produced hydrated matrix. Biofilms can develop on a biotic or abiotic surface, usually on solid substrates which are submerged in or exposed to an aqueous environment. When given a suitable environment for growth along with nutrients, biofilms can soon become macroscopic. Bacterial cells within biofilms can be organized into different structures, but primarily allow nutrients to come into the biofilm and wastes to escape. The hydrated matrix protects the bacterial cells from dehydration as well as from phagocytic cells like macrophages. It consists of exopolysaccharides, DNA, proteins.

Many microorganisms are capable of forming a biofilm. Common bacteria known to form biofilms include the Gram positive bacteria Enterococcus faecalis and Staphylococcus aureus; and Gram negative Escherichia coli, Klebsiella pneumonia, Proteus mirabilis and Pseudomonas aeruginosa (Nielson and Pupp 2006).

Biofilms have immense environmental significance and are ubiquitous; forming on rocks, pebbles at the bottom of the stream, inside water pipes leading to clogging and corrosion; however, biofilms can be helpful in certain ways. For instance, they help to eliminate petroleum oils from contaminated oceans and in sewage treatment plants.
among other things. Biofilms are helpful in bioremediation and are the major sites of carbon cycling and an indication of uptake of Biodegradable Dissolved Oxygen Carbon (BDOC) in river ecosystems (Romani et al. 2004).

Biofilms are important medically. They form on the teeth of most animals as dental plaque and are the basis for many bacterial infections. Biofilms readily form on abiotic surfaces such as catheters and implants as well as on biotic surfaces. *Pseudomonas aeruginosa* is an important opportunistic pathogen and is a model organism for the study of biofilms. It causes a range of systemic diseases bacteremia, dermatitis, ear and eye infections, respiratory system infections, bone infections, endocarditis (Costerton et al. 1999). *P. aeruginosa* is a ubiquitous organism and forms biofilms on sputum in the lungs of cystic fibrosis patients, arteriovenous shunts, sutures, catheters and even in distilled water.

In surgical patients, for instance in hip replacement patients, biofilms can cause latent infections, which can be detected only after several weeks to months (Bergamini et al. 1998; Nielson and Pupp 2006). Infections might respond to an initial course of antibiotics after which the bacteria gain resistance due to the development of biofilms (Bergamini et al. 1998; Nielson and Pupp 2006). Eventually surgical treatment might be necessary to remove the biofilms (Costerton et al. 1995). Biofilms stimulate an immune response in the body which can harm the surrounding host tissue and the biofilm itself (Cochrane et al. 1988). The bacteria embedded within clinically-relevant biofilms are resistant to both immunological and non-specific defense mechanisms of the body and the cells in bacterial biofilm expresse more virulent, phenotypes. Also, immune
phagocytic cells poorly penetrate the physical barrier of biofilm matrix (Zufferey et al. 1988; Bergamini et al. 1998; Shimabukuro K et al. 2004).

**Resistance of biofilms**

The mechanism of increased resistance of biofilms to antibiotics is not entirely clear. It has been attributed to the presence of exopolysaccharides, extracellular DNA and slow growth of cells in the biofilm matrix as well as up regulation of efflux systems that actively pump antibiotics out of cells (Lambert 2002; Whitchurch et al. 2002). However, the principal mechanism of resistance appears to be the appearance of “persister cells” which is a phenotypic variation. It has been suggested that these cells contribute to antibiotic resistance in planktonic cells as well as in biofilms. A recent study indicated that *Candida albicans* biofilms produce persister cells which are largely responsible for multidrug tolerance of fungal biofilms (LaFleur et al. 2006). Also, it has been shown that the emergence of ‘persister’ cells in *P. aeruginosa* populations, even in small numbers, may be a contributing factor in the time-dependent tolerance of both planktonic cells and biofilms to high concentrations of metal cations (Harrison et al. 2005).

Another phenotypic variant, termed “Small Colony Variants” (SCV), has been found to facilitate persistent and recurrent bacterial infections. These SCV are a slow growing sub population of bacteria with distinctive phenotypic and pathogenic traits (Proctor et al. 2006). Clinically, small colony variants of *S. aureus*, are better able to persist intracellularly in mammalian cells and are less susceptible to antibiotics than their wild-type counterparts, causing latent or recurrent infections upon emergence from the protective environment of the host cell (Proctor et al. 2006). There is a description of
SCV in an *E. coli* strain that is the etiological agent of chronic infections in prosthetic hip replacement and urinary tract infections (Roggenkamp *et al.* 1998; Tappe *et al.* 2006). In *P. aeruginosa*, SCV have been identified as hyperpilated morphotypes which are capable of increased adherence in cystic fibrosis lungs (Häußler *et al.* 2003). They exhibit increased fitness under stationary conditions compared to clonal wild types (Häußler *et al.* 2003). Also, it was found that SCV of *P. aeruginosa* exhibit increased twitching motility and the ability to form biofilms (Häußler *et al.* 2003). Rough SCV were found in static biofilms of *P. aeruginosa* strain PA14 when exposed to antibiotics (Drenkard and Ausubel 2002). Small, rough, strongly cohesive colony morphology variants from aging *P. aeruginosa* PAO1 biofilms were isolated which exhibited increased hydrophobicity and reduced motility compared to the wild-type parent strain (Kiristis *et al.* 2005). These variants form biofilms with significant three-dimensional structural difference and more biomass than the wild-type parent (Kiristis *et al.* 2005). These rough SCV generally showed increased expression of the *psl* and *pel* loci, which have been implicated in the adherence of *P. aeruginosa* to solid surfaces (Kiristis *et al.* 2005).

**Biofilm formation and development**

Bacteria that live in a biofilm have significantly different properties from free-floating, planktonic bacteria of the same species (Bergamini *et al.* 1998; Shimabukuro K *et al.* 2004; Nielson and Pupp 2006). The formation of biofilms can be divided into several stages, each of which has specific characteristics (Dunne 2002).

The first stage is adhesion which is divided further into two steps: reversible and irreversible adhesion. Reversible adhesion occurs when a bacterial cell comes in contact
with a surface closer than one nm either by chance or active motility. Hydrophobicity of the cell and the surface are key factors that influence attachment (Carpentier and Cerf 1993). Bacterial cell surface hydrophobicity, as determined by the bacterial adherence to octane and polystyrene, was the major parameter influencing the adhesion of *Lactobacillus* strains to sandy soil (Huysman and Verstraete 1992). Other factors that might contribute to attachment include electrostatic interactions, van der Waals forces, steric hindrance and temperature (Dunne 2002; Nielson and Pupp 2006). Another factor that can affect primary adhesion is surface conditioning, which involves various organic molecules that coat a surface. These coatings alter the physical forces between the surface and bacterial cells, hence resulting in less or more attraction between the two (Gottenbos *et al.* 1999).

Secondary or irreversible adhesion between a surface and bacterial cell can be mediated by appendages like pili or other adhesins on bacterial surface. In *Lactobacillus* strains, adhesion occurs within 30 sec of contact time (Huysman and Verstraete 1992). Secondary adhesion can occur between bacterial cells of the same or different species and may involve more than one adhesion (Dunne 2002). Calcium ions promote both specific and non-specific interactions with protein and polysaccharide adhesin molecules at the cell surface (Dunne 2002). Important, but less well understood is the calcium ions also influence the way microbial cells interact with different substrata (Geesey *et al.* 2000).

After bacterial cells attach, they begin to replicate and/or move along the surface to form microcolonies of cells. The formation of microcolonies is influenced by several factors which include temperature and pili as well as others (Boddey *et al.* 2006).
Finally, a mature biofilm is formed which is characterized by a mushroom shaped architecture. It consists of macrocolonies surrounded by fluid filled channels through which nutrients can enter and wastes can be removed from the biofilm. The biofilm at this stage consists of extracellular DNA, exopolysaccharides and proteins in a hydrated matrix.

**Development of *P. aeruginosa* biofilms**

*P. aeruginosa* is a useful model for the study of biofilms, because of its medical importance and well characterized biofilms. The stepwise development of biofilms in *P. aeruginosa*, in five stages, was first proposed by O’Toole *et al.* (2000). The genes responsible for these steps are shown in a step wise manner in Figure 1 (Caiazza and O’Toole 2004).

Reversible adhesion of the bacteria is mediated by bacterial sensing of the surface parameter(s) which initiates the transition from planktonic to sessile mode of growth (Sauer *et al.* 2002). Cells can most readily be removed from the smoothest, most hydrophilic, neutral surfaces, with removal becoming more difficult at longer attachment times (Pasmore *et al.* 2002). During this stage, the bacterium is attached to a surface at the pole (Sauer *et al.* 2002). *flgK*, encoding for flagellar hook protein, is required for initial cell-to-surface interactions (O’Toole and Kolter 2002). *fleR* and *rpoN*, a response regulator responsible for flagellar production and sigma factor respectively, are necessary for the transition from reversible to irreversible attachment (Caiazza and O’Toole 2004).

