The *Pseudomonas aeruginosa* Type IV Pilus-Associated Protein PilY1 is Required for Stable Fiber Formation and Host Attachment

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ABSTRACT

RYAN WILLIAM HEINIGER: The *Pseudomonas aeruginosa* Type IV Pilus-Associated Protein PilY1 is Required for Stable Fiber Formation and Host Attachment

(Under the direction of Dr. Matthew Wolfgang)

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that is a leading cause of hospital-acquired pneumonia and the primary pathogen associated with chronic respiratory infection in individuals with cystic fibrosis (CF). *P. aeruginosa* pathogenesis involves a wide variety of virulence factors; including secreted toxins, Type II and type III secretion systems, quorum sensing, biofilms, flagellum, and Type IV pili (TFP). TFP are long surface fibers composed primarily of pilin subunits, and are known to function in both a specialized form of movement known as twitching motility and attachment to host cells and tissues. An exposed epitope on the terminal pilin subunit of TFP is believed to mediate *P. aeruginosa* binding to host glycosphingolipids. We hypothesized that PilY1, a putative minor pilus-associated protein, may play a direct role in TFP-dependent bacterial adherence due to its homology to a known TFP-associated adhesin in *Neisseria gonorrhoeae,* PilC. We confirmed a previous report that *pilY1* is required for pilus biogenesis, though this requirement is dependant on the presence of PilT. We demonstrated PilY1 is required for adherence to injured Human Airway Epithelial (HAE) cells, indicating a potential role as a TFP-associated adhesin. This adherence requires the association of PilY1 with surface TFP fibers, a process that involves a set of pilin-like proteins (FimU, PilV, PilW, PilX, and PilE). Overall, our results indicate that adherence of *P. aeruginosa* to host cell requires both TFP and PilY1 and that PilY1 is a bi-functional protein with distinct roles in TFP biogenesis and adherence.

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AHL	N-acyl-L-homoserine lactone
Am	Ampicillin
BHL	N-butanoyl-L-homoserine lactone
cAMP	cyclic-adenosine-3',5'-monophosphate
Cb	Carbenicillin
CO_2	Carbon Dioxide
CDC	Centers for Disease Control and Prevention
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
cm	centimeter
CRP	cAMP receptor protein
^oC	degrees Celsius
Δ	deletion
DSL	disulfide-bonded loop
EPS	extracellular polymeric substance
Fab	fragment antigen binding
GFP	Green Fluorescent Protein
Gm	Gentamicin
GSL	glycosphingolipid
HAE	human airway epithelial cell cultures
HRP	horseradish peroxidase
HSL	homoserine lactone
IF	immunofluorescence microscopy
Ig	immunoglobulin

IL	interleukin
INF-γ	interferon γ
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	kilodalton
LB	Luria Bertani media
LDH	lactate dehydrogenase
LD_{50}	Lethal Dose, 50%
LPS	Lipopolysaccharide
μg	microgram
μl	microliter
μM	micromolar
ml	milliliter
MOI	multiplicity of infection
NIH	National Institutes of Health
NF-κB	nuclear factor kappa B
OD	optical density
OdDHL	N-3-oxododecanoyl-L-homoserine lactone
ORF	open reading frame
PAMP	pathogen-associated pattern recognition molecule
PCR	polymerase chain reaction
PvdS	pyoverdine sigma factor
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEM	transmission electron microscopy

TFP	Type IV Pili
TLR	toll-like receptor
T2SS	Type II secretion system
T3SS	type III secretion system
TNF-α	tumor necrosis factor α
QS	quorum sensing

CHAPTER 1

INTRODUCTION

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen, capable of causing a variety of life threatening infections in immunocompromised individuals (61). Recent statistics indicate that *P. aeruginosa* is responsible for greater than 10% of all hospital-acquired infections and nearly 22% of infections acquired in intensive care units (54). In addition, *P. aeruginosa* causes chronic lung infections in individuals with cystic fibrosis (CF), resulting in significant morbidity and mortality (78). Efforts to treat *P. aeruginosa* infections are hampered by the bacterium's high intrinsic antibiotic resistance and its ability to develop further resistance during antibiotic therapy (29). A unique feature of this organism is its versatility. *P. aeruginosa* can thrive in a wide range of natural environments (85) and can utilize over 50 different carbon sources (239). In aqueous environments, *P. aeruginosa* is highly motile; however, it can display complex multicellular phenotypes including the ability to form biofilms on a variety of biological and abiotic surfaces (39, 124). While *P. aeruginosa* is considered to be a strict aerobe, it can grow under hypoxic conditions using nitrite/nitrate as terminal electron acceptors (81). In addition to its metabolic and physiological flexibility, *P. aeruginosa* produces an abundance of surface associated and secreted virulence factors (129, 159), which allow it to infect many eukaryotic organisms such as plants, nematodes, insects and humans (43, 190, 196).

The ability of *P. aeruginosa* to inhabit diverse environments and cause disease in a variety of hosts is due in part to its relatively large and dynamic genome. To date, the genomes of five *P.*

aeruginosa strains have been determined (126, 144, 224). The genome of *P. aeruginosa* strains can vary between 6-7 million bases and is on average about 50% larger than the *Escherichia coli* genome. The genome of *P. aeruginosa* strain PAO1 is 6.3 million base pairs and contains 5,570 predicted open reading frames (ORFs). Moreover, the *P. aeruginosa* genome contains a high proportion of regulatory genes (8.4% of predicted ORFs) which is presumed to contribute to its adaptability, broad host range and high intrinsic resistance to a wide range of antimicrobial substances (79, 121, 224). In comparison, only around 5% of the genes in *E. coli* and *Bacillus subtilis* are regulatory genes (224). The pathogenic potential of this versatile bacterium is also reflected by the high number of genes encoding secreted virulence factors such as toxins and tissue degrading enzymes including proteases and phospholipases (224). Despite the large size of the *P. aeruginosa* genome, its core gene content appears to be relatively stable (254). Greater than 95% of the genes in strain PAO1 are found in strains collected from diverse habitats and various human infections. The conservation of a large genome may provide *P. aeruginosa* with the capacity to inhabit a wide range of environments. However, the ability of *P. aeruginosa* to further adapt and thrive in different or new environments depends on the ability of individual strains to acquire large novel genomic segments (144). These genomic segments can also play key roles in the infection process itself (250).

While *P. aeruginosa* is able to grow and survive in almost any environment, it rarely causes infections in healthy individuals. To initiate infection, *P. aeruginosa* often requires a substantial break in first-line host defenses. This usually involves a breach or bypass of normal cutaneous or mucosal barriers (trauma, surgery, or serious burns), disruption of the protective balance of normal mucosal flora by broad spectrum antibiotics, or alteration of the immune system (chemotherapy or CF). Once *P. aeruginosa* breaches these defenses, it can colonize the altered or damaged epithelium. Initial colonization requires the expression of bacterial surface fibers termed Type IV pili (TFP), which serve as an adhesin for host cells and tissue (51). Following colonization, *P. aeruginosa* can undergo transcriptional and mutational adaptation to the host environment facilitating either acute or

chronic infection. Acute infections are characterized by the production of numerous extracellular virulence factors. These factors (described in detail below) contribute to tissue damage, immune evasion, dissemination to the blood stream and peripheral organs, and frequently death. In contrast, chronic infection is characterized by mutational adaptations that lead to the production of an alginate capsular polysaccharide, biofilm formation, and reduced production or the loss of surface attached and secreted virulence factors. In chronic infections, tissue damage and disease progress is primarily due to unrelenting and non-productive inflammation. Overall, the pathogenesis of *Pseudomonas aeruginosa* infections is multifactorial due to the wide array of virulence factors possessed by the bacterium. This is to be expected given the wide range of diseases caused by *P. aeruginosa*; including septicemia, urinary tract infections, pneumonia, chronic lung infections, endocarditis, and dermatitis.

Acute Infections

Over the last few decades, *P. aeruginosa* has emerged as a major opportunistic human pathogen accounting for a high number of hospital-acquired infections, especially in intensive care units (138, 202). *P. aeruginosa* is responsible for 12% of hospital-acquired urinary tract infections, 8% of surgical wound infections, 10% of bloodstream infections, and 16% of nosocomial pneumonia cases (77). This bacterium often infects decubitus ulcers and burn wounds, thereby retarding healing or even causing enlargement of the wounds (42). *P. aeruginosa* infections are also commonly found among immune suppressed individuals such as AIDS patients or cancer patients undergoing chemotherapy.

Our current understanding of the pathogenesis of acute *P. aeruginosa* infection relies heavily on studies using animal infection models of pneumonia. Based on these studies, the picture of *P. aeruginosa* pathogenesis is complex, producing a result that depends on the multiple virulence factors displayed by the bacteria, as well as the host response. *P. aeruginosa* expresses both a flagellum and

polar TFP during its initial stage of infection. Flagella are critical for motility of the bacterium to the site of infection; however flagellin, the major structural subunit of the flagellum, is a potent pro-inflammatory signaling molecule that is detected by host toll-like receptor 5 (TLR5) (60). In fact, the proinflammatory role of flagellin has been shown to be a major factor in early death in a mouse model of acute pneumonia (10). Polar type IV pili play a crucial role in attachment of the bacteria to host epithelial cells (51), and may play a role in translocation of *P. aeruginosa* to tissue beyond the initial infection site via a specialized form of TFP-dependent movement known as twitching motility (1).

Once attached to lung epithelial cells, the *P. aeruginosa* type III secretion system can be activated, a process requiring type IV pilus mediated adherence (60, 83). The type III secretion system directly injects a set of effector proteins (ExoS, ExoT, ExoU, and ExoY) into the host cell. Following delivery to the host cell cytoplasm, the effector proteins can disrupt a variety of cellular processes and promote invasion of *P. aeruginosa* into the underlying tissue. Direct injection of ExoU has been shown to cause irreversible damage to cellular membranes and rapid necrotic death (203), while ExoS and ExoT disrupt cellular processes; including cell division, phagocytosis, and cell migration, by disrupting the actin cytoskeleton (57). Secretion of type III effector proteins can also act on immune cells to promote immune evasion. In human infections and mouse models of pneumonia, ExoU may specifically target and kill neutrophils, leading to localized areas of immune suppression (49, 56). This effector protein can also inhibit caspase-1-mediated pro-inflammatory cytokine production (228). Additionally, both ExoS and ExoT are known to inhibit cell migration, wound healing, and bacterial uptake by macrophages and neutrophils (72, 187).

The importance of type III secretion in *P. aeruginosa* virulence has been demonstrated in mouse models of infection. Intravenous administration of polyclonal antibodies against PcrV, a protein involved in translocation of type III secretion effector proteins from *P. aeruginosa*, resulted in

complete survival of the mice (210). Similar studies in a rabbit model of *P. aeruginosa*-induced septic shock showed that treatment with anti-PcrV IgG significantly reduced lung injury, bacteremia, and plasma TNF-α levels compared to animals treated with control IgG (210). Finally, in a human study, the relative risk of mortality was six-fold higher in acute infections involving *P. aeruginosa* strains that possessed a functional type III secretion system (82).

In addition to type III secretion and the associated effector molecules, *P. aeruginosa* expresses many other virulence factors that contribute to its pathogenicity in acute infections. Quorum sensing (QS) systems control a variety of virulence factors (71, 123, 177), and the associated quorum sensing molecules have the potential to directly modulate the immune system by inducing apoptosis of neutrophils and macrophages (230). These molecules are also known to increase the production of proinflammatory cytokines from airway epithelial cells and macrophages (215, 217, 231). Factors such as pili, flagella, and surface polysaccharides are involved in bacterial colonization, while factors such as elastase, alkaline proteases, hemolysins (phospholipase and lecithinase), and cytotoxins promote tissue invasion by *P. aeruginosa*. The elastases disrupt the integrity of the epithelial barrier by disrupting epithelial cell tight junctions and interfering with mucociliary clearance (141), while exotoxin A inhibits protein synthesis and promotes host cell death by catalyzing the ADP-ribosylation of eukaryotic elongation factor-2 (14, 260). Exotoxin A, in particular, is a potent virulence factor capable of being highly lethal in animal models of infection, and correlates with survival in studies of patients with *P. aeruginosa* sepsis (117, 118). All of these factors, as well as their role in *P. aeruginosa* infection, will be described in detail later in this introduction.

Chronic Infections

In general, acute *P. aeruginosa* infections develop rapidly, involve substantial inflammation, and if untreated lead quickly to sepsis and death. In contrast, chronic infections are characterized by a slower rate of disease progression. Early in the development of chronic infection, patients can be culture positive for *P. aeruginosa* without any obvious impact on health, and then gradually the clinical condition deteriorates as the infection persists (95). Early in chronic infections, *P. aeruginosa* strains appear to possess a full complement of virulence factors; however, eventually many of these factors including, flagella, type III secretion and TFP are lost due to mutation (94, 151, 216). It is generally believed that these "acute" virulence factors are not required once a chronic infection has been established and that they are counterproductive given their ability to be recognized by both the innate and adaptive immune systems (214). The best characterized chronic infection caused by *P. aeruginosa* occurs in cystic fibrosis (CF) patients, where *P. aeruginosa* is the main source of morbidity and mortality due to lung failure caused by prolonged *P. aeruginosa* infection (96, 112, 133).