The next stage of biofilm formation is microcolony formation or maturation-1 when the colonies exceed 10 μm. Analysis of two classes of surface attachment defective
(sad) mutants, defective in flagellar-mediated motility and biogenesis of the polar-localized type IV pili indicated that both flagella and pili play a role in the formation of microcolonies (O'Toole and Kolter 2002). This stage also requires the crc, gacA and pilB genes (Parkins et al. 2001; Sauer et al. 2002; Caiazza and O'Toole 2004). crc encodes for a catabolite regulatory protein, gacA encodes the response regulator for the GacA/S two component regulatory system and pilB encodes for the ATPase necessary for pili extrusion (Wolff et al. 1991; Parkins et al. 2001). Once established in microcolonies, it is very difficult to eradicate P. aeruginosa by antimicrobial treatment (Sriramulu et al. 2005). Also, several genes, including algD, oprF and lasR are required for tight microcolony formation (Sriramulu et al. 2005). The amino acids, lecithin, DNA, salt and low iron levels in sputum are also required for tight microcolony formation (Sriramulu et al. 2005). Formation of macrocolonies or maturation-2 follows the stage of microcolonies. This transition is mediated by the las quorum-sensing system.

The final stage is dispersal, where single cells or groups of cells are detached from the biofilm, either actively or passively. The filamentous bacteriophage, Pf1 which exists as prophage inside the genome of the cell, mediates cell death an important mechanism of differentiation inside macrocolonies that facilitates dispersal of a subpopulation of surviving cells (Webb et al. 2003). Dispersal of P. aeruginosa PAO1 from biofilms is inducible by a sudden increase in carbon substrate availability, especially succinate (Sauer et al. 2004). Gene families that are upregulated in dispersed cells include those for flagellar (fliC) and ribosomal proteins, kinases, and phage Pf1 (Sauer et al. 2004). There is a corresponding decrease in expression of pilus (pilA) genes in dispersed cells (Sauer et al. 2004). However, within the biofilm, genes in the denitrification
pathway and pilus biosynthesis are also upregulated (Sauer et al. 2004). Nutrient-induced dispersion is associated with an increase in the number of Ser/Thr-phosphorylated proteins within the newly dispersed cells, and inhibition of dephosphorylation reduces the extent of nutrient-induced dispersion (Sauer et al. 2004).

In *P. aeruginosa*, it has been proposed that rhamnolipid surfactants produced by the cells aid in the maintenance of biofilm architecture (Davey et al. 2003). Iron serves as a signal in the normal biofilm development of *P. aeruginosa* (Banin et al. 2005). It has been shown that in iron-sufficient medium, mutants that cannot obtain iron through the high-affinity pyoverdine iron acquisition system form thin biofilms which are similar to those formed by the wild type parent under low iron conditions (Banin et al. 2005).

Various stages of biofilm formation on abiotic surfaces by *P. aeruginosa* are mediated by extracellular appendages, such as type IV pili and flagella (Caiazza and O'Toole 2004; Vallet et al. 2004). The *cupA* (chaperone-usher pathway) gene cluster is required for biofilm formation on abiotic surfaces (Vallet et al. 2004). Additionally, MvaT (a negative regulator of *cupA* expression) might be an important regulatory component within a complex network that controls biofilm formation and maturation in *P. aeruginosa* (Vallet et al. 2004).

**Biofilms on medical devices**

The capacity of *P. aeruginosa* to form biofilms is an important requirement for chronic colonization of human tissues and for its persistence in implanted medical devices. The formation of biofilms on medical devices is similar to that on microtitre plates, due to the similarity in materials, except for the contribution of the host's immune
system. The tough hydrated exopolysaccahride and DNA rich matrix of the biofilm protects the bacterial cells from host defense mechanisms, especially phagocytosis by polymorphonuclear leukocytes. Planktonic cells are easily bound by antibiotics and marked for destruction. Macrophages are able to engulf and destroy planktonic cells of P. aeruginosa. Also, phagocytes produce enzymes in an attempt to break through the biofilm (Costerton et al. 1999). Antibiotics are efficient at killing the planktonic cells and reversing the symptoms, but not at destroying the cells encased in the biofilm (Marrie and Kwan 1982). Hence, the symptoms recur and often require the surgical removal of biofilms or the implanted medical devices (Costerton et al. 1995).

Biofilms in cystic fibrosis patients

Cystic fibrosis (CF, mucovoidosis, or mucoviscidosis) is a hereditary, autosomal recessive and congenital disease which affects the exocrine glands of the lungs, liver, pancreas, and intestines. It is caused by a mutation in the cystic fibrosis transmembrane regulator (CFTR gene) (Andersen 1938; Kerem et al. 1989). The product of this gene (CFTR), is a chloride ion channel important in creating sweat, digestive juices, and mucus. CF develops when neither allele can produce a functional CFTR protein.

P. aeruginosa forms resistant biofilms, thus making it the leading cause of mortality in these patients, with 80-95% of patients succumbing to lung failure (Lynzak et al. 2002). It was originally thought that in CF patients, biofilms are formed on epithelial lining of airways; however, they typically form in and on the mucous that is moved along the epithelial surface. In normal individuals, the mucous layer is composed of two parts: the top layer is more viscous and traps the bacterial cells and foreign bodies
and the bottom layer is less viscous which allows the cilia to move the mucous along the surface of the epithelial cells. This helps in clearing the mucous and associated bacterial cells. The mutation of CFTR gene renders this process of clearing debris dysfunctional. The CFTR transporter regulates apical ion transport. Defects in the gene lead to defects in Cl⁻ and Na⁺ transport which in turn leads to abnormal fluid secretion and a thickening of the mucous. Under these conditions it is difficult for the cilia to beat and clear the airways (Lynzak et al. 2002). The result is the formation of thick resistant P. aeruginosa biofilms.

Type IV pili and flagella are initially used to colonize the mucous in CF infections which is similar to the attachment steps in laboratory biofilm formation described above. Formation of microcolonies and macrocolonies require the quorum sensing system and also type IV pili. Activated neutrophils produce oxygen radicals that cause mutations in the bacterial genome. Mutations in the mucA gene take place that lead to increased alginate production, thus protecting the cells from antibiotics and neutrophils. Lysed neutrophils release DNA which further adds to the matrix. It has been proposed that P. aeruginosa in biofilms in airway mucous of CF patients respire anaerobically and NO₃ acts as terminal electron acceptor (Figure 2) (Hassett et al. 2002; Hoiby 2002). The outer membrane protein, OprF, and rhl quorum sensing circuit are two important cellular factors that are required for optimal anaerobic biofilm viability (Hassett et al. 2002). Thus, with increasing age of CF patients, mucoid, anaerobic populations predominate in the inspissated airway mucus.
**Quorum sensing and biofilm formation**

Quorum sensing (QS) is the ability of bacteria to communicate and coordinate behavior via signaling molecules. Opportunistic bacteria can grow within a host without harming it, until they reach a certain population density, i.e., a quorum. When their numbers are sufficient to overcome the host's immune system, they change their behavior and cause disease.

Bacteria which use quorum sensing produce and secrete signaling compounds called Quorum Sensing Molecules (QSMs) which differ in chemical structure but are often referred to as autoinducers. These bacteria also have a receptor which can specifically bind and detect the QSM. When the QSM binds to its cognate receptor, it activates transcription of certain genes, including those for inducer synthesis. When only a few other bacteria of the same kind are in the vicinity, diffusion reduces the concentration of the inducer in the surrounding medium, so the bacteria produce less inducer. With high concentrations of bacteria, the concentration of the inducer passes a threshold, so more inducer is synthesized. This forms a positive feedback loop, and the receptor becomes fully activated and results in the activation or deactivation of QS regulated genes.

As shown in Figures 1 and 2, QS signaling is required for normal biofilm formation. In *P. aeruginosa*, QS is mediated by two signaling systems, the las and rhl systems. These are homologous to the first system discovered in *Vibrio fischeri*, the lux system.

In the las system, LasR is the receptor/transcriptional regulator which is encoded by *lasR* gene, LasI is a synthase which produces the QSMs which are small diffusible
acyl homoserine lactone (AHL) molecules. When an AHL molecule binds to the transcriptional regulator LasR it is activated and is able to bind to specific DNA sequences leading to transcription of the genes necessary for tissue damage and colonization of the bacteria. Similar sequence of activation of the genes takes place in the rhl system. However, the system is more complicated in that the two systems are hierarchical; the las system controls the rhl system.

A third type of bacterial signaling molecule, 2-heptyl-3-hydroxy-4-quinolone, the Pseudomonas Quinolone Signal (PQS), has been identified in this bacterium. PQS acts as a link between the las and rhl quorum-sensing systems and this signal is not involved in directly sensing cell density (McKnight et al. 2000). PQS was recently found to mediate the packaging of AHLs into membrane vesicles, which appear to traffic between neighboring cells (Mashburn and Whiteley 2005). Increased expression of genes involved in the biosynthesis of PQS during growth in reconstituted sputum was observed in a cystic fibrosis patient. This supports that PQS is involved in biofilm formation (Palmer et al. 2005).

PQS production is negatively regulated by the rhl quorum-sensing system and positively regulated by the las quorum-sensing system and PqsR (also known as MvfR) (Wade et al. 2005). It was shown that PqsR binds the promoter of the pqsA gene and that this binding increases markedly in the presence of PQS, which implies that PQS acts as a coinducer with PqsR (Wade et al. 2005). Also, pqsR is under the control of LasR and RhlR and where PqsR in turn controls pqsABCDE, which is required for the production of PQS (Wade et al. 2005). pqsH mutant cells, which produce HHQ (4-hydroxy-2-heptylquinoline, precursor of PQS), but completely lack PQS are incapable of producing
pyocyanin which is a virulence factor (Xiao et al. 2006). Increased expression of genes involved in the biosynthesis of PQS in reconstituted sputum was observed in a cystic fibrosis patient which supports the idea that PQS is involved in biofilm formation (Palmer et al. 2005). Also, no AHL production was detected under these conditions, suggesting that PQS might play an important signaling role in medically relevant biofilms (Spoering and Gilmore 2006).

The PQS molecule regulates rhl-dependent genes at the onset of stationary phase (Diggle et al. 2003). PQS also revealed a marked upregulation of genes belonging to the tightly interdependent functional groups of the iron acquisition and the oxidative stress response (Bredenbruch et al. 2006). Most of the differentially regulated genes, as well as the induction of rhlR, was shown to be due to an iron-chelating effect of PQS (Bredenbruch et al. 2006).

**Polysaccharides in biofilms**

The extracellular polysaccharide (EPS) in bacterial biofilms is an important virulence factor against the host immune system and antibiotics. The biofilm matrix protects it against dehydration as well as other ecological hazards. The EPS of *P. aeruginosa* is produced by three loci: *psl*, *pel* and *algD* (Evans and Linker 1973; Friedman and Kolter 2004; Matsukawa and Greenberg 2004).

The *psl* gene cluster is comprised of 15 cotranscribed genes in *P. aeruginosa*. The *pslA* and *pslB* genes of *P. aeruginosa* PAO1 play a role in attachment and biofilm formation, but not in lipopolysaccharide (LPS) production or motility (Friedman and Kolter 2004; Overhage et al. 2005). Psl also plays a role in cell–surface and cell–cell
interactions. Psl is also required for adherence to mucin-coated surfaces and airway epithelial cells; biotic surfaces that are clearly relevant to CF (Ma et al. 2006). It has been predicted from the protein motifs involved in polysaccharide synthesis that psl genes encode a pathway for exopolysaccharide synthesis and extrusion. A gene cluster of P. aeruginosa, PA2231 was identified which appears to encode functions for EPS biosynthesis (Matsukawa and Greenberg 2004). A mutant of this gene cluster formed thin unstructured abnormal biofilms (Matsukawa and Greenberg 2004).

Alginate is a capsular polysaccharide that confers a selective advantage for P. aeruginosa in the CF airway and is therefore an important virulence factor. It protects P. aeruginosa from the consequences of inflammation since it scavenges free radicals released by activated macrophages in vitro and appears to provide protection from phagocytic clearance (Govan and Deretic 1996; Pier 1998). It is a major component of EPS in mucoid strains while it is neither necessary for biofilm formation nor a significant component of the EPS in non-mucoid P. aeruginosa strains such as PAO1 or PA14 (Wozniak et al. 2003) while it has also been proven that algD mutants form thin and frail biofilms and pellicles.

Alginate biosynthesis and regulation involves at least 30 genes (Figure 3) and the system is not completely understood (Ramsey and Wozniak 2005). The biosynthetic pathway begins with glucose and ends in alginate production (Gacesa 1998). The biosynthetic operon begins with algD, responsible for the production of GDP mannose dehydrogenase, which catalyzes the transformation of GDP mannose to GDP manuronate (Deretic et al. 1987).
In mucoid strains, the \textit{algR} gene encodes for an enzyme involved in both LPS and alginate production and is responsible for expression of both \textit{algD} as well as \textit{algC} (Zielinski \textit{et al.} 1991; Fujiwara \textit{et al.} 1993). Also, an \textit{algR} mutant is deficient in the formation of biofilms. Overproduction of alginate has been shown to provide protection for the biofilm from several factors. It leads to an altered biofilm structure that is rougher and thicker than its wild type parent strain (Hentzer \textit{et al.} 2001). These biofilms exhibited increased antibiotic resistance to tobramycin (Hentzer \textit{et al.} 2001). Treating biofilms with alginate lyase prior to antibiotic treatment led to better antibiotic efficacy (Alkawash \textit{et al.} 2006). Alginate has also been shown to protect from macrophages (Leid \textit{et al.} 2005).

**DNA in biofilms**

DNA is another important component in the extracellular matrix of biofilms. In Gram positive bacteria, the release of DNA is linked to QS (Spoering and Gilmore 2006). It has been clarified that in \textit{Streptococci pneumoniae}, CSP (Competence Signaling peptide), a peptide based signal for QS, plays a role in the release of extracellular DNA in the biofilms. When the CSP reaches a threshold of 1–10 ng/ml in the medium, a subpopulation of the bacteria lyse, and the released DNA adds to the biofilm biomass and protects the remaining population (Steinmoen \textit{et al.} 2002). This flux of exogenous DNA is believed to be an important mechanism for horizontal gene transfer in naturally competent bacteria (Thomas and Nielsen 2005).

DNA plays a role in the formation of bacterial biofilms. It has been suggested that competence-mediated release of DNA through mutacin IV, a bacteriocin could affect biofilm formation (van der Ploeg 2005).
In petri dish biofilms of *P. aeruginosa* PAO1, DNA was found to be the most abundant polymer, occurring in quantities of 500 mg of DNA per 100 mg of protein (Whitchurch *et al.* 2002). Also, *P. aeruginosa* biofilm formation was inhibited by the addition of DNase I. However, there was no inhibition in planktonic bacterial growth (Webb *et al.* 2003). In Gram negative bacteria, the mechanism is still unclear but direct secretion, lysis of a subpopulation by prophage and release of small membrane vesicles are among viable hypotheses (Allesen-Holm *et al.* 2006). It has also been suggested that the DNA might be used as a physical scaffold by twitching cells as they migrate to form mushroom caps (Allesen-Holm *et al.* 2006).

It has been recently shown that extracellular DNA increases the antibiotic resistance by chelating cations such as magnesium from the tissues which resulted in induction of the PhoPQ- and PmrAB-regulated cationic antimicrobial peptide resistance operon *PA3552–PA3559* in *P. aeruginosa* (Mulcahy *et al.* 2008). Furthermore, DNA-induced expression of this operon resulted in up to 2560-fold increased resistance to cationic antimicrobial peptides and 640-fold increased resistance to aminoglycosides (Mulcahy *et al.* 2008).

**Iron and biofilms**

Iron serves as a signal in *Pseudomonas aeruginosa* biofilm development. The functional iron signal for *P. aeruginosa* biofilm development is active transport of chelated iron or the level of internal iron (Banin *et al.* 2005). If the signal is internal iron levels, then a factor likely to be involved in iron signaling is the cytoplasmic ferric uptake
regulator protein, Fur, which controls expression of iron-responsive genes (Banin et al. 2005).

Iron helps in the stability of biofilms. It was shown in Candida albicans that daughter cells from iron-limited biofilms were significantly more susceptible to amphotericin B than those from glucose-limited biofilms (Baillie and Douglas 1998). Catalase is a key enzyme involved in oxidative stress response.

In P. aeruginosa, it was shown that iron availability, but not oxygen availability, is a major factor affecting catalase expression in biofilms (Frederick et al. 2001). However, iron-stimulated catalase activity in biofilms was only about one-third that in planktonic cells (Frederick et al. 2001). In contrast, it has been shown that iron deficiency leads to inhibition of oxygen transfer. Also, it led to enhancement of secretion of proteins into the culture medium, formation of virulence factor, elastase. From this, it was concluded that for lung infection in a cystic fibrosis patient by P. aeruginosa, the site of limited availability of iron (in Hemoglobin) is the site of infection (Kim et al. 2003).

Bacteriophage and biofilms

We are beginning to understand that phage play an important role in biofilm development, conditioning of bacterial cells for attachment and dispersal. Effect of bacteriophage on biofilm formation has been shown in three bacteria: P. aeruginosa, E. coli and S. aureus. Induction of the filamentous Pfl like prophage, which resides in several bacterial genomes, is linked to lysis of subpopulations of bacterial cells in the late stage of biofilm formation (Webb et al. 2003). Increase in carbon availability leads to upregulation of Pfl like genes (Sauer et al. 2004). The Pfl like prophage in P.
aeruginosa is designated as Pf4 and the prophage genes are highly upregulated during biofilm development. The release of Pf4 phage particles results in the formation of Small Colony Variants (SCVs), a phenotypic variant that exhibits increased attachment (Webb et al. 2004). Further, the Pf4 sequence also contains what may be a host addiction module, or toxin-antitoxin module, which ensures the prophage is maintained in the population.

Phage have been used experimentally as a method to prevent and/or treat biofilm formation with varying degrees of success. Prevention of biofilm formation is a better strategy than the eradication of already formed biofilms. In S. epidermis, biofilms were reduced 2.34 log-CFU when hydrogel coated catheters were pretreated with Phage 456 and reduced 4.47 log-CFU (from 7.01 log-CFU) when treated with Phage 456 plus divalent cations (Curtin and Donlan 2006). Listeria monocytogenes is a Gram positive, intracellular parasite that infects immunocompromised individuals. When stainless steel was pretreated with phage specific for the L-form of L. monocytogenes, biofilm formation was reduced 3 log-CFU, similar to stainless steel treated with acid (Hibma et al. 1997). A recent study proved the high efficiency of phage φ1BB-PF7A in removal of P. fluorescens biofilms (Sillankorva et al. 2008).