CF is an autosomal recessive disease and according to Centers for Disease Control and Prevention (CDC), the incidence rate is 1:3300 among non-Hispanic Caucasians and 1:9000 and 1:15,300 among Hispanic Caucasians and African Americans, respectively (133). The disease is caused by dysfunction of a cAMP-regulated chloride channel, CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) and more than 1500 different mutations have been identified in the CFTR gene. CFTR is localized to the apical plasma membrane of epithelia cells including those located in the respiratory, gastrointestinal, hepatobiliary and reproductive systems where it is involved in electrolyte secretion and absorption across the epithelium. As the name implies the CFTR protein is a "conductance regulator" and thereby it affects many important transport systems involved in the maintenance of the basal state fluid balance. Inactivating mutations in the CFTR gene result in an

increased resorption and a decreased secretion of water, which among other serious effects leads to dehydration of mucosal surfaces. Reduced surface liquid volume in the airways of CF patients impairs ciliary function thereby reducing mucociliary clearance and creating the basis for recurrent infections (133). In young CF patients a variety of bacteria such as *Haemophilus influenzae, Staphylococcus aureus* and *Streptococcus pneumonia* cause pulmonary infections. From the age 10-14, *P. aeruginosa* becomes the predominant pathogen in the CF lung, sometimes followed by co-infection by *Burkholderia cepacia* (64).

In order to adapt to environments like the CF lung, *P. aeruginosa* can coordinately express a variety of genes in a bacterial cell/density-dependent manner. This response is controlled by quorum sensing systems, which are complex regulatory circuits involving cell-to-cell signaling (46, 177). This type of signaling allows for gene regulation in response to bacterial density through the production of small, diffusible autoinducers known as homoserine lactones (HSLs) (68). These molecules are secreted, accumulate in the external environment, and upon reaching a critical concentration, are sensed by the bacteria, triggering various responses. One such response is the formation of biofilms. Activation of quorum sensing cascades promotes formation of biofilms, which can make conditions more favorable for survival in the "thickened" mucus environment of the CF lung (23). Beyond survival due to conditions within the lung, biofilms can also contribute significantly to the resistance of *P. aeruginosa* to aggressive antimicrobial therapy (222). While technical difficulties exist in studying the contribution of biofilms to pathogenesis within the CF lung, studies have shown the presence of quorum sensing molecules in CF sputum, which has been used as evidence that *P. aeruginosa* forms biofilms in the CF lung (213). Further, treatment with azithromycin, which interferes with quorum sensing signaling and alginate production (described below), helps clear *P. aeruginosa* from a mouse model of chronic lung infection (92).

The most prominent feature of *P. aeruginosa* infecting CF patients is its conversion to a mucoid phenotype, where it overproduces the exopolysaccharide alginate (78). Mucoid strains of *P. aeruginosa* have been associated with the establishment of the chronic phase of respiratory infections in CF (133), protection from phagocytosis (208), and protection from reactive oxygen intermediates (125, 212). Further, alginate overproduction has been shown to improve the resistance of *P. aeruginosa* to the innate clearance mechanisms in the lung of a mouse model of respiratory infection (22).

Other factors besides reduced bacterial clearing caused by high mucus viscosity and overproduction of alginate might predispose CF patients to chronic bacterial colonization. It has been proposed that CFTR functions as an epithelial cell receptor for *P. aeruginosa* being involved in bacterial internalization and clearance from the lung. The absence of apical CFTR in CF patients may be a critical factor for the hypersusceptibility of these patients to *P. aeruginosa* chronic lung infections (188, 189). The increased bacterial survival in the CF lung may in part be caused by impaired phagocytic killing by neutrophils due to the elevated chloride concentration (229). Finally, the ability of *P. aeruginosa* to grow anaerobically may be an important determinant in its ability to cause a chronic infection within the CF lung. *P. aeruginosa* CF lung infections are often modeled under aerobic laboratory conditions. However, direct oxygen measurements within the CF lung sputum *in situ* indicated that sputum contains hypoxic and potentially anaerobic regions (255). Additionally, strict anaerobic bacteria have been detected in sputum samples from multiple CF patients, suggesting that an environment capable of sustaining these species is present *in vivo* (104, 197, 235). The presence of antibodies to components of two putative anaerobic nitrate reductases of *P. aeruginosa* in sera of CF patients (15), suggests that the ability of *P. aeruginosa* to grow anaerobically may be an important adaptation during chronic CF infections. Beyond factors important in acute or chronic infections, there is another aspect of *P. aeruginosa* that plays an important role in general virulence; antibiotic resistance.

Antibiotic Resistance

P. aeruginosa has an intrinsic high resistance against most antibiotics, which significantly contributes to eradication failure, especially in patients with CF. Different mechanisms accounts for this inherent multi-drug resistance against a range of structurally and functionally different antibiotics such as chloramphenicol, kanamycin, imipenem, tetracycline and many dyes (113, 114, 152, 194).

First, the permeability of the *P. aeruginosa* cell membrane is 10 to 100 fold lower than for other gram-negative bacteria, and this protects it from harmful substances entering the cell (161). Other important factors adding to *P. aeruginosa*'s ability to tolerate high doses of antibiotics are the constitutively expressed multidrug efflux pumps such as MexAB-OprM and Mex pumps induced by the presence of antibiotics such as MexXY–OprM, MexCD–OprJ and MexEF–OprN (161, 191). Efflux is usually not considered to confer high levels of resistance, but it may narrow the choice antibiotics in the clinic (131). Induced efflux of antibiotics may also favor the emergence of target-specific mutations due to lowered intra-bacterial antibiotic concentrations (160). Predictions based on the sequenced PAO1 genome suggest that there may be as many as 30 efflux systems in *P. aeruginosa* (224).

In addition to efflux mechanisms, many clinical strains of *P. aeruginosa* produce enzymes that target the activity of antibiotics such as β-lactamase and aminoglycoside acetyltransferase (47). *P. aeruginosa* can also develop high-level resistance by altering the targets of antibiotics. A number of clinical strains have acquired a novel enzyme that can methylate the 16S rRNA subunit, resulting in resistance to various aminoglycosides that inhibit protein synthesis (50). Furthermore, *P. aeruginosa* frequently develops high-level resistance to fluoroquinolones by acquiring mutation in genes encoding its targets (DNA gyrase and the topoisomerase IV) (119, 158, 261).

While antibiotic resistance is a major roadblock in the treatment of *P. aeruginosa* infections, relatively few novel antibiotics with antipseudomonal activity are currently being developed (74, 152). This is troubling, as infections caused by resistant strains are associated with a three-fold higher rate of mortality, a nine-fold higher rate of secondary bacteremia, and a two-fold increase in the length of hospital stay (74). Thus, besides the personal consequences for the patients, resistant strains also considerably increase healthcare costs. Unfortunately it seems that the frequencies of multidrug-resistant *P. aeruginosa* (defined as resistance to at least three main classes of antipseudomonal agents such as β-lactams, carbapenems, aminoglycosides and fluoroquinolones) are increasing worldwide, reaching frequencies of up to 20 % in intensive care units in the USA and above 30 % in Asia (149, 152, 167). As such, the development of new therapeutic strategies, including drugs acting on new targets is urgently needed (152).

Virulence Mechanisms

Extensive studies have shown that *P. aeruginosa* is armed with a large arsenal of virulence factors, which enabling it to colonize host tissue, avoid the innate immune system, intoxicate and damage host cells, and modulate adaptive immune mechanisms.

Secreted Proteins and Toxic Molecules

P. aeruginosa is able to secrete a variety of toxins, enzymes and reactive molecules that cause extensive damage to host tissues, as well as allow the bacteria to escape clearance via immune evasion (18). Exotoxin A is a cytotoxic enzyme, which is able to ADP-ribosylate the eukaryotic elongation factor II, thereby inhibiting protein synthesis within the target host cell leading to cell death (99). Expression of Exotoxin A (encoded by *toxA*) is complex and involves a cascade of regulators that results in the maximal production of Exotoxin A in the stationary growth phase. Besides being dependent on low iron concentration, the *toxA* expression is presumed to be regulated

by factors such as PvdS (pyoverdine sigma factor) and connected to cell density and growth phase by the *las* encoded quorum sensing system and *vfr* (71, 132, 168, 244).

The *P. aeruginosa* elastase, LasB, (also called pseudolysin) degrades matrix proteins including elastin, laminin, fibrin, and collagen types III and IV (16, 88, 205). Consequently, LasB has the capacity to cause extensive tissue damage. Elastase is also a potent anti-inflammatory factor in mouse models (258). Besides the destruction of structural proteins, elastase has the capacity to inactivate crucial immune system components such as immunoglobulin, serum complement factors and the α_1-proteinase inhibitor (87, 97, 156). Together with alkaline protease (described below), elastase inactivates the human cytokines interferon γ (INF-γ) and tumor necrosis factor α (TNF-α) (176).

Another protease, LasA, enhances the elastolytic activity of other elastases, including human leukocyte elastase and LasB, in a dose-dependent manner (184, 185). LasA, also known as staphylolysin, can lyse *S. aureus* cells by cleaving the peptidoglycan pentaglycine interpeptides (107). LasA enhances the shedding of syndecan-1 from the surface of epithelial cells (175). Shedding of cell surface molecules as soluble extracellular domains is one of the host responses activated during tissue injury, and it seems that *P. aeruginosa* exploits this mechanism to promote its establishment within the host (174). Together with LasB, LasA was one of the first factors of *P. aeruginosa* found to be controlled by quorum sensing (70, 123, 233).

The endoprotease PrpL has elastolytic activity (248). Besides cleaving elastin, PrpL is able to degrade casein and proteins of the transferrin family, including lactoferrin and decorin. PrpL contributes to *P. aeruginosa´*s ability to persist in a rat chronic pulmonary infection model (248). PrpL production is controlled by quorum sensing like most other secreted virulence factors of *P. aeruginosa* (7, 163).

Alkaline protease (AprA) is a metalloprotease and a member of the serralysin family. AprA has maximal enzymatic activity at alkaline pH, hence the name (170). AprA activity is controlled by AprI, a small protease inhibitor (AprI) (55). The inhibitory protein consists of 131 residues, including a signal peptide of 25 residues that is cleaved off during secretion into the periplasm. The inhibitor is entirely located in the periplasm of *P. aeruginosa*, where its presumed physiological function is to protect the periplasmic proteins against the secreted protease (55, 89). AprA, together with LasB, inhibits the activity of Natural Killer cells and reduces lymphocyte proliferation, probably via cleavage of IL-2 (181, 232). The two proinflammatory cytokines, IL-6 and IL-8 are degraded by the two proteases AprA and LasB (145).

The release of hydrogen peroxide and other reactive oxygen species (ROS) by phagocytes represents an important defense against bacterial infection. However, many bacterial pathogens, including *P. aeruginosa*, exploit superoxide dismutase and catalytic enzyme systems to survive the oxidative stress within the host. *P. aeruginosa* produces at least four catalases; KatA, KatB, KatE and KatN and two superoxide dismutases SodB and SodM (also known as SodA) (224). KatA and, to a lesser extent, KatB, but not KatE, have been shown to be required for resistance to peroxide and osmotic stresses in *P. aeruginosa* strain PA14 (127). Both superoxide dismutase and catalase have been found to be required for full virulence of *P. aeruginosa* in different *in vivo* models (100, 127).

A characteristic of *P. aeruginosa* compared to other fluorescent pseudomonads is the production of substantial quantities of the phenazine-derivative pyocyanin. The production of pyocyanin is controlled by quorum sensing, iron and phosphate availability (103). Pyocyanin displays redox activity and induces oxidative stress in cellular systems and thus has the potential to impair tissue repair (26). Pyocyanin has been isolated from sputum of infected patients at levels above 100 μM and is reported to modify several host cell responses including altering the platelet eicosanoid metabolism and inhibit prostacyclin release (106, 115, 157, 249). When pyocyanin is

reduced by the reaction with NAD(P)H, the reduced pigment immediately reacts with molecular oxygen and thereby produces superoxide and by dismutation, hydrogen peroxide (48). The ability of *P. aeruginosa* to generate phenazines is critical for pathogenicity in both a worm model (*Caenorhabditis elegans*) and a murine model of septicemia (136).

P. aeruginosa isolated from burn wounds produces high levels of hydrogen cyanide, suggesting that cyanide might play a role in *P. aeruginosa* septicemia in burn patients (130). This has been supported by the finding that cyanide is the mediating factor in the paralytic killing model of *C. elegans* by *P. aeruginosa* (69). The toxicity of the cyanide ion is caused by its ability to bind to cytochrome c oxidase, thereby inhibiting the enzyme and disrupting the electron transport chain. In *P. aeruginosa* hydrogen cyanide biosynthesis is catalyzed by the membrane-bound enzyme HCN synthase, which forms hydrogen cyanide and CO_2 from glycine (30). Besides being controlled by quorum sensing the synthesis of hydrogen cyanide seems to be partly regulated by oxygen availability via the anaerobic regulator ANR (183).