Recently, it has been proposed that using engineered enzymatic bacteriophage to reduce bacterial biofilms of E. coli might be beneficial. DspB-expressing T7DspB phage was engineered to express a biofilm-degrading enzyme during infection to simultaneously attack the bacterial cells in the biofilm and the biofilm matrix. There was
decrease in bacterial biofilm cell counts by $\approx 4.5$ orders of magnitude (≈99.997% removal). The two prong attack strategy is shown in Figure 4 (Lu and Collins 2007).

When phage come in contact with biofilms, further interactions are dependent on the susceptibility of the biofilm bacteria to phage and to the availability of receptor sites. If the phage also possess polysaccharide-degrading enzymes, or if considerable cell lysis is effected by the phage, the integrity of the biofilm may rapidly be destroyed. Alternatively, coexistence between phage and host bacteria within the biofilm may develop.

*E. coli* environmental phages have been shown to induce a subpopulation of cells that also exhibit increased attachment as well as an upregulation of fimbriae genes (Lacqua *et al.* 2006). UT1 is an environmental phage of *P. aeruginosa*. UT1 was isolated from Ft. Louden lake water in Knoxville, which is a flowing freshwater lake polluted by agricultural and industrial waste (Saye *et al.* 1990). It is a member of Myoviridae family and is a generalized transduction (GT) phage. It is capable of infecting environmental and laboratory strains of *P. aeruginosa*. It has not been well characterized; however, it has been used study of gene transfer in aquatic environment (Ripp *et al.* 1994). UT1 shares some sequence homology with the lytic *P. aeruginosa* phage E79 and is predominantly virulent. However, it is known to be temperate as well (Saye *et al.* 1990; Ogunseitan *et al.* 1992). In its temperate form, particularly under starvation conditions, UT1 is pseudolysogenic, a state of unstable lysogeny in which phage genome does not integrate into bacterial genome. In lake water, most *Pseudomonas* cells exist in a state of
starvation; therefore, most UT1 is found in the pseudolysogenized condition (Saye et al. 1990).

While we now understand a little about how Pf4 prophage affect biofilm formation by *P. aeruginosa*, we know substantially less about how exogenous and environmental phage affect the process. The hypothesis has been that we can use phage to destroy biofilms but our work has shown that under certain conditions phage can have the opposite effect. It may be that infection with exogenous phage, like UT1, leads to an induction of Pf4. Although phage have been proposed as a means of destroying or controlling biofilms, the technology for this has not yet been successfully developed.
Fig. 1. Development of *P. aeruginosa* biofilms. The five stages of biofilm formation and genes responsible for the transitions in the developmental pathway. Reproduced from Caiazza *et al.*, 2004.
Fig. 2. Development of *P. aeruginosa* biofilm in bronchioles of a cystic fibrosis lung. Cells attach in the viscous gel above the epithelial cells. Polymorphonucleocytes (PMN) are represented by white spheres and the yellow spheres attached are oxygen radicals and elastase released by the PMNs. Inside the biofilm *P. aeruginosa* cells are anaerobic and utilize NO₃ as the terminal electron acceptor. Reproduced from Hoiby 2002.
Fig. 3. Biosynthesis and assembly of alginate in *P. aeruginosa*. There are over 30 genes involved in alginate synthesis, export and regulation. This illustration shows the localization of alginate biosynthesis in a cell. Reproduced from Ramsey and Woznaik 2005.
Fig. 4. Two-pronged attack strategy for biofilm removal with enzymatically active DspB-expressing T7DspB phage. Initial infection of *E. coli* biofilm results in rapid multiplication of phage and expression of DspB. Both phage and DspB are released upon lysis, leading to subsequent infection as well as degradation of a crucial biofilm EPS component. Reproduced from Lu and Collins 2007.
MATERIALS AND METHODS

Preparation of UT1 phage. Phage were prepared using top agar technique. 100 µL of a known dilution of phage was mixed with an equal volume of overnight broth grown culture of PAO1. The mixture was immediately added to 4 mL of top agar in (0.7%). The top agars were poured onto LB agar plates (1.5%) and the plates were incubated overnight at 37 °C. Control plates were also prepared which contained bacterial cells that had not been infected with phage. Phage were harvested by resuspending the soft agar in LB (4mL), vortexing to release the phage and then incubating on ice for 30 minutes. Bacterial cells were removed by centrifugation for 10 minutes at 10,000 x g. the supernatant was then filtered through a 0.2 µm filter and stored at 4 °C. Sterility of the phage was determined by plating 100 µL on a LB plate and incubating it overnight at 37 °C.

Phage preparations that were shown to be free of bacterial host cells were used in subsequent assays.

Biofilm Assays. The protocol developed by O'Toole and Kolter (1998) was modified as follows.

*Pseudomonas aeruginosa* PAO1 wild-type and mutant strains were grown in Minimal Salts Medium (MSM) with 40% glucose to achieve a final concentration of
0.4% (See Appendix) as the source of carbon at 37 °C. Overnight cultures were then diluted with fresh media to give A₅₉₀ of 0.15. Phage were added to the diluted cells at an MOI of 0.01. Parallel control cell suspensions did not receive phage. Cell suspensions were aliquoted, 100μL per well into 96 well microtitre plates made of PVC (Polyvinyl Chloride, a common catheter material) and allowed to incubate, without shaking, overnight at 37 °C. Biofilm formation was measured by staining the cells adhering to the walls of a microtitre plate with crystal violet (0.25 % w/v) and allowing it to stain for 30 minutes at room temperature. After the 30 min incubation with crystal violet, the wells were washed five times with standing MilliQ water and then allowed to dry overnight at room temperature. The following day the crystal violet stained cells were resuspended by adding 200 μL of 95% ethanol to each well and transferring the stained cells to a microtitre plate made of Polystyrene. The absorbance at wavelength of 590 nm was measured in a Victor² plate reader.

Results reported are the average and standard deviation of 12 replicate wells after 24 hours.

**The effect of 2, 2’ dipyridyl on biofilm formation.** Growth of cultures and preparation of cell suspensions were as described in the previous section.

Appropriate volumes of a 50mM stock solution of 2, 2’ dipyridyl dissolved in ethanol were added to microtitre plate wells containing 100 μL of cell suspensions to achieve final concentrations of (0.5 μM, 2.5 μM, 50 μM and 100 μM) Samples were then incubated overnight at 37 °C in 96 well PVC microtitre plate. Cells receiving no 2, 2’ dipyridyl acted as controls. Phage were added at an MOI of 0.01 to duplicate plates.
On the following day, the protocol for CV staining was followed as described above. Results reported are the average and standard deviation of 12 replicate wells after 24 hours.

**Measurements of extracellular DNA in Biofilms.** DNA release was measured in biofilm assays to which propidium iodide was added as a stain for DNA. Propidium iodide with initial concentration of 1 mM was diluted to a concentration of 0.05 mM and was added to minimal salts medium with glucose and all the bacterial cells with and without phage were grown in this media.

The samples were allowed to incubate overnight at 37 °C. The amount of DNA released was measured using Victor² plate reader set to a wavelength of 490nm and 590nm.

All plates contained replicates of 12 wells and the amount of DNA released was measured using the Victor² plate reader.

**Measurement of DNase and SDS effect on Biofilms.** The initial set up for biofilms was as described above in the biofilm assay section. In this case duplicate plates were prepared for cells only and cells plus UT1 (MOI = 0.01). Following the overnight incubation at 37 °C, DNase was added to each well and allowed to incubate for 45 minutes at room temperature. 1 mg of powder DNase I was mixed in 1ml of DNase Digestion buffer (1M Tris/ 50 mM MgCl₂, pH adjusted to 8.3) to give a concentration of 100 µg/mL. The plates were subjected to the CV staining after the DNase treatment.
To test the effect of SDS on biofilms treated with DNase microtitre plates were submerged in Tupperware with 0.01% solution of SDS followed by shaking for two hours at 63 rpm on a rotary shaker at room temperature. Following the exposure to SDS, excess detergent was removed by shaking the plates and biofilms were stained with Crystal Violet as described above.

In all cases measurements of the absorbance of each well using a Victor plate reader were taken. Results reported are average and standard deviation of 12 replicate wells.

**Measurement of DNA in biofilms using PicoGreen.** Overnight cultures of *P. aeruginosa* PAO1 wild type and PAO1 plus UT1 were allowed to incubate with Minimal Salts Medium (MSM) with 40% glucose as the source of carbon at 37 °C on a shaker. Overnight cultures were then washed twice with fresh media for 2 minutes each at 10,000 x g using a microcentrifuge and then diluted with fresh media to give A_{590} of 0.15. PAO1 with/ without UT1 phage (MOI=0.01) was incubated overnight in 96 well microtitre plates made of PVC. The supernatant was shook off the plate and 100 µL/ well of fresh media was added to resuspend the cells. The mixture was then centrifuged for 2 minutes at 10,000 x g to remove bacterial cells. The supernatant was transferred to a polystyrene microtitre plate and to which freshly prepared PicoGreen (200-fold diluted dimethylsulfoxide, DMSO in TE buffer, 20 µL/ well) was added. The mixture was allowed to incubate at room temperature for three min protected from light.
The measurements of florescence of each well were taken using a Victor² plate reader set to wavelengths 485nm/ 535 nm for 0.1 sec. Results reported are the average and standard deviation of four replicate wells.