P. aeruginosa produces different lectins that contribute to virulence. The galactophilic lectin PA-IL was the first to be characterized. It is a broad-spectrum hemagglutinin and agglutinates all types of erythrocytes from humans and various animals, as well as thrombocytes and leukocytes. Lectins are presumed to participate in the initial infection process where they facilitate binding to the respiratory, gastrointestinal or other epithelial surfaces (75, 193). It has been demonstrated that quorum sensing controls expression of lectins (251).

Type II secretion

P. aeruginosa possesses a number of dedicated secretion mechanisms for delivering toxins and enzymes to the extracellular environment. One such system, the type II secretion system (T2SS), is conserved and found among a wide variety of bacterial species (62). Secretion via the T2SS is a

two-step process. First, initial inner membrane translocation of the secreted protein is achieved in a Sec-dependent manner. Once in the periplasm, the mature polypeptide is then a substrate for the type II secretion machinery, which is able to discriminate between exoproteins or periplasmic/outer membrane proteins (62).

P. aeruginosa contains two independent T2SSs: Xcp and Hxc. Both systems are comprised of 11 proteins each (XcpP to XcpZ, HxcP to HxcZ). The XcpT, XcpU, XcpV, and XcpW proteins (and presumably their Hxc counterparts) are synthesized as precursor proteins with a short, positively charged leader peptide followed by a hydrophobic domain, a conserved feature that is shared with pilin (PilA) the major structural subunit of the Type IV pilus (225). Thus, these proteins are referred to as pilin-like proteins. Proteolytic processing of the pilin-like proteins to their mature and functional form requires the presence of a prepilin peptidase PilD (XcpA) (9, 62, 165). Xcp pilin-like proteins are mainly localized in the cell envelope of *P. aeruginosa*, though this localization was found to change under physiological conditions (11, 166). XcpR is a putative ATP-binding protein that is required for Xcp-mediated secretion (200, 237). XcpQ localizes to the outer membrane where it forms a ring-shaped structure consisting of 12 to 15 subunits with a central opening through which exoproteins may be secreted to the extracellular environment (19, 80). As with many of the secreted proteins described above, expression of the *xcp* genes is under the positive control of quorum sensing (32, 242). The Xcp system is required for secretion of the majority of the proteins describe above, including exotoxin A, elastase (LasB), LasA, PrpL and alkaline protease (AprA). In contrast, Hxc is functional only under phosphate limitation, and was described to secrete one enzyme, phosphatase LapA (9, 62).

type III secretion

In addition to extracellular proteins and type II-mediated secretion, *P. aeruginosa* uses a type III secretion system (T3SS) to promote severe illness via secretion of effector molecules directly into

the cytoplasm of target cells. type III secretion plays a role both in antiphagocytosis and in dissemination through effector-mediated damage to host cells (117). Four effector molecules have been described in *P. aeruginosa*: ExoS, ExoT, ExoU, and ExoY. ExoU is a cytotoxin with phospholipase A2 activity (186, 204). ExoS and ExoT are bifunctional enzymes that encode both GTPase-activating properties and ADP-ribosyltransferase activity (76, 111). ExoY is a cytotoxin with adenylate cyclase activity (259). The importance of these effector molecules, as well as their roles in virulence, has been established using mutants with specific disruptions in genes encoding portions of the type III secretion apparatus (219).

Deletion of *exoU* significantly reduces virulence and development of pathology in the lung (63, 84). ExoS has an intermediate effect on pathogenesis in a mouse model of acute pneumonia due to its ADP-ribosyltransferase activity (209). ExoS and ExoT inhibit phagocytosis by disrupting signal transduction cascades, focal adhesins, and preventing cytoskeletal rearrangement (12). Expression of the T3SS genes is induced upon intimate contact of *P. aeruginosa* with host cells *in vivo* and low extracellular calcium concentrations *in vitro* (66, 238). Attachment of *P. aeruginosa* to host cells via type IV pili is a prerequisite for contact-dependent activation of the T3SS (227).

Alginate

One distinctive feature of *P. aeruginosa* pathogenicity is the existence of the mucoid phenotype, a condition where bacterial cells overproduce the extracellular polysaccharide alginate during chronic colonization of the CF airways. Presumably, alginate can be expressed at low levels under currently unidentified conditions, yet hyper-production of alginate and the resulting mucoid phenotype is only known to occur in the CF lung. The mucoid phenotype results from mutation in the *mucA* gene (22), which is located within a gene cluster (*algU mucABCD*) that appears to encode regulators of alginate production in *P. aeruginosa* (78). These regulators form a signal detection system that senses stressful conditions in the surrounding environment, including situations of

anaerobic and oxidative stress (78, 154). The *mucA* gene codes an anti-sigma factor of AlgU, while AlgU is an alternative sigma factor that directs the transcription of alginate biosynthetic genes at the *algD* promoter (142, 143, 207). Mutations in *mucA* are specifically enriched within the CF lung and lead to a cascade of events resulting in overproduction of alginate and a mucoid phenotype (22).

It has been suggested that alginate may enhance *P. aeruginosa* virulence or survival in several ways (78). First, alginate serves as a barrier for bacterial cells against phagocytes and antibodies. Alginate was found to interfere with both opsonic phagocytosis and nonopsonic phagocytosis (116, 153, 211). It is also believed that alginate is capable of quenching oxidative radicals released by phagocytic cells (125). Second, alginate may function as an immunomodulatory molecule. It has been reported that alginate, when used at low doses (close to physiological concentration), can stimulate the oxidative burst of neutrophils, while at high concentrations it has an inhibitory effect (108, 125). Alginate also suppresses lymphocyte functions and can act as a mitogen together with LPS, which may contribute to hypergammaglobulinemia associated with CF exacerbation (41, 139). Finally, alginate may play an important role in biofilm formation associated with chronic CF lung infection (256).

Biofilms

An important factor contributing to *P. aeruginosa* pathogenesis in clinical settings is the biofilm mode of growth involved in chronic as well as in acute infections (206). The Centers for Disease Control and Prevention (CDC) and National Institute of Health (NIH) estimate that 65-80 % of all human bacterial infections involve biofilms. Biofilms are defined as sessile microbial communities, characterized by cells that are irreversibly attached to a substratum or interface or to each other, and embedded in a matrix of self-produced extracellular polymeric substances (EPS). Biofilms exhibit an altered phenotype with respect to growth rate and gene transcription (52). The biofilm consists of heterogeneously distributed bacterial cells (cells constitute 5-20 % of the biofilm

volume) embedded in an EPS matrix. The exact composition of the matrix depends on the phenotype of the bacteria producing the EPS. Mucoid *P. aeruginosa* strains overproduce the exopolysaccharide alginate (composed of uronic acid β-D-mannuronate and its C-5 epimer α-L-guluronate), whereas in the non-mucoid matrix the predominant exopolysaccharides are polymers of glucose and rhamnose (256). Also extracellular products such as proteins, nucleic acids, metabolites and absorbed nutrients are parts of the biofilm matrix (96, 247).

Biofilm development (**Figure 1.1**) begins from individual planktonic cells, which initiate interactions with a surface (biotic or abiotic) in response to cues such as bacterial cell density, nutrient availability, and energy sources present in the environment (172, 192). Functional flagella are important for this initial stage of development, as a mutation in *flgK* has been shown to prevent attachment of planktonic cells to an abiotic surface (171). Once attached to the surface, *P. aeruginosa* cells begin to attach to each other and form small microcolonies, a process that requires functional type IV pili (171). The development of microcolonies gives the biofilm a three-dimensional structure with distinct architectural and physical/chemical properties (38). Further, biofilm-grown cells differ from planktonic cells in increased expression of exopolysaccharides (44, 45) and in increased resistance to antibiotics (98). In contrast to surface-attached biofilms, *P. aeruginosa* forms "raft-like" biofilms in dehydrated CF mucus (255). It has been established that *P. aeruginosa* biofilms in CF are anaerobic given the low oxygen tension of the mucus environment (4, 255, 262). Once the structured biofilm is formed, bacterial cells can be actively dispersed back into the environment through a process known as "seeding dispersal" (195). This dispersal mechanism is characterized by "hollowing" of the mature biofilm, which is caused by highly motile cells departing the interior of the structure and cell death in a subpopulation of the interior cells (195). Significantly, *P. aeruginosa* released by seeding dispersal exhibit phenotype and functional diversification, with some variants showing an increased ability to disseminate while others manifest accelerated biofilm development (110, 243).

Common components of *P. aeruginosa* biofilms are the polysaccharides, of which there are three sub-types: Pel, Psl, and Alginate. Pel was originally identified from a PA14 transposon library as being necessary for maintenance of biofilm structure (67). While mutation of the *pel* genes did not affect biofilm initiation, colony morphology and the ability of the mutants to bind Congo Red was affected (67). Further analysis demonstrated the requirement for the *pel* genes for biofilm initiation and structure in a pilus-deficient strain, indicating Pel might play a similar role to type IV pili in the development of small microcolonies (240). The Pel polysaccharide is biochemically and genetically distinct from Psl. Psl is a second biofilm-associated polysaccharide that is essential for biofilm formation in strains PAO1 and ZK2870 (102, 67, 134, 146). Inactivation of the *psl* cluster of genes leads to a significant defect in cell-to-surface and cell-to-cell interactions. Additionally, Psl is required for adherence to mucin-coated surfaces and airway epithelial cells, both of which may be relevant in the CF lung (134). After attachment, Psl was shown to be required for the maintenance of biofilm structure, indicating this polysaccharide might play a role in holding biofilms together (134). While both Psl and Pel are required for biofilm development, there is no data as to their contribution to virulence. As mentioned above, alginate plays a distinct role in the virulence of *P. aeruginosa* in the CF lung. In fact, there is a distinct correlation between the appearance of alginate overproducing *P. aeruginosa* and a worsening clinical prognosis for CF patients (78). However, studies have shown that alginate is not required for biofilm development *in vitro* (162, 221, 256). Alginate-producing and -deficient *P. aeruginosa* strains form morphologically similar biofilms with differing antibiotic resistance characteristics.

P. aeruginosa cells growing as biofilms tolerate 100-1000 times more antibiotics than their planktonic counterparts (6, 31, 98). However, most of the studies reporting elevated resistance of biofilm cells describe an increased tolerance to killing rather than growth inhibition (220). The mechanisms for the increased tolerance of biofilm cells towards antibiotics are not fully elucidated. Several factors may contribute to the protection of cells in the biofilm mode of growth, perhaps also

depending on the properties of various antibiotics. The matrix is assumed to reduce the diffusion of some antibiotics in a rate depending on the nature of the applied compounds and the biofilm matrix composition (135). Decreased diffusion combined with a rapid (enzymatic) degradation of the antimicrobial compound could inhibit the penetration of active antibiotics (5). Finally, the accumulation of waste products or the depletion of oxygen and/or nutrients within the biofilm may cause the bacteria to enter a non-growing state, and thereby protect them against killing by antibiotics that require active growth to be effective (236). When the antibiotic treatment is ended, surviving cells can proliferating and restore the biofilm population. This is reasonable given that the switch to a biofilm mode of growth is considered to be a survival strategy for the bacterium (96).

Quorum sensing

In order to be able to survive under changing conditions and in hostile environments, bacteria have evolved systems to monitor and respond appropriately to environmental stimuli such as nutrients availability, stress and, for some bacteria, cell density. The latter depends on small diffusible signaling molecules constitutively produced by the bacteria, which interact with transcriptional activators to couple gene expression with cell population density. This enables the bacteria to function as a group and to some degree exhibit multicellular behavior. This form of cell to cell communication is termed quorum sensing. In Gram-negative bacteria this phenomenon is mainly mediated by *N*-acyl L-homoserine lactones (AHLs), consisting of a conserved homoserine lactone ring with an *N*-acyl side chain. AHL structures vary based on the presence or absence of a keto- or hydroxy- group on the C3 of the acyl chain and the length and saturation of this chain.

Two AHL-mediated quorum sensing circuits have been identified in *P. aeruginosa*: the LasR-LasI and the RhlR-RhlI system. The final step in the synthesis of OdDHL (*N*-3-oxododecanoyl-L-homoserine lactone) and BHL (*N*-butanoyl-L-homoserine lactone) are mediated by LasI and RhlI, respectively. Each system modulates a regulon comprising an overlapping set of genes. However, it

is presumed that a regulatory hierarchy exists in which LasR-OdDHL activates the transcription of *rhlR* (122). Hence, genes controlled by the RhlI-RhlR system require a functional LasI-LasR system for full activation. Under phosphate-limiting conditions various transcriptional activators, including the global regulatory gene Vfr, the alternative sigma factor (σ^{54}) and RhlR itself participate in the regulation of *rhlR* expression (150). In a heterologous genetic background (*E. coli*), OdDHL is able to compete with BHL for the binding site of RhlR, and thus inhibit the translation of RhlR activated genes, indicating that LasI, through OdDHL, controls RhlR activity at a posttranslational level (182). In addition, RhlR forms homodimers both in the presence and absence of BHL, whereas OdDHL induce monomerization of the RhlR homodimer (241). LasR forms multimers only in the presence of OdDHL and this form is presumed to be the one responsible for its ability to function as a transcriptional activator (21, 109).