Measurement of DNA in supernatant of biofilms using PicoGreen. The initial set up of the biofilms is same as described above under the measurement of quantification of DNA in biofilms using PicoGreen section.

The following day, overnight cultures were washed twice with fresh media for two minutes each at 10,000 x g and then diluted with fresh media to give A₅₉₀ of 0.15. MSG and PAO1 in two rows of wells were set up initially. For each time point, 10 µL/ well of freshly prepared UT1 phage (MOI=0.01) was added to the second row of PAO1 cells. After the required incubation period at 37 °C, the supernatant was carefully pipetted out into micro centrifuge tubes and spun for 2 minutes at 10,000 x g. The supernatant was then carefully pipetted to a polystyrene microtitre plate to which freshly prepared PicoGreen was added. The mixture was then incubated for 3 minutes at room temperature protected from light.

The measurements of florescence of each well were taken using a Victor² plate reader (485nm/ 535 nm for 0.1 sec). Results reported are the average and standard deviation of four replicate wells.

Enumeration of viable cells in biofilms. The initial set up of biofilms is same as described above under the Biofilm Assay section.
The following day after the formation of biofilms in the PVC microtitre plate, the supernatant was discarded by shaking it off. Fresh media (100 μL/ well) was added and the attached cells were resuspended in it. Any two wells (preferably towards the center of the plate) were picked and the contents 50 μL/ well) was used to prepare serial dilutions of $10^{-3}$, $10^{-4}$, $10^{-5}$. The dilutions were then plated on LB Agar plates (1.5%) and/or MSG Agar plates (1.5%) and allowed to incubate at 37 °C overnight. Plates with dilutions which formed 30-300 cfu/ plate were selected to enumerate the viable cells with/without UT1.

Results reported are the average of three replicate plates of a single dilution.

To determine the ability of Crystal Violet to stain DNA. Absorbance at 260nm of genomic DNA (10 μL in 990 μL of sterile water) was determined using Methacrylate cuvettes. Later, genomic DNA was serially diluted with water in the ratio of 1:5. 100 μL/ well of each dilution were plated in a 96 welled PVC microtitre plate. Sterile water acted as a control. The plate was then sealed using a sealing film and incubated overnight at 37 °C. The following day, the protocol for staining using CV was followed and the absorbance of each well was determined using a Victor$^2$ plate reader was set to 490 nm.

Results reported are the average and standard deviation of 12 replicate wells after 24 hours.

Mutant library screening and antibiotic testing of the mutants. A transposon mutant library of P. aeruginosa was obtained. A plate from the mutant library was selected.
Using a sterile 96 prong replicator the contents were replicated onto a sterile polystyrene plate with LB (100 μL/well) and allowed to incubate overnight at 37 °C. The plate was then scored for any growth or no growth in the wells. The mutants were then transferred to 96 deep welled microtitre plates with LB (635 mL/well) and Glycerol (375 mL/well) and stored at (-)70 °C for future use.

A plate from the freezer stocks was selected and using the sterile 96 prong replicator, mutants were replicated to two sterile PVC microtitre plates with 100 μL/well of minimal salts media with glucose (MSG). Sterilization of PVC plates was done by submerging the plates in Ethanol for 30 minutes and later air drying in the laminar hood for 30 minutes. Absorbance of the mutants in the wells was measured using Victor² plate reader set to 590nm wavelength. If the average absorbance of the plate was less than 0.15, then it was allowed to incubate at 37 °C, and if the greater than 0.15, the wells were diluted using fresh MSG. When the desired absorbance was achieved, 10 μL/well of UT1 phage (MOI=0.01) was added to the second plate. Later, both the plates were incubated overnight at 37 °C. Both the plates were then subjected the CV staining and resuspension of the stained biofilms in ethanol. The absorbances of both the plates were taken using Victor² plate reader set to wavelength 490 nm.

The ratio of the absorbance of the plate with phage to no phage was determined to select the mutants which showed induction ratios greater than three and equal to or less than one.

These mutants were then subjected to the “Biofilm” assay using CV staining.

All the selected mutants were also tested for antibiotic resistance to determine the source of the transposon. 10 μL each of the mutants were plated on LB plates with
Gentamycin (100 µg/mL), Kanamycin (300 µg/mL) and Tetracycline (25 µg/mL) and incubated overnight at 37 °C.

The mutants which showed decreased biofilm with phage and showed resistance to a single antibiotic will be sequenced to identify the gene that is mutated by transposon. The protocol for the library screening is shown in the flowchart below.
Replicate the plate into LB, Grow O/N for growth scoring

Replicate plate into 2 sterile PVC plates with MSG
Check for OD 590nm

If < 0.15, allow it to grow
If > 0.15, dilute with MSG
for ___ mins/hrs

2 sterile PVC microtitre plates

Without phage

With phage

CV Assay

Determine the ratio of the plate with phage to no phage

Select mutants of interest

10 μL of selected mutants on antibiotic plates

Mutants which show decreased biofilm and resistance to any single antibiotic will be sequenced to identify the gene
RESULTS

Influence of phage infection with UT1 on biofilms formed by P. aeruginosa PAO1 wild type and mutant cells. We have previously shown that exposure of P. aeruginosa PAO1 cultures to various phage (UT1, D3 and D3112) led to an increase in biofilm formation. The induction of biofilm formation by UT1 infected bacterial cells is shown in Figure 5. (Buemer and Robinson 2006). This effect was observed when cultures were grown on minimal salts medium supplemented with glucose, but not in minimal salts medium supplemented with succinate or Luria broth. Further the induction by phage infection was independent of growth phase and evident at MOIs ranging from 5 to 0.001. Phage that were inactivated by UV light pretreatment did not induce greater biofilm production. Tests comparing wild type PAO1 cells with isogenic mutants in the las and rhl quorum sensing (QS) systems showed the QS mutants were unaltered in their response to phage.

In this study we extended the investigation by examining the effect of phage UT1 on biofilm formation by wild type PAO1 and various mutants in genes known to be involved in biofilm formation, using a standard microtitre dish assay. While the las and rhl QS systems are not necessary, P. aeruginosa has a third known quorum sensing system, the Pqs system. To determine whether the Pqs system is necessary for the
observed increase in biofilm induced by infection with UT1 we compared wild type and several pqs mutants. In this assay the pqsA, H and R mutants produced similar levels of biofilm formation when infected with UT1 as observed with wild type cells (Figure 6). The pqsH mutant formed slightly less abundant biofilms compared to wild-type but levels of induction by the phage were statistically significant. These data show that none of the known QS systems of *P. aeruginosa* PAO1 are necessary for the observed phage induced increase in biofilm formation.

Polysaccharides form a major component of the extracellular hydrated matrix of biofilms. Previous studies in our lab showed a increase in acidic polysaccharides of UT1 infected biofilms as evidenced by staining with calcofluor white (Buemer and Robinson 2006). To determine whether polysaccharides known to play a role in biofilm formation are necessary for the induction of biofilm formation by UT1 we tested algD and psl mutants. Alginate is a capsular polysaccharide virulence factor that confers a selective advantage for *P. aeruginosa* in the CF airway. Also, it is a major component of EPS in mucoid strains. The algD mutant cells infected with UT1 exhibited a substantially reduced response to UTI compared with PAO1 wild type cells; however, the response was still statistically significant (p value=0.01) (Figure 7). Similarly, induction of biofilm formation by phage was less pronounced for the psl mutant cells compared to wild type cells yet remained statistically significant (p value=0.002) (Figure 8). Psl plays a role in attachment and biofilm formation (Friedman and Kolter. 2004; Overhage *et al.* 2005). Psl also plays a role cell–surface and cell–cell interactions. These data show that none of the known polysaccharides involved in stable biofilms of *P. aeruginosa* PAO1 are necessary for the phage induced increase in biofilm formation.
Iron limitation affects biofilm formation by *P. aeruginosa* PAO1 infected by UTI phage. Because iron availability influences biofilm formation (Banin *et al.* 2005), we investigated the effects of iron limitation on phage induced biofilms. Biofilm assays were performed using *P. aeruginosa* PAO1 wild-type cells. Cultures were grown and prepared as described above. To achieve iron limitation during biofilm formation, 2, 2’ dipyridyl, an iron chelator, was added to the wells at final concentrations equal to 0.5, 2.5, 50 and 100 μM. A statistically significant increase in biofilm formation was observed at 0.5 μM (p value=0.003) and significantly reduced at 2.5 μM (p value= 0.011) 2, 2’ dipyridyl (Figure 9).

The addition of 2, 2’ dipyridyl at either 100 or 250 μM to PAO1 cells infected with UT1 did not eliminate biofilm induction by the phage (p value= 0.001 for both concentrations); however, the fold change was reduced (Figure 10).

**Extracellular DNA levels in supernatant and attached cells of biofilms of wild type PAO1 cells infected by UT1.** DNA is released in late-log phase growth and during biofilm development by *P. aeruginosa* PAO1 and serves as a connective matrix in biofilms (Whitchurch *et al.* 2002; Allesen-Holm *et al.* 2006). A role for environmental exogenous phage in this programmed DNA release has not yet been established. Because phage infection would likely lead to lysis of some cells in the population and concomitant release of DNA we investigated whether there was enhanced DNA release during biofilm formation from cells infected with UT1.
Initial experiments involved using propidium iodide (PI) to stain DNA in supernatants and biofilms. PI (0.05 mM) was added to cell cultures, with and without UT1, at the onset of biofilm formation. Following overnight incubation at 37 °C, the biofilm supernatants were removed and transferred to fresh microtitre plates. The DNA concentration in the supernatants was determined by measuring the absorbance of PI at 490 nm and cell density was measured by optical density at 590 nm. Both values are corrected by subtracting background absorbance due to MSG. Cell density was found to be lower in the supernatants in the biofilms formed by UT1 infected bacterial cells. Cell density was almost the same in the attached cells with or without UT1 phage. Also, PI in MSG alone, even in the absence of DNA does not show a higher value than MSG alone (Figures 11 and 12).

The amount of DNA released on a per cell basis was then derived from the above data. These data are summarized in Table 2. In the supernatants, the amount of DNA released per cell with UT1 is almost equal to that of without UT1. In contrast, the amount of DNA released per cell with UT1 was lower than that released without UT1 in the attached cell population.

We sought to determine the timing of DNA release during biofilm formation by assaying for DNA using PicoGreen, which is a more sensitive dye than PI. Release of DNA into the supernatants of biofilms formed by the bacterial cells was significant at eight hours (p value= 0.025) and in overnight biofilms (p value= 0.0). Additionally, DNA release was significant immediately after the addition of UT1 phage (p value= 0.032) with levels gradually increasing up to eight hours before leveling off (Figure 13). However, the DNA levels were substantially lower in supernatants of biofilms formed by
uninfected cells. In attached biofilms DNA levels were almost eight times higher when cells were infected with phage (Figure 14).

**Effect of DNase and/or SDS on biofilms formed.** In order to determine the effect of phage infection on biofilm stability and subsequent release of DNA, the biofilms were subjected to treatment with either SDS, DNase or both. Biofilms formed by uninfected wild type PAO1 cells were unaffected by DNase treatment. In contrast, biofilms formed by PAO1 cells infected with UT1 were significantly disrupted by treatment with DNase. Treatment of biofilms with SDS resulted in a significant disruption of biofilms formed by PAO1 cultures infected with UT1 as well as the uninfected biofilms. Treatment with both SDS and DNase did not result in greater disruption than treatment with SDS alone regardless of infection with UT1 (Figure 15). This shows that the biofilms formed with the cells infected with phage are unstable compared to those formed by cells alone.

**Infection with UT1 results in a reduction of viable cell counts and appearance of Small Colony Variants (SCV).** In previous studies in our laboratory infection by UT1 did not lead to a reduction in total viable cell counts in biofilms assayed after 24 hours when plated onto MSG plates (Buemer and Robinson 2006). Further, *in situ* staining with LIVE/DEAD® stain confirmed that the majority of cells in the phage infected biofilms were alive. However, these biofilms were formed on glass slides immersed in cell cultures and not in microtitre plates as described here. The reduction in optical density observed upon exposure to UT1 prompted us to conduct a careful analysis of the numbers of viable cells in biofilms exposed to UT1 compared to uninfected biofilms. Attached
cells from 24 hour biofilms were resuspended in MSG media and plated on either LB or MSG agar (1.5%). On both media there was a decrease in the number of colony forming units formed by the infected cells compared to the uninfected biofilms; however, the reduction was greater on the MSG agar plates. Additionally, Small Colony Variants (SCV) appeared on LB agar plates from the biofilms formed by PAO1 cells infected with UT1 and on MSG plates with and without UT1 (Figure 16).

Attached material and supernatant of the biofilms formed bacterial cells and cells infected by UT1 after one hour of infection were plated on LB agar (1.5%). It was observed that the number of colony forming units formed by both infected and uninfected cells after one hour were almost the same in both the supernatant and the attached material (Table 3). Four hours post infection, the number of colony forming units were decreased in both the supernatant and attached cells (Table 3). The number of colony forming units formed by infected and uninfected cells at one, four and eight hours is shown in Table 3. We also observed that the appearance of the SCV in the plates formed by infected cells emerged at eight hours post infection (Table 3).

We selected several SCV from each plate and streaked them onto fresh LB media in order to determine whether this phenotypic conversion was stable. In all cases the isolated microcolonies gave rise to only microcolonies (data not shown).

Macrocolonies and SCV recovered from infected and uninfected biofilms were recovered and compared in biofilm assays to wild type PAO1 “naïve” cells that had not been put through a biofilm assay. Naïve wild type PAO1 cells were induced to greater biofilm formation by UT1 as shown previously. In contrast, the macrocolonies and the SCV recovered on LB plates from UT1 infected biofilms exhibited enhanced biofilms
formation even in the absence of the exogenous addition of UT1 (Figure 17). Addition of UT1 to these biofilms did not result in a significant increase (p values= 0.389, 0.485 respectively).

**Screening of the Transposon mutant library of *P. aeruginosa*.** In order to determine any and all genes responsible for the induction of formation of biofilms by infected bacterial cells, screening of a transposon mutant library was initiated. The mutant library is in 115 96-welled microtitre plate and each well contains a mutant of *P. aeruginosa*. Further screening of such microtitre plates was done to determine the response on interaction with UT1 phage. Few mutants which showed large induction with UT1 phage and also those which showed minimal or no effect with phage were isolated. These mutants were allowed to form biofilms and the quantification of biofilms was done using a crystal violet assay. The mutants were also tested for resistance to antibiotics, tetracycline, gentamycin and tobramycin, in order to determine the source of the transposon. And the mutants which showed resistance to one antibiotic were marked for further studies. So, far, one such mutant from three 96 welled microtitre plate has been isolated. This mutant was tetracycline resistant and was not induced by UT1 phage (Mutant # 3 in Figure 18).
Fig. 6. Comparison of biofilms formed by wild-type (WT) PAO1 and pqs isogenic mutants infected with UT1 phage. Overnight cultures grown in minimal salts medium supplemented with 40% glucose were diluted with fresh media to an absorbance at 590nm of 0.15. UT1 phage were added to the diluted cells to achieve an MOI = 0.01. Parallel control cell suspensions did not receive phage. Cell suspensions were aliquoted into 96 well PVC microtitre plate wells and allowed to incubate overnight, without shaking, at 37°C. The resulting biofilms were stained with crystal violet (0.25% w/v) for 30 minutes at room temperature. Following solubilization in 95% ethanol the crystal violet stained biofilms were quantified by measuring absorbance at 590nm using a Victor3 plate reader. Error bars represent S.D. Values were corrected for background absorbance due to the medium.

* The mean difference is significant (p value = 0.05) compared with uninfected cells.
Fig. 7. Comparison of biofilms of wild-type (WT) PAO1 and algD mutant cells upon infection with UT1 phage. Static microtitre plate biofilm assays were performed as described in Figures 5 and 6.

* The mean difference is significant at the 0.05 level compared to uninfected cells.
Fig. 8. Comparison of biofilms of wild-type (WT) PAO1 and psl mutant cells upon infection with UT1 phage. Static microtitre plate biofilm assays were performed as described in Figures 5 and 6.

* The mean difference is significant at the 0.05 level compared with uninfected cells.
Fig. 9. Biofilms formed in the presence of different concentrations of 2-2' dipyridyl. Static microtitre plate biofilm assays were performed as described in Figures 5 and 6. Absorbances of CV stained biofilms formed by wild type PAO1 cells in the presence of 2, 2’ dipyridyl were measured at 590 nm using Victor² plate reader. Error bars represent S.D. Control plates received no 2, 2’ dipyridyl.

* The mean difference is significant at the 0.05 level compared with cells that received no 2, 2’ dipyridyl.
Fig. 10. Biofilm assay comparing wild-type with/without UT1 with different concentrations of 2, 2’ dipyridyl. Static microtitre plate biofilm assays were performed as described in Figures 5 and 6. Absorbances of CV stained biofilms formed by the cells were measured at 590 nm using Victor² plate reader. Error bars represent S.D.