Both LasRI and RhlRI function in concert to control the expression of an array of genes, many of which encode known virulence factors (71, 123, 177). LasRI regulates the production of elastase, alkaline protease, and toxin A, while RhlRI regulates the production of rhamnolipid, pyocyanin, cyanide, lipase, alkaline protease, and elastase (26, 71, 123, 169, 179, 180). A third LasR/RhlR homolog in *P. aeruginosa*, QscR, induces hypervirulence when mutated (35). Activation of virulence factors by the quorum sensing systems is crucial for successful virulence of *P. aeruginosa* in a mouse model of acute pneumonia (128, 178). Further, the quorum sensing molecules themselves have the ability to modulate the immune system. *P. aeruginosa* AHL can induce COX-2 production through activation of NF-κB, leading to the induction of mucus secretion, vasodilation, and edema (218). AHL molecules can also induce apoptosis of neutrophils and macrophages (230), and can increase the production of inflammatory cytokines from airway cells and macrophages (215, 217, 231).

In addition to acute infections, quorum sensing also plays a role in the successful establishment of chronic infections in the CF lung. *P. aeruginosa* quorum sensing is active in the CF lungs, as both quorum sensing systems are up regulated in CF samples (223) and AHLs are detected in both sputum samples and lung extracts from CF patients (58, 73). Quorum sensing affects both the development of *P. aeruginosa* biofilms and the tolerance of these biofilms to antibiotics (46, 90). In fact, the ratio of AHLs detected in the CF lung has been used to support the hypothesis that *P. aeruginosa* is present in the lungs in a biofilm (213). Disruption of quorum sensing, specifically mutations in *lasI* or *rhlI*, result in *P. aeruginosa* that are less virulent than wild type in an animal model of chronic lung infection (93, 257). Combined with the data that suggests AHL receptors (*lasR*/*rhlR*) are necessary for successful colonization of the lung (20), these results seem to indicate that a functional QS system is critical for the establishment of chronic infection by *P. aeruginosa* (34). This would seem to be an inaccurate claim given the frequency of QS mutants obtained from the CF lung (3% to 50%) (91), or the evidence that whole-genome sequencing of early- and late-stage isolates of *P. aeruginosa* from a CF patient showed that *lasR* was inactivated in the late-stage infection isolates (216). However, these results can be accounted for by data demonstrating a mutation in *lasR* from *P. aeruginosa* after over 100 generations can give the bacterium a growth advantage over other cells in the same culture (201). Additionally, these mutants are able to use the quorum sensing molecules produced by other cells in population to continue QS gene expression (201).

Flagellum

Another group of virulence factors are attachment and motility organelles, including flagella (swimming) and Type IV pili (twitching). These factors give the bacterium the ability to attach to host cells and maneuver around the host environment, facilitating colonization and immune avoidance (8, 78, 140, 147). *P. aeruginosa* usually possesses a single polar flagellum, responsible for the

primary function of bacterial motility (swimming). A non-flagellated mutant was defective in various infection and invasion studies (53, 65, 155). Interestingly, the flagellar cap protein FliD is involved in the adherence of *P. aeruginosa* to the human respiratory mucin, which is important for the initial colonization of the CF airways (8).

Beyond movement and adherence, the major component of the flagellum, flagellin, is a known Pathogen Associated Pattern Recognition Molecule (PAMP) which can promote inflammation during *P. aeruginosa* infection by signaling through TLR5 (10, 86). In fact, the absence of motility does not affect the LD_{50} of a mouse model of acute pneumonia, while production of excess amounts of flagellin lowers the LD_{50}. Given this role as a potent stimulator of the immune system, it is not surprising that flagellin expression is lost during chronic infection (59, 137, 253).

Type IV pili

Type IV pili (TFP) also are polarly localized motility and adherence organelles in *P. aeruginosa.* TFP are responsible for a specialized form of movement known as twitching motility, specific adherence to eukaryotic host cells, and nonspecific binding to other surfaces where these pili could mediate intimate contact and bacterial colonization (147). The *pilA* gene codes for the major structural subunit of TFP in *P. aeruginosa*, and is essential for Type IV pilus expression and function (245). Pilin (PilA) is transcribed as a pre-protein and contains an amino-terminal short leader sequence that is cleaved prior to assembly of the mature protein into the pilus fiber. Cleavage of the leader sequence is carried out by the prepilin peptidase PilD (226). PilD is also required for processing the structurally related Xcp pilin-like proteins involved in type II secretion (described above). The resulting mature pilin protein contains an extended, highly conserved, and hydrophobic N-terminal α-helical region followed by a globular C-terminal domain terminating in a disulphide-bonded loop (DSL) (40, 173). The DSL varies in size between different pilin alleles (120). The mature pilin subunits are predicted to assemble such that the N-terminal α-helical regions form the

core of the pilus structure, with the globular C-terminal domains on the outside and exposed to the environment (28). Assembly of pilin subunits occurs on the periplasmic face of the inner membrane and is an energy dependent process that requires the ATPase PilB. As the pilus fiber grows it passes through a pore in the outer membrane. The pore is composed of PilQ, a protein that belongs to the super family of secretins, which forms a hexameric ring-like structure (36, 252) (**Figure 1.2**). PilB is an ATPase that is localized to the poles of the inner membrane of *P. aeruginosa*, where it is involved in the assembly of the pilin monomers into the mature pilus structure (33, 148). A homolog of PilB, PilT is another TFP-associated ATPase in *P. aeruginosa* that is involved in disassembly and subsequent retraction of the pilus fiber (245). Loss-of-function mutations in *pilB* result in a non-piliated phenotype (164). Conversely, a loss-of-function mutation in *pilT* results in hyperpiliation, a loss in the ability of the bacteria to undergo twitching motility on a solid surface (246), defective type III secretion dependent cytotoxicity *in vitro*, and reduced virulence *in vivo* (37). This reduction in virulence is likely due to a defect in dissemination, as *pilT* mutants are able to efficiently infect mouse lungs, but are unable to spread to peripheral organs (263).

TFP are required for *P. aeruginosa* adherence to host cells and tissue (51). Currently, pilin is believed act as both the structural subunit and the adhesin. The *in vitro* binding of TFP to asialo-G_{M1} and asialo-G_{M2} implicated the exposed C-terminal disulfide-bonded loop, located within residues 128-144 of the pilin monomer on the tip of the pilus fiber, as the TFP-associated adhesin (27, 51, 101, 199). However, the nature of *P. aeruginosa* binding to epithelial cells, as well as recent work demonstrating the lack of live *P. aeruginosa* bacteria binding to asialo-G_{M1}/G_{M2}, have raised questions as to the role of pilin in TFP-mediated adherence (28). In response to this new data, we have hypothesized that another pilus-associated protein (PilY1) is the pilus-associated adhesin.

The PilY1 gene was originally identified in a transposon screen (3) as being required for twitching motility and surface TFP expression in *P. aeruginosa*. *pilY1* is located in an operon with

six other TFP-associated genes (*fimUpilVWXY1Y2E*). The PilY1 protein is approximately 117 kDa and localizes to sheared pilus fractions (3); however, little is known about the role PilY1 plays in *P. aeruginosa* TFP biogenesis. Interestingly, the C-terminus of PilY1 shares 27% amino acid identity with the C-terminus of PilC2 from *Neisseria gonorrhoeae.* PilC2 has been identified as the TFP-associated adhesion in *N. gonorrhoeae* due to its ability to bind cells of human origin, and competitively block the adherence of piliated gonococci (198).

TFP assembly requires the presence of both cAMP and the global transcription factor, Vfr (13, 105). Vfr is a homologue of the *E. coli* catabolite repressor protein CRP (cAMP receptor protein), and has been shown to bind to a CRP-binding consensus sequence (CCS) located at the promoter region of *lasR* and to positively regulate expression of exotoxin A and protease production (2, 244). In addition to Vfr, the two-component regulatory system AlgZ/AlgR is required for TFP assembly in *P. aeruginosa*. Phosphorylation of AlgR results in activation of the *fimU-pilVWXY1Y2E* operon and, consequently, biogenesis of a functional pilus structure (17). While it has been demonstrated that pilin expression is controlled by PilS/PilR (24, 25) and RpoN (234), the effects of AlgZ/AlgR and of cAMP/Vfr are specific to the *pilY1* operon (17, 105). This would suggest that there are multiple modes of TFP regulation and that, under certain conditions, *pilY1* expression can be regulated independent of pilin.

The aim of the work presented in this document was to further characterize the role of PilY1 in *P. aeruginosa* TFP biogenesis and function. How does PilY1 control TFP expression? Given the homology between PilY1 and PilC in *N. gonorrhoeae*, could PilY1 act as the TFP-associated adhesin in *P. aeruginosa*? Answers to both questions would not only lead to further insight into the mechanism of TFP biogenesis, but could also provide a novel target for inhibiting *P. aeruginosa* colonization of epithelial surfaces and subsequent infection.

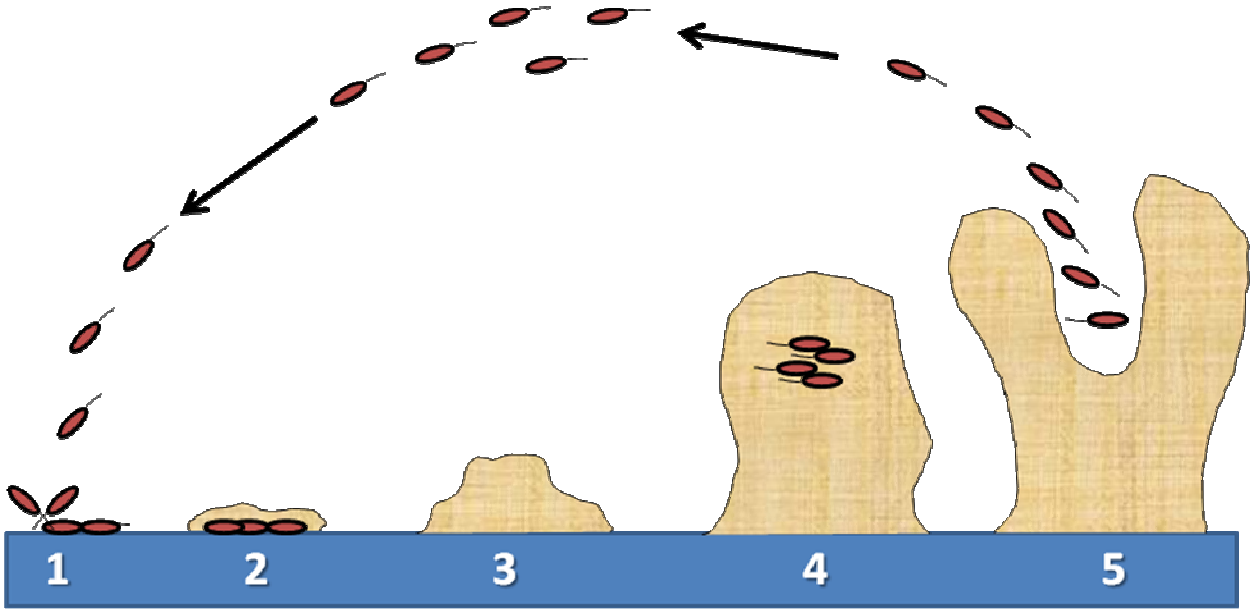

Figure 1.1. Biofilm development in *Pseudomonas aeruginosa*. 1) Initial attachment to substratum, **2)** the bacteria adhere to each other **3)** forming small microcolonies, **4)** the three-dimensional structure of the mature biofilm develops, and **5)** cells are released (sloughing off) and re-colonize new areas of substratum via "seeding dispersal". (Adapted from graphic by Peg Dirckx and David Davies, Center for Biofilm Engineering, Montana State University, Bozeman.)

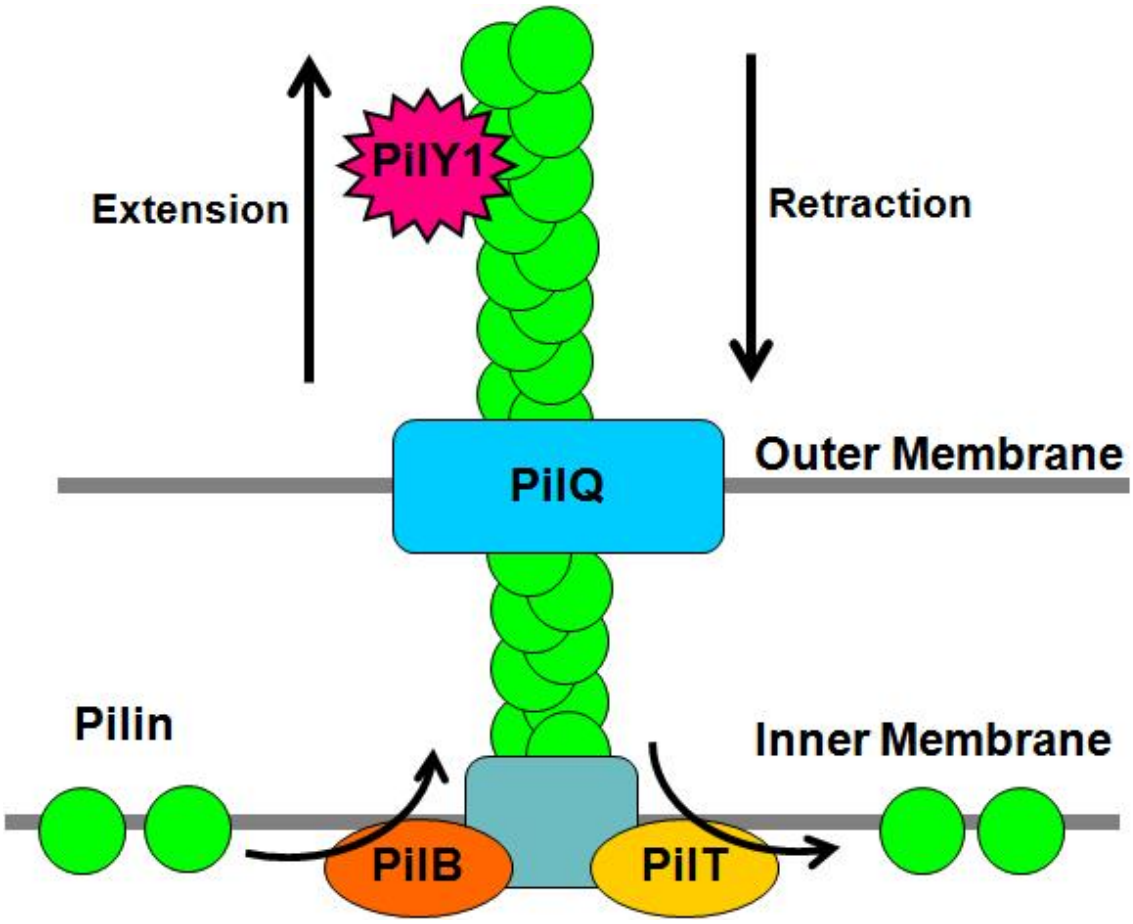

Figure 1.2. Overview of *Pseudomonas aeruginosa* Type IV pili. Pilin subunits (green circles) are localized to the inner membrane after cleavage of their leader sequence by PilD (not pictured). The pilin subunits are then assembled into a mature pilus structure through the actions of the ATPase PilB (orange). The assembled fiber extends through the outer membrane via a PilQ pore, and can be retracted by the PilT-dependant removal of pilin subunits from the base of the assembled fiber. PilY1 (pink) associates with the mature pilus fiber, but it is unclear how it is localized to this structure from the cytoplasm.

References

1. **Alarcon, I., D.J. Evans, and S.M. Fleiszig**. 2009. Twitching Motility is Required for *Pseudomonas aeruginosa* to Exit Corneal Epithelial Cells After Internalization and to Translocate a Multilayered Epithelium. Invest Ophthalmol Vis Sci.

2. **Albus, A.M., E.C. Pesci, L.J. Runyen-Janecky, S.E. West, and B.H. Iglewski**. 1997. Vfr controls quorum sensing in *Pseudomonas aeruginosa.* J Bacteriol **179**(12): p. 3928-35.

3. **Alm, R.A. and J.S. Mattick**. 1996. Identification of two genes with prepilin-like leader sequences involved in type 4 fimbrial biogenesis in *Pseudomonas aeruginosa.* J Bacteriol **178**(13): p. 3809-17.

4. **Alvarez-Ortega, C. and C.S. Harwood**. 2007. Responses of *Pseudomonas aeruginosa* to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. Mol Microbiol **65**(1): p. 153-65.

5. **Anderl, J.N., M.J. Franklin, and P.S. Stewart**. 2000. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrob Agents Chemother **44**(7): p. 1818-24.

6. **Anwar, H., M.K. Dasgupta, and J.W. Costerton**. 1990. Testing the susceptibility of bacteria in biofilms to antibacterial agents. Antimicrob Agents Chemother **34**(11): p. 2043-6.

7. **Arevalo-Ferro, C., M. Hentzer, G. Reil, A. Gorg, S. Kjelleberg, M. Givskov, K. Riedel, and L. Eberl**. 2003. Identification of quorum-sensing regulated proteins in the opportunistic pathogen *Pseudomonas aeruginosa* by proteomics. Environ Microbiol **5**(12): p. 1350-69.

8. **Arora, S.K., B.W. Ritchings, E.C. Almira, S. Lory, and R. Ramphal**. 1998. The *Pseudomonas aeruginosa* flagellar cap protein, FliD, is responsible for mucin adhesion. Infect Immun **66**(3): p. 1000-7.

9. **Ball, G., E. Durand, A. Lazdunski, and A. Filloux**. 2002. A novel type II secretion system in *Pseudomonas aeruginosa.* Mol Microbiol **43**(2): p. 475-85.

10. **Balloy, V., A. Verma, S. Kuravi, M. Si-Tahar, M. Chignard, and R. Ramphal**. 2007. The role of flagellin versus motility in acute lung disease caused by *Pseudomonas aeruginosa.* J Infect Dis **196**(2): p. 289-96.

11. **Bally, M., A. Filloux, M. Akrim, G. Ball, A. Lazdunski, and J. Tommassen**. 1992. Protein secretion in *Pseudomonas aeruginosa*: characterization of seven *xcp* genes and processing of secretory apparatus components by prepilin peptidase. Mol Microbiol **6**(9): p. 1121-31.

12. **Barbieri, J.T. and J. Sun**. 2004. *Pseudomonas aeruginosa* ExoS and ExoT. Rev Physiol Biochem Pharmacol **152**: p. 79-92.

13. **Beatson, S.A., C.B. Whitchurch, J.L. Sargent, R.C. Levesque, and J.S. Mattick**. 2002. Differential regulation of twitching motility and elastase production by Vfr in *Pseudomonas aeruginosa.* J Bacteriol **184**(13): p. 3605-13.

14. **Beattie, B.K. and A.R. Merrill**. 1996. *In vitro* enzyme activation and folded stability of *Pseudomonas aeruginosa* exotoxin A and its C-terminal peptide. Biochemistry **35**(28): p. 9042-51.

15. **Beckmann, C., M. Brittnacher, R. Ernst, N. Mayer-Hamblett, S.I. Miller, and J.L. Burns**. 2005. Use of phage display to identify potential *Pseudomonas aeruginosa* gene products relevant to early cystic fibrosis airway infections. Infect Immun **73**(1): p. 444-52.

16. **Bejarano, P.A., J.P. Langeveld, B.G. Hudson, and M.E. Noelken**. 1989. Degradation of basement membranes by *Pseudomonas aeruginosa* elastase. Infect Immun **57**(12): p. 3783-7.

17. **Belete, B., H. Lu, and D.J. Wozniak**. 2008. *Pseudomonas aeruginosa* AlgR regulates type IV pilus biosynthesis by activating transcription of the *fimU-pilVWXY1Y2E* operon. J Bacteriol **190**(6): p. 2023-30.

18. **Bitter, W.** 2003. Secretins of *Pseudomonas aeruginosa*: large holes in the outer membrane. Arch Microbiol **179**(5): p. 307-14.

19. **Bitter, W., M. Koster, M. Latijnhouwers, H. de Cock, and J. Tommassen**. 1998. Formation of oligomeric rings by XcpQ and PilQ, which are involved in protein transport across the outer membrane of *Pseudomonas aeruginosa.* Mol Microbiol **27**(1): p. 209-19.

20. **Bjarnsholt, T.*, et al.*** 2005. *Pseudomonas aeruginosa* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. Microbiology **151**(Pt 2): p. 373-83.

21. **Bottomley, M.J., E. Muraglia, R. Bazzo, and A. Carfi**. 2007. Molecular insights into quorum sensing in the human pathogen *Pseudomonas aeruginosa* from the structure of the virulence regulator LasR bound to its autoinducer. J Biol Chem **282**(18): p. 13592-600.

22. **Boucher, J.C., H. Yu, M.H. Mudd, and V. Deretic**. 1997. Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: characterization of *muc* mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. Infect Immun **65**(9): p. 3838-46.

23. **Boucher, R.C.** 2004. New concepts of the pathogenesis of cystic fibrosis lung disease. Eur Respir J **23**(1): p. 146-58.

24. **Boyd, J.M., T. Koga, and S. Lory**. 1994. Identification and characterization of PilS, an essential regulator of pilin expression in *Pseudomonas aeruginosa.* Mol Gen Genet **243**(5): p. 565-74.

25. **Boyd, J.M. and S. Lory**. 1996. Dual function of PilS during transcriptional activation of the *Pseudomonas aeruginosa* pilin subunit gene. J Bacteriol **178**(3): p. 831-9.

26. **Brint, J.M. and D.E. Ohman**. 1995. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. J Bacteriol **177**(24): p. 7155-63.

27. **Bryan, R., D. Kube, A. Perez, P. Davis, and A. Prince**. 1998. Overproduction of the CFTR R domain leads to increased levels of asialoGM1 and increased *Pseudomonas aeruginosa* binding by epithelial cells. Am J Respir Cell Mol Biol **19**(2): p. 269-77.

28. **Burrows, L.L.** 2005. Weapons of mass retraction. Mol Microbiol **57**(4): p. 878-88.

29. **Carmeli, Y., N. Troillet, G.M. Eliopoulos, and M.H. Samore**. 1999. Emergence of antibiotic-resistant *Pseudomonas aeruginosa*: comparison of risks associated with different antipseudomonal agents. Antimicrob Agents Chemother **43**(6): p. 1379-82.

30. **Castric, P.A.** 1977. Glycine metabolism by *Pseudomonas aeruginosa*: hydrogen cyanide biosynthesis. J Bacteriol **130**(2): p. 826-31.

31. **Ceri, H., M.E. Olson, C. Stremick, R.R. Read, D. Morck, and A. Buret**. 1999. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol **37**(6): p. 1771-6.

32. **Chapon-Herve, V., M. Akrim, A. Latifi, P. Williams, A. Lazdunski, and M. Bally**. 1997. Regulation of the *xcp* secretion pathway by multiple quorum-sensing modulons in *Pseudomonas aeruginosa*. Mol Microbiol **24**(6): p. 1169-78.

33. **Chiang, P., M. Habash, and L.L. Burrows**. 2005. Disparate subcellular localization patterns of *Pseudomonas aeruginosa* Type IV pilus ATPases involved in twitching motility. J Bacteriol **187**(3): p. 829-39.

34. **Christensen, L.D., C. Moser, P.O. Jensen, T.B. Rasmussen, L. Christophersen, S. Kjelleberg, N. Kumar, N. Hoiby, M. Givskov, and T. Bjarnsholt**. 2007. Impact of *Pseudomonas aeruginosa* quorum sensing on biofilm persistence in an *in vivo* intraperitoneal foreign-body infection model. Microbiology **153**(Pt 7): p. 2312-20.

35. **Chugani, S.A., M. Whiteley, K.M. Lee, D. D'Argenio, C. Manoil, and E.P. Greenberg**. 2001. QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A **98**(5): p. 2752-7.

36. **Collins, R.F., L. Davidsen, J.P. Derrick, R.C. Ford, and T. Tonjum**. 2001. Analysis of the PilQ secretin from *Neisseria meningitidis* by transmission electron microscopy reveals a dodecameric quaternary structure. J Bacteriol **183**(13): p. 3825-32.

37. **Comolli, J.C., A.R. Hauser, L. Waite, C.B. Whitchurch, J.S. Mattick, and J.N. Engel**. 1999. *Pseudomonas aeruginosa* gene products PilT and PilU are required for cytotoxicity in vitro and virulence in a mouse model of acute pneumonia. Infect Immun **67**(7): p. 3625-30.

38. **Costerton, J.W., Z. Lewandowski, D.E. Caldwell, D.R. Korber, and H.M. Lappin-Scott**. 1995. Microbial biofilms. Annu Rev Microbiol **49**: p. 711-45.

39. **Costerton, J.W., P.S. Stewart, and E.P. Greenberg**. 1999. Bacterial biofilms: a common cause of persistent infections. Science **284**(5418): p. 1318-22.

40. **Craig, L.*, et al.*** 2003. Type IV pilin structure and assembly: X-ray and EM analyses of *Vibrio cholerae* toxin-coregulated pilus and *Pseudomonas aeruginosa* PAK pilin. Mol Cell **11**(5): p. 1139-50.

41. **Daley, L., G.B. Pier, J.D. Liporace, and D.D. Eardley**. 1985. Polyclonal B cell stimulation and interleukin 1 induction by the mucoid exopolysaccharide of *Pseudomonas aeruginosa* associated with cystic fibrosis. J Immunol **134**(5): p. 3089-93.