* The mean difference is significant at the 0.05 level compared with cells receiving no 2, 2’ dipyridyl.
Fig. 11. Propidium Iodide stained biofilm supernatants. Supernatants from 24 hour biofilms were carefully removed and 50μL was aliquoted into a fresh microtitre plate and the absorbance of the propidium iodide (490 nm) and cell density (590 nm) measured using a Victor² plate reader. Error bars represent S.D.
Fig. 12. Propidium iodide stained attached cells of biofilms. Supernatants were removed from microtitre plate wells following 24 hours of biofilm formation and remaining biofilm biomass resuspended in 100μL of MSG. Propidium iodide absorbance (490 nm) and cell density (590 nm) was measured in a Victor² plate reader. Error bars represent S.D.
Fig. 13. Time course of DNA levels in biofilm supernatants of UT1 infected and uninfected cells stained with PicoGreen. Overnight cultures grown in minimal salts medium supplemented with 40% glucose were washed twice with fresh media and then diluted with the same medium to an OD at 590 nm of 0.15. UT1 phage were added to the diluted cells to achieve an MOI = 0.01 and allowed to incubate at 37 °C and sampled at various time points up to 24 h. Parallel control cell suspensions did not receive phage. The supernatant from each well was carefully pipetted out and further centrifuged to remove the cells prior to the addition of PicoGreen. Cell free supernatant was then transferred to a microtitre plate to which PicoGreen was added. Following a three minute incubation at room temperature, florescence was measured using Victor² plate reader at 485 nm and 535 nm. Error bars represent S.D.

* The mean difference is significant at the 0.05 level compared to the cells before the addition of UT1 phage.

The red arrow indicates the florescence of PAO1 cells immediately after infection with UT1 phage.
Fig. 14. Quantification of DNA in attached cells of biofilms of UT1 infected and uninfected bacterial cells using PicoGreen. Overnight cultures grown in minimal salts medium supplemented with 40% glucose were washed twice with fresh media and then diluted with the same to an OD at 590 nm of 0.15. UT1 phage were added to the diluted cells to achieve an MOI = 0.01 and allowed to incubate overnight, without shaking, at 37 °C. Parallel control cell suspension did not receive phage. The supernatant from each well was removed by shaking. Biofilms were then resuspended in fresh media and further centrifuged to remove the cells prior to the addition of PicoGreen. Cell free supernatants were then transferred to a microtitre plate to which PicoGreen was added. Following a three minute incubation at room temperature, florescence was measured using Victor² plate reader at 485 nm and 535 nm. Error bars represent S.D.
**Fig. 15. Biofilms treated with DNase, SDS or both.** The initial set up for biofilms was as described in Fig. 6. Duplicate plates were prepared for cells only and cells plus UTI (MOI = 0.01). Following the overnight incubation at 37 °C, DNase was added to each well to a concentration of 100 μg/mL and allowed to incubate for 45 minutes at room temperature. The plates were subjected to the CV staining after the DNase treatment. To test the effect of SDS on biofilms treated with DNase, microtitre plates were submerged in Tupperware with 0.01% solution of SDS followed by shaking for two hours at 63 rpm on a rotary shaker at room temperature. Following the exposure to SDS, excess detergent was removed by shaking the plates and biofilms were stained with Crystal Violet as described in Figures 5 and 6. Parallel control cell suspensions were not subjected to any treatment. In all cases, absorbances at 590 nm were measured using a Victor² plate reader. Error bars represent S.D.
Fig. 16. Cell counts of attached cells of overnight biofilms. Colonies formed by bacterial cells and bacterial cells infected by UT1 at $10^3$ dilution which were resuspended in MSG media and later plated on either LB agar (1.5%) for 24 hrs and on MSG agar (1.5%) for 48 hrs. SCV are encircled in red.
Fig. 17. Comparison of biofilms formed by wild type naive PAO1 and isolates of UT1 infected biofilms. Both macrocolonies and SCV isolated from UT1 infected biofilms along with macrocolonies isolated from uninfected wild-type PAO1 biofilms on LB agar (1.5%) plates were used in these assays. These variants were further infected with UT1 phage (MOI = 0.01). Static microtitre plate biofilm assays were performed as described in Figures 5 and 6. Absorbances of CV stained biofilms formed by the cells were measured at 590 nm using Victor² plate reader. Error bars represent S.D.

* The mean difference is significant at the 0.05 level compared with cell suspensions that did not receive a fresh inoculum of UT1 phage (MOI = 0.01).
Fig. 18. Comparison of biofilms formed by wild-type (WT) PAO1 and mutants isolated from transposon library infected with UT1 phage. Static microtitre plate biofilm assays were performed as described in Figures 5 and 6. Numbers represent the mutants.
Table 1. Strains and bacteriophage used in this study with relevant features.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Relevant feature(s)</th>
<th>Phage sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>Wild type <em>P. aeruginosa</em></td>
<td>UT1, D3, D3112</td>
</tr>
<tr>
<td><em>pqsA</em></td>
<td>Mutant unable to synthesize all quinolones</td>
<td>ND</td>
</tr>
<tr>
<td><em>pqsH</em></td>
<td>Mutant unable to synthesize PQS, a Quinolone, synthesis of pyocyanin</td>
<td>ND</td>
</tr>
<tr>
<td><em>pqsR</em></td>
<td>Mutant unable to make PQS receptor</td>
<td>ND</td>
</tr>
<tr>
<td><em>algD</em></td>
<td>Alginate deficient mutant of PAO1</td>
<td>UT1, D3, D3112</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bacteriophage</th>
<th>Relevant feature(s)</th>
<th>Phage sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT1</td>
<td>Pseudolysogenic. Receptor is unknown.</td>
<td>NA</td>
</tr>
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Table 2. DNA and cell Biomass accumulation in biofilms stained with propidium iodide

<table>
<thead>
<tr>
<th></th>
<th>Supernatant</th>
<th>Attached cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without phage (A)</td>
<td>With phage (B)</td>
</tr>
<tr>
<td>Amount of DNA $^1$</td>
<td>0.448</td>
<td>0.151</td>
</tr>
<tr>
<td>Cell biomass $^2$</td>
<td>0.694</td>
<td>0.210</td>
</tr>
<tr>
<td>DNA/Cell</td>
<td>0.650</td>
<td>0.720</td>
</tr>
<tr>
<td>DNA/Cell (B/A)</td>
<td>1.11</td>
<td>0.639</td>
</tr>
</tbody>
</table>

$^1$ absorbance at 490 nm, corrected for background absorbance at 490 nm

$^2$ absorbance at 590 nm, corrected for background absorbance at 590 nm
### Table 3. Time course of the Colony forming units per mL (CFU/mL) of supernatants and attached cells of biofilms formed by UT1 phage infected and uninfected *P. aeruginosa* on LB and MSG agar (1.5%)

<table>
<thead>
<tr>
<th></th>
<th>Supernatant of biofilms</th>
<th>Attached cells of biofilms</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1hr</td>
<td>4hrs</td>
<td>8hrs</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>SCV</td>
<td>Total</td>
</tr>
<tr>
<td>LB</td>
<td>PAO1</td>
<td>1.2x10⁵</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PAO1+UT1</td>
<td>1.43x10⁵</td>
<td>0</td>
</tr>
<tr>
<td>MSG*</td>
<td>PAO1</td>
<td>3.87x10⁵</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PAO1+UT1</td>
<td>5.57x10⁵</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>ND</td>
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<td>ND</td>
</tr>
</tbody>
</table>

*MSG cell counts were after 48 hrs
DISCUSSION

With the increasing emergence of antibiotic resistance in the treatment of *Pseudomonas aeruginosa* infections, alternate methods of treatment such as phage therapy are being considered. Since the use of phage therapy is in its infancy and before phage can be used to treat infections in human beings, mechanistic studies are required to determine precisely how *P. aeruginosa* biofilms respond to exogenous phage.

Previous work in our lab had shown that exposure of *P. aeruginosa* PAO1 cultures to various phage (UT1, D3 and D3112) led to an increase in biofilm formation (Buemer and Robinson 2006). Tests comparing wild type PAO1 cells with isogenic mutants in the *las* and *rhl* quorum sensing (QS) systems showed the QS mutants were unaltered in their response to phage. Similar results were found with the *algD* polysaccharide mutant.

In this study we extend the investigation of the effect of phage UT1 on biofilm formation by wild type PAO1 and various mutants. Mutants, *pqsA*, *H* and *R*, of a third quorum sensing signaling system, the Pqs system were tested. PQS is involved in biofilm formation (Palmer *et al.* 2005). It acts as a link between the *las* and *rhl* quorum-sensing systems. It was found that all of them produced similar levels of biofilm formation when infected with UT1 as observed with wild type cells (Figure 6). This data shows that the
Pqs system of *P. aeruginosa* PAO1 is not essential for the phage induced increase in biofilm formation.

Extracellular polysaccharide (EPS) in the biofilms is an important virulence factor against the host immune system and antibiotics. The EPS of *P. aeruginosa* is produced by three loci: *psl, pel* and *alg* (Evans and Linker 1973; Friedman and Kolter 2004; Matsukawa and Greenberg 2004). Alginate is a capsular polysaccharide virulence factor that confers a selective advantage for *P. aeruginosa* in the CF airway. It is a major component of EPS in mucoid strains. Previous experiments in our laboratory have shown that in the biofilms of bacterial cells exposed to phage, there was an increase of exopolysaccharide production which was indicated by increase of calcoflour staining (a dye known to stain all the polysaccharides linked by a β-1, 4-glycoside bond) (Buemer and Robinson 2006). A crystal violet assay comparing the biofilms formed by wild-type *P. aeruginosa* and isogenic mutant, *algD*, with and without bacteriophage UT1 was done. It was found that *algD* mutant formed better biofilms than wild-type, however, phage infection did not induce greater biofilm formation in the *algD* mutant. However, in this study, it was found that *algD* produced similar level of biofilm formation when infected with UT1 as observed with wild type cells (p value = 0.01 at 0.05 significance level, Figure 7). One possible explanation for the different results is, both the *algD* mutants were obtained from different sources.