42. **Daltrey, D.C., B. Rhodes, and J.G. Chattwood**. 1981. Investigation into the microbial flora of healing and non-healing decubitus ulcers. J Clin Pathol **34**(7): p. 701-5.

43. **D'Argenio, D.A., L.A. Gallagher, C.A. Berg, and C. Manoil**. 2001. Drosophila as a model host for *Pseudomonas aeruginosa* infection. J Bacteriol **183**(4): p. 1466-71.

44. **Davies, D.G., A.M. Chakrabarty, and G.G. Geesey**. 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa.* Appl Environ Microbiol **59**(4): p. 1181-6.

45. **Davies, D.G. and G.G. Geesey**. 1995. Regulation of the alginate biosynthesis gene *algC* in *Pseudomonas aeruginosa* during biofilm development in continuous culture. Appl Environ Microbiol **61**(3): p. 860-7.

46. **Davies, D.G., M.R. Parsek, J.P. Pearson, B.H. Iglewski, J.W. Costerton, and E.P. Greenberg**. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280**(5361): p. 295-8.

47. **Davies, J. and G.D. Wright**. 1997. Bacterial resistance to aminoglycoside antibiotics. Trends Microbiol **5**(6): p. 234-40.

48. **Davis, G. and P.J. Thornalley**. 1983. Free radical production from the aerobic oxidation of reduced pyridine nucleotides catalysed by phenazine derivatives. Biochim Biophys Acta **724**(3): p. 456-64.

49. **Diaz, M.H., C.M. Shaver, J.D. King, S. Musunuri, J.A. Kazzaz, and A.R. Hauser**. 2008. *Pseudomonas aeruginosa* induces localized immunosuppression during pneumonia. Infect Immun **76**(10): p. 4414-21.

50. **Doi, Y. and Y. Arakawa**. 2007. 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. Clin Infect Dis **45**(1): p. 88-94.

51. **Doig, P., T. Todd, P.A. Sastry, K.K. Lee, R.S. Hodges, W. Paranchych, and R.T. Irvin**. 1988. Role of pili in adhesion of *Pseudomonas aeruginosa* to human respiratory epithelial cells. Infect Immun **56**(6): p. 1641-6.

52. **Donlan, R.M. and J.W. Costerton**. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev **15**(2): p. 167-93.

53. **Drake, D. and T.C. Montie**. 1988. Flagella, motility and invasive virulence of *Pseudomonas aeruginosa.* J Gen Microbiol **134**(1): p. 43-52.

54. **Driscoll, J.A., S.L. Brody, and M.H. Kollef**. 2007. The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. Drugs **67**(3): p. 351-68.

55. **Duong, F., A. Lazdunski, B. Cami, and M. Murgier**. 1992. Sequence of a cluster of genes controlling synthesis and secretion of alkaline protease in *Pseudomonas aeruginosa*: relationships to other secretory pathways. Gene **121**(1): p. 47-54.

56. **El Solh, A.A., M.E. Akinnusi, J.P. Wiener-Kronish, S.V. Lynch, L.A. Pineda, and K. Szarpa**. 2008. Persistent infection with *Pseudomonas aeruginosa* in ventilator-associated pneumonia. Am J Respir Crit Care Med **178**(5): p. 513-9.

57. **Engel, J. and P. Balachandran**. 2009. Role of *Pseudomonas aeruginosa* type III effectors in disease. Curr Opin Microbiol **12**(1): p. 61-6.

58. **Erickson, D.L., R. Endersby, A. Kirkham, K. Stuber, D.D. Vollman, H.R. Rabin, I. Mitchell, and D.G. Storey**. 2002. *Pseudomonas aeruginosa* quorum-sensing systems may control virulence factor expression in the lungs of patients with cystic fibrosis. Infect Immun **70**(4): p. 1783-90.

59. **Ernst, R.K., E.C. Yi, L. Guo, K.B. Lim, J.L. Burns, M. Hackett, and S.I. Miller**. 1999. Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa.* Science **286**(5444): p. 1561-5.

60. **Feldman, M., R. Bryan, S. Rajan, L. Scheffler, S. Brunnert, H. Tang, and A. Prince**. 1998. Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. Infect Immun **66**(1): p. 43-51.

61. **Fergie, J.E., S.J. Shema, L. Lott, R. Crawford, and C.C. Patrick**. 1994. *Pseudomonas aeruginosa* bacteremia in immunocompromised children: analysis of factors associated with a poor outcome. Clin Infect Dis **18**(3): p. 390-4.

62. **Filloux, A.** 2004. The underlying mechanisms of type II protein secretion. Biochim Biophys Acta **1694**(1-3): p. 163-79.

63. **Finck-Barbancon, V., J. Goranson, L. Zhu, T. Sawa, J.P. Wiener-Kronish, S.M. Fleiszig, C. Wu, L. Mende-Mueller, and D.W. Frank**. 1997. ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. Mol Microbiol **25**(3): p. 547-57.

64. **FitzSimmons, S.C.** 1993. The changing epidemiology of cystic fibrosis. J Pediatr **122**(1): p. 1-9.

65. **Fleiszig, S.M., S.K. Arora, R. Van, and R. Ramphal**. 2001. FlhA, a component of the flagellum assembly apparatus of *Pseudomonas aeruginosa*, plays a role in internalization by corneal epithelial cells. Infect Immun **69**(8): p. 4931-7.

66. **Frank, D.W.** 1997. The exoenzyme S regulon of *Pseudomonas aeruginosa.* Mol Microbiol **26**(4): p. 621-9.

67. **Friedman, L. and R. Kolter**. 2004. Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. Mol Microbiol **51**(3): p. 675-90.

68. **Fuqua, C. and E.P. Greenberg**. 2002. Listening in on bacteria: acyl-homoserine lactone signalling. Nat Rev Mol Cell Biol **3**(9): p. 685-95.

69. **Gallagher, L.A. and C. Manoil**. 2001. *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. J Bacteriol **183**(21): p. 6207-14.

70. **Gambello, M.J. and B.H. Iglewski**. 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. J Bacteriol **173**(9): p. 3000-9.

71. **Gambello, M.J., S. Kaye, and B.H. Iglewski**. 1993. LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (*apr)* and an enhancer of exotoxin A expression. Infect Immun **61**(4): p. 1180-4.

72. **Garrity-Ryan, L., S. Shafikhani, P. Balachandran, L. Nguyen, J. Oza, T. Jakobsen, J. Sargent, X. Fang, S. Cordwell, M.A. Matthay, and J.N. Engel**. 2004. The ADP ribosyltransferase domain of *Pseudomonas aeruginosa* ExoT contributes to its biological activities. Infect Immun **72**(1): p. 546-58.

73. **Geisenberger, O., M. Givskov, K. Riedel, N. Hoiby, B. Tummler, and L. Eberl**. 2000. Production of N-acyl-L-homoserine lactones by *P. aeruginosa* isolates from chronic lung infections associated with cystic fibrosis. FEMS Microbiol Lett **184**(2): p. 273-8.

74. **Giamarellou, H.** 2002. Prescribing guidelines for severe Pseudomonas infections. J Antimicrob Chemother **49**(2): p. 229-33.

75. **Gilboa-Garber, N. and N. Garber**. 1989. Microbial lectin cofunction with lytic activities as a model for a general basic lectin role. FEMS Microbiol Rev **5**(3): p. 211-21.

76. **Goehring, U.M., G. Schmidt, K.J. Pederson, K. Aktories, and J.T. Barbieri**. 1999. The N-terminal domain of *Pseudomonas aeruginosa* exoenzyme S is a GTPase-activating protein for Rho GTPases. J Biol Chem **274**(51): p. 36369-72.

77. **Gordon, S.M., J.M. Serkey, T.F. Keys, T. Ryan, C.A. Fatica, S.K. Schmitt, J.A. Borsh, D.M. Cosgrove, 3rd, and J.P. Yared**. 1998. Secular trends in nosocomial bloodstream infections in a 55-bed cardiothoracic intensive care unit. Ann Thorac Surg **65**(1): p. 95-100.

78. **Govan, J.R. and V. Deretic**. 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia.* Microbiol Rev **60**(3): p. 539-74.

79. **Hancock, R.E. and D.P. Speert**. 2000. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. Drug Resist Updat **3**(4): p. 247-255.

80. **Hardie, K.R., S. Lory, and A.P. Pugsley**. 1996. Insertion of an outer membrane protein in *Escherichia coli* requires a chaperone-like protein. EMBO J **15**(5): p. 978-88.

81. **Hassett, D.J., *et al.*** 2002. Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. Adv Drug Deliv Rev **54**(11): p. 1425-43.

82. **Hauser, A.R., E. Cobb, M. Bodi, D. Mariscal, J. Valles, J.N. Engel, and J. Rello**. 2002. type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by *Pseudomonas aeruginosa.* Crit Care Med **30**(3): p. 521-8.

83. **Hauser, A.R., S. Fleiszig, P.J. Kang, K. Mostov, and J.N. Engel**. 1998. Defects in type III secretion correlate with internalization of *Pseudomonas aeruginosa* by epithelial cells. Infect Immun **66**(4): p. 1413-20.

84. **Hauser, A.R., P.J. Kang, and J.N. Engel**. 1998. PepA, a secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence. Mol Microbiol **27**(4): p. 807-18.

85. **Haussler, S.** 2004. Biofilm formation by the small colony variant phenotype of *Pseudomonas aeruginosa.* Environ Microbiol **6**(6): p. 546-51.

86. **Hayashi, F., K.D. Smith, A. Ozinsky, T.R. Hawn, E.C. Yi, D.R. Goodlett, J.K. Eng, S. Akira, D.M. Underhill, and A. Aderem**. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature **410**(6832): p. 1099-103.

87. **Heck, L.W., P.G. Alarcon, R.M. Kulhavy, K. Morihara, M.W. Russell, and J.F. Mestecky**. 1990. Degradation of IgA proteins by *Pseudomonas aeruginosa* elastase. J Immunol **144**(6): p. 2253-7.

88. **Heck, L.W., K. Morihara, W.B. McRae, and E.J. Miller**. 1986. Specific cleavage of human type III and IV collagens by *Pseudomonas aeruginosa* elastase. Infect Immun **51**(1): p. 115-8.

89. **Hege, T., R.E. Feltzer, R.D. Gray, and U. Baumann**. 2001. Crystal structure of a complex between *Pseudomonas aeruginosa* alkaline protease and its cognate inhibitor: inhibition by a zinc-NH2 coordinative bond. J Biol Chem **276**(37): p. 35087-92.

90. **Hentzer, M., *et al.*** 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J **22**(15): p. 3803-15.

91. **Heurlier, K., V. Denervaud, and D. Haas**. 2006. Impact of quorum sensing on fitness of *Pseudomonas aeruginosa.* Int J Med Microbiol **296**(2-3): p. 93-102.

92. **Hoffmann, N., B. Lee, M. Hentzer, T.B. Rasmussen, Z. Song, H.K. Johansen, M. Givskov, and N. Hoiby**. 2007. Azithromycin blocks quorum sensing and alginate polymer formation and increases the sensitivity to serum and stationary-growth-phase killing of *Pseudomonas aeruginosa* and attenuates chronic *P. aeruginosa* lung infection in Cftr(-/-) mice. Antimicrob Agents Chemother **51**(10): p. 3677-87.

93. **Hoffmann, N., T.B. Rasmussen, P.O. Jensen, C. Stub, M. Hentzer, S. Molin, O. Ciofu, M. Givskov, H.K. Johansen, and N. Hoiby**. 2005. Novel mouse model of chronic *Pseudomonas aeruginosa* lung infection mimicking cystic fibrosis. Infect Immun **73**(4): p. 2504-14.

94. **Hogardt, M., C. Hoboth, S. Schmoldt, C. Henke, L. Bader, and J. Heesemann**. 2007. Stage-specific adaptation of hypermutable *Pseudomonas aeruginosa* isolates during chronic pulmonary infection in patients with cystic fibrosis. J Infect Dis **195**(1): p. 70-80.

95. **Hoiby, N., E.W. Flensborg, B. Beck, B. Friis, S.V. Jacobsen, and L. Jacobsen**. 1977. *Pseudomonas aeruginosa* infection in cystic fibrosis. Diagnostic and prognostic significance of *Pseudomonas aeruginosa* precipitins determined by means of crossed immunoelectrophoresis. Scand J Respir Dis **58**(2): p. 65-79.

96. **Hoiby, N., H. Krogh Johansen, C. Moser, Z. Song, O. Ciofu, and A. Kharazmi**. 2001. *Pseudomonas aeruginosa* and the *in vitro* and in vivo biofilm mode of growth. Microbes Infect **3**(1): p. 23-35.

97. **Hong, Y.Q. and B. Ghebrehiwet**. 1992. Effect of *Pseudomonas aeruginosa* elastase and alkaline protease on serum complement and isolated components C1q and C3. Clin Immunol Immunopathol **62**(2): p. 133-8.

98. **Hoyle, B.D. and J.W. Costerton**. 1991. Bacterial resistance to antibiotics: the role of biofilms. Prog Drug Res **37**: p. 91-105.

99. **Iglewski, B.H. and D. Kabat**. 1975. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. Proc Natl Acad Sci U S A **72**(6): p. 2284-8.

100. **Iiyama, K., Y. Chieda, J.M. Lee, T. Kusakabe, C. Yasunaga-Aoki, and S. Shimizu**. 2007. Effect of superoxide dismutase gene inactivation on virulence of *Pseudomonas aeruginosa* PAO1 toward the silkworm, Bombyx mori. Appl Environ Microbiol **73**(5): p. 1569-75.

101. **Irvin, R.T., P. Doig, K.K. Lee, P.A. Sastry, W. Paranchych, T. Todd, and R.S. Hodges**. 1989. Characterization of the *Pseudomonas aeruginosa* pilus adhesin: confirmation that the pilin structural protein subunit contains a human epithelial cell-binding domain. Infect Immun **57**(12): p. 3720-6.

102. **Jackson, K.D., M. Starkey, S. Kremer, M.R. Parsek, and D.J. Wozniak**. 2004. Identification of *psl*, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. J Bacteriol **186**(14): p. 4466-75.

103. **Jensen, V., D. Lons, C. Zaoui, F. Bredenbruch, A. Meissner, G. Dieterich, R. Munch, and S. Haussler**. 2006. RhlR expression in *Pseudomonas aeruginosa* is modulated by the Pseudomonas quinolone signal via PhoB-dependent and -independent pathways. J Bacteriol **188**(24): p. 8601-6.

104. **Jewes, L.A. and R.C. Spencer**. 1990. The incidence of anaerobes in the sputum of patients with cystic fibrosis. J Med Microbiol **31**(4): p. 271-4.

105. **Jones, A.K., R.W. Heiniger, H.C. Winther-Larsen, P.M. Holiday, N.B. Fulcher, N. Roos, M.J. Cann, and M.C. Wolfgang**. 2009. Intracellular cyclic AMP controls Vfr autoregulation and virulence factor expression in *Pseudomonas aeruginosa*. Submitted for Publication.

106. **Kamath, J.M., B.E. Britigan, C.D. Cox, and D.M. Shasby**. 1995. Pyocyanin from *Pseudomonas aeruginosa* inhibits prostacyclin release from endothelial cells. Infect Immun **63**(12): p. 4921-3.

107. **Kessler, E., M. Safrin, J.C. Olson, and D.E. Ohman**. 1993. Secreted LasA of *Pseudomonas aeruginosa* is a staphylolytic protease. J Biol Chem **268**(10): p. 7503-8.

108. **Kharazmi, A., C. Rechnitzer, P.O. Schiotz, T. Jensen, L. Baek, and N. Hoiby**. 1987. Priming of neutrophils for enhanced oxidative burst by sputum from cystic fibrosis patients with *Pseudomonas aeruginosa* infection. Eur J Clin Invest **17**(3): p. 256-61.

109. **Kiratisin, P., K.D. Tucker, and L. Passador**. 2002. LasR, a transcriptional activator of *Pseudomonas aeruginosa* virulence genes, functions as a multimer. J Bacteriol **184**(17): p. 4912-9.

110. **Kirisits, M.J., L. Prost, M. Starkey, and M.R. Parsek**. 2005. Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. Appl Environ Microbiol **71**(8): p. 4809-21.

111. **Knight, D.A., V. Finck-Barbancon, S.M. Kulich, and J.T. Barbieri**. 1995. Functional domains of *Pseudomonas aeruginosa* exoenzyme S. Infect Immun **63**(8): p. 3182-6.

112. **Koch, C. and N. Hoiby**. 1993. Pathogenesis of cystic fibrosis. Lancet **341**(8852): p. 1065-9.

113. **Kohler, T., S.F. Epp, L.K. Curty, and J.C. Pechere**. 1999. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. J Bacteriol **181**(20): p. 6300-5.

114. **Kohler, T., M. Michea-Hamzehpour, U. Henze, N. Gotoh, L.K. Curty, and J.C. Pechere**. 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol Microbiol **23**(2): p. 345-54.

115. **Kong, F., L. Young, Y. Chen, H. Ran, M. Meyers, P. Joseph, Y.H. Cho, D.J. Hassett, and G.W. Lau**. 2006. *Pseudomonas aeruginosa* pyocyanin inactivates lung epithelial vacuolar ATPase-dependent cystic fibrosis transmembrane conductance regulator expression and localization. Cell Microbiol **8**(7): p. 1121-33.

116. **Krieg, D.P., R.J. Helmke, V.F. German, and J.A. Mangos**. 1988. Resistance of mucoid *Pseudomonas aeruginosa* to nonopsonic phagocytosis by alveolar macrophages *in vitro*. Infect Immun **56**(12): p. 3173-9.

117. **Kurahashi, K., O. Kajikawa, T. Sawa, M. Ohara, M.A. Gropper, D.W. Frank, T.R. Martin, and J.P. Wiener-Kronish**. 1999. Pathogenesis of septic shock in *Pseudomonas aeruginosa* pneumonia. J Clin Invest **104**(6): p. 743-50.

118. **Kurahashi, K.*, et al.*** 2004. Effect of lung-protective ventilation on severe *Pseudomonas aeruginosa* pneumonia and sepsis in rats. Am J Physiol Lung Cell Mol Physiol **287**(2): p. L402-10.

119. **Kureishi, A., J.M. Diver, B. Beckthold, T. Schollaardt, and L.E. Bryan**. 1994. Cloning and nucleotide sequence of *Pseudomonas aeruginosa* DNA gyrase *gyrA* gene from strain PAO1 and quinolone-resistant clinical isolates. Antimicrob Agents Chemother **38**(9): p. 1944-52.

120. **Kus, J.V., E. Tullis, D.G. Cvitkovitch, and L.L. Burrows**. 2004. Significant differences in type IV pilin allele distribution among *Pseudomonas aeruginosa* isolates from cystic fibrosis (CF) versus non-CF patients. Microbiology **150**(Pt 5): p. 1315-26.

121. **Labaer, J., Q. Qiu, A. Anumanthan, W. Mar, D. Zuo, T.V. Murthy, H. Taycher, A. Halleck, E. Hainsworth, S. Lory, and L. Brizuela**. 2004. The *Pseudomonas aeruginosa* PA01 gene collection. Genome Res **14**(10B): p. 2190-200.

122. **Latifi, A., M. Foglino, K. Tanaka, P. Williams, and A. Lazdunski**. 1996. A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol Microbiol **21**(6): p. 1137-46.

123. **Latifi, A., M.K. Winson, M. Foglino, B.W. Bycroft, G.S. Stewart, A. Lazdunski, and P. Williams**. 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. Mol Microbiol **17**(2): p. 333-43.

124. **Lawrence, J.R., D.R. Korber, B.D. Hoyle, J.W. Costerton, and D.E. Caldwell**. 1991. Optical sectioning of microbial biofilms. J Bacteriol **173**(20): p. 6558-67.

125. **Learn, D.B., E.P. Brestel, and S. Seetharama**. 1987. Hypochlorite scavenging by *Pseudomonas aeruginosa* alginate. Infect Immun **55**(8): p. 1813-8.

126. **Lee, D.G.*, et al.*** 2006. Genomic analysis reveals that Pseudomonas aeruginosa virulence is combinatorial. Genome Biol **7**(10): p. R90.

127. **Lee, V.T., R.S. Smith, B. Tummler, and S. Lory**. 2005. Activities of *Pseudomonas aeruginosa* effectors secreted by the type III secretion system *in vitro* and during infection. Infect Immun **73**(3): p. 1695-705.

128. **Lesprit, P., F. Faurisson, O. Join-Lambert, F. Roudot-Thoraval, M. Foglino, C. Vissuzaine, and C. Carbon**. 2003. Role of the quorum-sensing system in experimental pneumonia due to *Pseudomonas aeruginosa* in rats. Am J Respir Crit Care Med **167**(11): p. 1478-82.

129. **Liu, P.V.** 1974. Extracellular toxins of *Pseudomonas aeruginosa.* J Infect Dis **130 Suppl**(0): p. S94-9.

130. **Liu, P.V., Y. Abe, and J.L. Bates**. 1961. The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. J Infect Dis **108**: p. 218-28.

131. **Lomovskaya, O., A. Lee, K. Hoshino, H. Ishida, A. Mistry, M.S. Warren, E. Boyer, S. Chamberland, and V.J. Lee**. 1999. Use of a genetic approach to evaluate the consequences of inhibition of efflux pumps in *Pseudomonas aeruginosa.* Antimicrob Agents Chemother **43**(6): p. 1340-6.

132. **Lory, S.** 1986. Effect of iron on accumulation of exotoxin A-specific mRNA in *Pseudomonas aeruginosa.* J Bacteriol **168**(3): p. 1451-6.

133. **Lyczak, J.B., C.L. Cannon, and G.B. Pier**. 2002. Lung infections associated with cystic fibrosis. Clin Microbiol Rev **15**(2): p. 194-222.

134. **Ma, L., K.D. Jackson, R.M. Landry, M.R. Parsek, and D.J. Wozniak**. 2006. Analysis of *Pseudomonas aeruginosa* conditional *psl* variants reveals roles for the *psl* polysaccharide in adhesion and maintaining biofilm structure postattachment. J Bacteriol **188**(23): p. 8213-21.

135. **Mah, T.F., B. Pitts, B. Pellock, G.C. Walker, P.S. Stewart, and G.A. O'Toole**. 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. Nature **426**(6964): p. 306-10.

136. **Mahajan-Miklos, S., M.W. Tan, L.G. Rahme, and F.M. Ausubel**. 1999. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model. Cell **96**(1): p. 47-56.

137. **Mahenthiralingam, E., M.E. Campbell, J. Foster, J.S. Lam, and D.P. Speert**. 1996. Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. J Clin Microbiol **34**(5): p. 1129-35.

138. **Mahmood, A.** 2001. Blood stream infections in a medical intensive care unit: spectrum and antibiotic susceptibility pattern. J Pak Med Assoc **51**(6): p. 213-5.

139. **Mai, G.T., W.K. Seow, G.B. Pier, J.G. McCormack, and Y.H. Thong**. 1993. Suppression of lymphocyte and neutrophil functions by *Pseudomonas aeruginosa* mucoid exopolysaccharide (alginate): reversal by physicochemical, alginase, and specific monoclonal antibody treatments. Infect Immun **61**(2): p. 559-64.

140. **Manson, M.D., J.P. Armitage, J.A. Hoch, and R.M. Macnab**. 1998. Bacterial locomotion and signal transduction. J Bacteriol **180**(5): p. 1009-22.

141. **Mariencheck, W.I., J.F. Alcorn, S.M. Palmer, and J.R. Wright**. 2003. *Pseudomonas aeruginosa* elastase degrades surfactant proteins A and D. Am J Respir Cell Mol Biol **28**(4): p. 528-37.

142. **Martin, D.W., B.W. Holloway, and V. Deretic**. 1993. Characterization of a locus determining the mucoid status of *Pseudomonas aeruginosa*: AlgU shows sequence similarities with a Bacillus sigma factor. J Bacteriol **175**(4): p. 1153-64.

143. **Martin, D.W., M.J. Schurr, H. Yu, and V. Deretic**. 1994. Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in *Pseudomonas aeruginosa*: relationship to sigma E and stress response. J Bacteriol **176**(21): p. 6688-96.

144. **Mathee, K., *et al.*** 2008. Dynamics of *Pseudomonas aeruginosa* genome evolution. Proc Natl Acad Sci U S A **105**(8): p. 3100-5.

145. **Matheson, N.R., J. Potempa, and J. Travis**. 2006. Interaction of a novel form of *Pseudomonas aeruginosa* alkaline protease (aeruginolysin) with interleukin-6 and interleukin-8. Biol Chem **387**(7): p. 911-5.

146. **Matsukawa, M. and E.P. Greenberg**. 2004. Putative exopolysaccharide synthesis genes influence *Pseudomonas aeruginosa* biofilm development. J Bacteriol **186**(14): p. 4449-56.

147. **Mattick, J.S.** 2002. Type IV pili and twitching motility. Annu Rev Microbiol **56**: p. 289-314.

148. **Mattick, J.S., C.B. Whitchurch, and R.A. Alm**. 1996. The molecular genetics of type-4 fimbriae in *Pseudomonas aeruginosa*--a review. Gene **179**(1): p. 147-55.

149. **McGowan, J.E., Jr.** 2006. Resistance in nonfermenting gram-negative bacteria: multidrug resistance to the maximum. Am J Infect Control **34**(5 Suppl 1): p. S29-37; discussion S64-73.

150. **Medina, G., K. Juarez, R. Diaz, and G. Soberon-Chavez**. 2003. Transcriptional regulation of *Pseudomonas aeruginosa rhlR*, encoding a quorum-sensing regulatory protein. Microbiology **149**(Pt 11): p. 3073-81.

151. **Mena, A., E.E. Smith, J.L. Burns, D.P. Speert, S.M. Moskowitz, J.L. Perez, and A. Oliver**. 2008. Genetic adaptation of *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients is catalyzed by hypermutation. J Bacteriol **190**(24): p. 7910-7.