Another polysaccharide, Psl, aids in attachment of bacteria to a surface and in biofilm formation. It also plays a role cell–surface and cell–cell interactions. A biofilm assay comparing the biofilms of wild-type PAO1 and isogenic mutant *psl* with and without UT1 showed a similar level of biofilm formation (p value = 0.002 at 0.05
significance level, Figure 8). Hence, it can be concluded from this data that although polysaccharides are a major component in the biofilms, they are not necessary for the induction of biofilm formation on interaction with UT1 phage.

Since, encouraging results were not obtained with QS and polysaccharide mutants, screening of a Transposon mutant library was started to identify any and all genes that are crucial for biofilm formation as well as the subset of those genes that mediate the induction of biofilm formation upon the bacterial cells exposure to UT1 phage. So, far, one such likely candidate has been identified (Figure 18).

Other factors which might be responsible for the UT1 phage induced were also investigated. Iron serves as a signal in *Pseudomonas aeruginosa* biofilm development. The functional iron signal for *P. aeruginosa* biofilm development is active transport of chelated iron or the level of internal iron (Banin *et al.* 2005). 2, 2′ dipyridyl is a chelator of iron. Biofilms assays were performed by growing wild-type *P. aeruginosa* PAO1 in different final concentrations of 2, 2′ dipyridyl, 0.5, 2.5, 50 and 100 μM. It was found that biofilm formation was statistically significant for cells with 0.5 μM (p value=0.003 at 0.05 significance level) and 2.5 μM (p value= 0.011 at 0.05 significance level) 2, 2′ dipyridyl, but was not statistically significant with 50 μM and 100 μM concentrations (Figure 9). Hence higher concentrations of 2, 2′ dipyridyl was considered for further biofilm assays with phage. However, the addition of 2, 2′ dipyridyl at either 100 or 250 μM to PAO1 cells infected with UT1 did not eliminate biofilm induction by the phage (p value= 0.001 for both concentrations at 0.05 significance level) (Figure 10).

Experiments were done previously in our lab to determine whether the in biofilms formed in response to phage were composed of live cells, or lysed cells and cell debris.
LIVE/DEAD® Baclight staining (Molecular Probes) was done to determine this. Biofilms were grown for 24 hrs. on glass slides which were partially in air and partially in tubes with MSG media containing *P. aeruginosa* PAO1 wild-type cells with or without UT1 phage. Slides were analyzed using an epifluorescence microscopy at 60x magnification using a FITC filter on a Nikon model microscope. It was found that in biofilms of *P. aeruginosa* PAO1 exposed to the lytic phage UT1 were primarily composed of live cells. Additionally, a hazy red material surrounding these biofilms was observed which was attributed to extracellular DNA stained by propidium iodide present in the LIVE/DEAD® Baclight stain. Biofilms of uninfected cells revealed a mix of live and dead cells and little DNA matrix. Further tests were done to determine the effect of phage on cell numbers and colony morphology. It was shown that equivalent numbers of cells were recovered from both infected and uninfected biofilms which were plated on MSG agar (1.5%). Therefore, it was concluded that the increase in DNA in biofilms formed by infected cells is not due to lysis of cells. It was also found that the colony morphologies were unchanged as a result of phage infection.

In this study, we sought to quantify the levels of DNA in the supernatants and in the attached material of biofilms. We initially used propidium iodide. It was found that in the supernatants, the amount of DNA released per cell with UT1 is almost equal to that of without UT1. In contrast, the amount of DNA released per cell with UT1 was lower than that released without UT1 in the attached cell population. PI enters cells with compromised membrane and hence the quest for another stain was done.

A more sensitive stain of DNA, PicoGreen was used in subsequent assays to determine the mechanism and timing of DNA release in the biofilms. It was found that
there was an eight fold increase in the amount of DNA release in the attached cells of overnight biofilms (Figure 14). In order to confirm that the induction was DNA, DNase and SDS assays were performed. These assays attribute DNA to the eight fold increase in the intensity of fluorescence and also suggest that the biofilms formed by infected bacterial cells are unstable and hence more susceptible to SDS detergent (Figure 15).

Time course of the release of DNA into supernatant of biofilms formed by the bacterial cells was significant at eight hours (p value = 0.025) and in overnight biofilms (p value = 0.0). And that formed by PAO1 was significant immediately after adding UT1 phage (p value = 0.032) and following four, six, eight, ten, 12 hours and in overnight biofilms (p value = 0.0 in all treatments, Figure 13). The increase of DNA immediately after the addition of UT1 phage indicates that DNA aids in the protection of the bacteria. It can also be concluded that on interaction with UT1 phage, the bacterial cells release increased levels of DNA compared to wild-type bacterial cells alone.

It has been clarified that in *Streptococci pneumoniae*, CSP (Competence Signaling peptide), a peptide based signal for QS, plays a role in the release of extracellular DNA in the biofilms. When the CSP reaches a threshold of 1–10 ng/ml in the medium, a subpopulation of the bacteria lyse, and the released DNA adds to the biofilm biomass and protects the remaining population (Steinmoen *et al.* 2002).

In Gram negative bacteria, the mechanism of DNA release is still unclear but direct secretion, lysis of a subpopulation by prophage and release of small membrane vesicles are among viable hypotheses (Allesen-Holm *et al.* 2006). But there are no studies indicating the interaction of phage and DNA release. Experiments were hence performed to determine the mechanism of DNA release in the biofilms formed by UT1
infected bacterial cells. Enumeration of cell counts of the supernatants and attached cells of biofilms on LB agar (1.5%) indicated that there is a lysis of a subpopulation of cells on interaction with UT1 phage (Table 3). Further, it has been shown that the lysis of a subpopulation of cells occurs between one to four hours post infection with UT1 phage.

Another interesting finding was the emergence of a phenotypic variant, SCV on the LB agar (1.5%) plates with attached material of infected bacterial cells. It was previously shown that rough SCV were found in the biofilms of \textit{P. aeruginosa} strain PA14 exposed to antibiotics (Drenkard and Ausubel 2002). However, emergence of SCV was also noticed when the attached material of biofilms formed by uninfected cells were plated on MSG agar (1.5%). It was previously shown that induction of filamentous Pfl like prophage, which resides in the bacterial genome, is linked to lysis of subpopulations of bacterial cells in the late stage biofilms (Webb \textit{et al.} 2003). Also, the increase in carbon availability upregulated Pfl like genes (Sauer \textit{et al.} 2002). Hence, it can be concluded that the emergence of the SCV is dependant on the carbon source (LB or MSG agar) and also the exogenous infection with UT1 phage.

Characterization of the SCV revealed that they retained their morphology on subsequent streaking for isolation. Further, the SCV which were formed by infected bacterial cells along with the macrocolonies isolated from the biofilms of infected and uninfected cells were subjected to biofilm assays with and without phage. It was found that the cells which were previously exposed to UT1 did not show the usual induction on subsequent exposure to UT1 phage (Figure 17).
FUTURE DIRECTIONS

We have demonstrated that the exposure and infection by an environmental exogenous phage leads to greater biofilm production by *P. aeruginosa*. Our new hypothesis is that infection by induces phenotypic variants (SCV) and the release of DNA from a subpopulation of cells. The DNA serves to create an environment that is conducive for biofilm formation.

The source of the increased exogenous DNA in the biofilms of UT1 infected bacterial cells has to be determined. It is also essential to determine if pseudolysogeny is involved in the emergence of SCV and to determine if UT1 induces prophage Pf4. Further characterization of the SCV would also be interesting since the emergence of SCV appears to be linked to carbon source and also infection with UT1 phage. Another interesting finding would be the determination of the receptor of UT1.

Phage induction of biofilms has important implications for phage therapy. Since encouraging results were not achieved with QS and polysaccharide mutants, screening of a transposon mutant library which was started has to be accomplished, since the question “What are phage inducing in the bacterial host” has to be appropriately answered.
Composition of Mineral Salts Medium (MSM)

1) To one liter of MilliQ water, the following was added

<table>
<thead>
<tr>
<th>Name of the Chemical</th>
<th>Weight (in gms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium phosphate dibasic, K₂HPO₄</td>
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</tr>
<tr>
<td>Potassium phosphate monobasic, KH₂PO₄</td>
<td>3.0</td>
</tr>
<tr>
<td>Ammonium sulphate, (NH₄)₂SO₄</td>
<td>1.0</td>
</tr>
<tr>
<td>Magnesium sulphate heptahydrate, MgSO₄·7H₂O</td>
<td>0.05</td>
</tr>
<tr>
<td>Ferric chloride hexahydrate, FeCl₃·6H₂O</td>
<td>0.0025</td>
</tr>
</tbody>
</table>

2) 10 mL of MSM was supplemented with 100 μL of 40% Glucose to achieve a final concentration of 0.4% (w/v).