152. **Mesaros, N., *et al.*** 2007. *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millennium. Clin Microbiol Infect **13**(6): p. 560-78.

153. **Meshulam, T., N. Obedeanu, D. Merzbach, and J.D. Sobel**. 1984. Phagocytosis of mucoid and nonmucoid strains of *Pseudomonas aeruginosa*. Clin Immunol Immunopathol **32**(2): p. 151-65.

154. **Missiakas, D. and S. Raina**. 1998. The extracytoplasmic function sigma factors: role and regulation. Mol Microbiol **28**(6): p. 1059-66.

155. **Montie, T.C., D. Doyle-Huntzinger, R.C. Craven, and I.A. Holder**. 1982. Loss of virulence associated with absence of flagellum in an isogenic mutant of *Pseudomonas aeruginosa* in the burned-mouse model. Infect Immun **38**(3): p. 1296-8.

156. **Morihara, K., H. Tsuzuki, and K. Oda**. 1979. Protease and elastase of *Pseudomonas aeruginosa*: inactivation of human plasma alpha 1-proteinase inhibitor. Infect Immun **24**(1): p. 188-93.

157. **Muller, M. and T.C. Sorrell**. 1997. Modulation of neutrophil superoxide response and intracellular diacylglyceride levels by the bacterial pigment pyocyanin. Infect Immun **65**(6): p. 2483-7.

158. **Nakano, M., T. Deguchi, T. Kawamura, M. Yasuda, M. Kimura, Y. Okano, and Y. Kawada**. 1997. Mutations in the *gyrA* and *parC* genes in fluoroquinolone-resistant clinical isolates of *Pseudomonas aeruginosa.* Antimicrob Agents Chemother **41**(10): p. 2289-91.

159. **Nicas, T.I. and B.H. Iglewski**. 1985. The contribution of exoproducts to virulence of *Pseudomonas aeruginosa.* Can J Microbiol **31**(4): p. 387-92.

160. **Niga, T., H. Ito, Y. Oyamada, J. Yamagishi, M. Kadono, T. Nishino, N. Gotoh, and M. Inoue**. 2005. Cooperation between alteration of DNA gyrase genes and over-expression of MexB and MexX confers high-level fluoroquinolone resistance in *Pseudomonas aeruginosa* strains isolated from a patient who received a liver transplant followed by treatment with fluoroquinolones. Microbiol Immunol **49**(5): p. 443-6.

161. **Nikaido, H.** 1998. Multiple antibiotic resistance and efflux. Curr Opin Microbiol **1**(5): p. 516-23.

162. **Nivens, D.E., D.E. Ohman, J. Williams, and M.J. Franklin**. 2001. Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. J Bacteriol **183**(3): p. 1047-57.

163. **Nouwens, A.S., S.A. Beatson, C.B. Whitchurch, B.J. Walsh, H.P. Schweizer, J.S. Mattick, and S.J. Cordwell**. 2003. Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudomonas aeruginosa* PAO1. Microbiology **149**(Pt 5): p. 1311-22.

164. **Nunn, D., S. Bergman, and S. Lory**. 1990. Products of three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. J Bacteriol **172**(6): p. 2911-9.

165. **Nunn, D.N. and S. Lory**. 1991. Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin leader peptidase. Proc Natl Acad Sci U S A **88**(8): p. 3281-5.

166. **Nunn, D.N. and S. Lory**. 1993. Cleavage, methylation, and localization of the *Pseudomonas aeruginosa* export proteins XcpT, -U, -V, and -W. J Bacteriol **175**(14): p. 4375-82.

167. **Obritsch, M.D., D.N. Fish, R. MacLaren, and R. Jung**. 2005. Nosocomial infections due to multidrug-resistant *Pseudomonas aeruginosa*: epidemiology and treatment options. Pharmacotherapy **25**(10): p. 1353-64.

168. **Ochsner, U.A., Z. Johnson, I.L. Lamont, H.E. Cunliffe, and M.L. Vasil**. 1996. Exotoxin A production in *Pseudomonas aeruginosa* requires the iron-regulated *pvdS* gene encoding an alternative sigma factor. Mol Microbiol **21**(5): p. 1019-28.

169. **Ochsner, U.A. and J. Reiser**. 1995. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A **92**(14): p. 6424-8.

170. **Okuda, K., K. Morihara, Y. Atsumi, H. Takeuchi, S. Kawamoto, H. Kawasaki, K. Suzuki, and J. Fukushima**. 1990. Complete nucleotide sequence of the structural gene for alkaline proteinase from *Pseudomonas aeruginosa* IFO 3455. Infect Immun **58**(12): p. 4083-8.

171. **O'Toole, G.A. and R. Kolter**. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol **30**(2): p. 295-304.

172. **O'Toole, G.A. and R. Kolter**. 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol **28**(3): p. 449-61.

173. **Parge, H.E., K.T. Forest, M.J. Hickey, D.A. Christensen, E.D. Getzoff, and J.A. Tainer**. 1995. Structure of the fibre-forming protein pilin at 2.6 A resolution. Nature **378**(6552): p. 32-8.

174. **Park, P.W., G.B. Pier, M.T. Hinkes, and M. Bernfield**. 2001. Exploitation of syndecan-1 shedding by *Pseudomonas aeruginosa* enhances virulence. Nature **411**(6833): p. 98-102.

175. **Park, P.W., G.B. Pier, M.J. Preston, O. Goldberger, M.L. Fitzgerald, and M. Bernfield**. 2000. Syndecan-1 shedding is enhanced by LasA, a secreted virulence factor of *Pseudomonas aeruginosa.* J Biol Chem **275**(5): p. 3057-64.

176. **Parmely, M., A. Gale, M. Clabaugh, R. Horvat, and W.W. Zhou**. 1990. Proteolytic inactivation of cytokines by *Pseudomonas aeruginosa.* Infect Immun **58**(9): p. 3009-14.

177. **Passador, L., J.M. Cook, M.J. Gambello, L. Rust, and B.H. Iglewski**. 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science **260**(5111): p. 1127-30.

178. **Pearson, J.P., M. Feldman, B.H. Iglewski, and A. Prince**. 2000. *Pseudomonas aeruginosa* cell-to-cell signaling is required for virulence in a model of acute pulmonary infection. Infect Immun **68**(7): p. 4331-4.

179. **Pearson, J.P., L. Passador, B.H. Iglewski, and E.P. Greenberg**. 1995. A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A **92**(5): p. 1490-4.

180. **Pearson, J.P., E.C. Pesci, and B.H. Iglewski**. 1997. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J Bacteriol **179**(18): p. 5756-67.

181. **Pedersen, B.K. and A. Kharazmi**. 1987. Inhibition of human natural killer cell activity by *Pseudomonas aeruginosa* alkaline protease and elastase. Infect Immun **55**(4): p. 986-9.

182. **Pesci, E.C., J.P. Pearson, P.C. Seed, and B.H. Iglewski**. 1997. Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa.* J Bacteriol **179**(10): p. 3127-32.

183. **Pessi, G. and D. Haas**. 2000. Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa.* J Bacteriol **182**(24): p. 6940-9.

184. **Peters, J.E. and D.R. Galloway**. 1990. Purification and characterization of an active fragment of the LasA protein from *Pseudomonas aeruginosa*: enhancement of elastase activity. J Bacteriol **172**(5): p. 2236-40.

185. **Peters, J.E., S.J. Park, A. Darzins, L.C. Freck, J.M. Saulnier, J.M. Wallach, and D.R. Galloway**. 1992. Further studies on *Pseudomonas aeruginosa* LasA: analysis of specificity. Mol Microbiol **6**(9): p. 1155-62.

186. **Phillips, R.M., D.A. Six, E.A. Dennis, and P. Ghosh**. 2003. *In vivo* phospholipase activity of the *Pseudomonas aeruginosa* cytotoxin ExoU and protection of mammalian cells with phospholipase A2 inhibitors. J Biol Chem **278**(42): p. 41326-32.

187. **Pielage, J.F., K.R. Powell, D. Kalman, and J.N. Engel**. 2008. RNAi screen reveals an Abl kinase-dependent host cell pathway involved in *Pseudomonas aeruginosa* internalization. PLoS Pathog **4**(3): p. e1000031.

188. **Pier, G.B., M. Grout, and T.S. Zaidi**. 1997. Cystic fibrosis transmembrane conductance regulator is an epithelial cell receptor for clearance of *Pseudomonas aeruginosa* from the lung. Proc Natl Acad Sci U S A **94**(22): p. 12088-93.

189. **Pier, G.B., M. Grout, T.S. Zaidi, J.C. Olsen, L.G. Johnson, J.R. Yankaskas, and J.B. Goldberg**. 1996. Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. Science **271**(5245): p. 64-7.

190. **Plotnikova, J.M., L.G. Rahme, and F.M. Ausubel**. 2000. Pathogenesis of the human opportunistic pathogen *Pseudomonas aeruginosa* PA14 in Arabidopsis. Plant Physiol **124**(4): p. 1766-74.

191. **Poole, K. and R. Srikumar**. 2001. Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. Curr Top Med Chem **1**(1): p. 59-71.

192. **Pratt, L.A. and R. Kolter**. 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol Microbiol **30**(2): p. 285-93.

193. **Prince, A.** 1992. Adhesins and receptors of *Pseudomonas aeruginosa* associated with infection of the respiratory tract. Microb Pathog **13**(4): p. 251-60.

194. **Pumbwe, L. and L.J. Piddock**. 2000. Two efflux systems expressed simultaneously in multidrug-resistant *Pseudomonas aeruginosa.* Antimicrob Agents Chemother **44**(10): p. 2861-4.

195. **Purevdorj-Gage, B., W.J. Costerton, and P. Stoodley**. 2005. Phenotypic differentiation and seeding dispersal in non-mucoid and mucoid *Pseudomonas aeruginosa* biofilms. Microbiology **151**(Pt 5): p. 1569-76.

196. **Rahme, L.G., *et al.*** 2000. Plants and animals share functionally common bacterial virulence factors. Proc Natl Acad Sci U S A **97**(16): p. 8815-21.

197. **Rogers, G.B., M.P. Carroll, D.J. Serisier, P.M. Hockey, G. Jones, and K.D. Bruce**. 2004. Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol **42**(11): p. 5176-83.

198. **Rudel, T., I. Scheurerpflug, and T.F. Meyer**. 1995. Neisseria PilC protein identified as type-4 pilus tip-located adhesin. Nature **373**(6512): p. 357-9.

199. **Saiman, L. and A. Prince**. 1993. *Pseudomonas aeruginosa* pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. J Clin Invest **92**(4): p. 1875-80.

200. **Sandkvist, M., M. Bagdasarian, S.P. Howard, and V.J. DiRita**. 1995. Interaction between the autokinase EpsE and EpsL in the cytoplasmic membrane is required for extracellular secretion in *Vibrio cholerae*. EMBO J **14**(8): p. 1664-73.

201. **Sandoz, K.M., S.M. Mitzimberg, and M. Schuster**. 2007. Social cheating in *Pseudomonas aeruginosa* quorum sensing. Proc Natl Acad Sci U S A **104**(40): p. 15876-81.

202. **Santucci, S.G., S. Gobara, C.R. Santos, C. Fontana, and A.S. Levin**. 2003. Infections in a burn intensive care unit: experience of seven years. J Hosp Infect **53**(1): p. 6-13.

203. **Sato, H. and D.W. Frank**. 2004. ExoU is a potent intracellular phospholipase. Mol Microbiol **53**(5): p. 1279-90.

204. **Sato, H., *et al.*** 2003. The mechanism of action of the *Pseudomonas aeruginosa*-encoded type III cytotoxin, ExoU. EMBO J **22**(12): p. 2959-69.

205. **Saulnier, J.M., F.M. Curtil, M.C. Duclos, and J.M. Wallach**. 1989. Elastolytic activity of *Pseudomonas aeruginosa* elastase. Biochim Biophys Acta **995**(3): p. 285-90.

206. **Schaber, J.A., W.J. Triffo, S.J. Suh, J.W. Oliver, M.C. Hastert, J.A. Griswold, M. Auer, A.N. Hamood, and K.P. Rumbaugh**. 2007. *Pseudomonas aeruginosa* forms biofilms in acute infection independent of cell-to-cell signaling. Infect Immun **75**(8): p. 3715-21.

207. **Schurr, M.J., H. Yu, J.M. Martinez-Salazar, J.C. Boucher, and V. Deretic**. 1996. Control of AlgU, a member of the sigma E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. J Bacteriol **178**(16): p. 4997-5004.

208. **Schwarzmann, S. and J.R. Boring**. 1971. Antiphagocytic Effect of Slime from a Mucoid Strain of *Pseudomonas aeruginosa*. Infect Immun **3**(6): p. 762-767.

209. **Shaver, C.M. and A.R. Hauser**. 2004. Relative contributions of *Pseudomonas aeruginosa* ExoU, ExoS, and ExoT to virulence in the lung. Infect Immun **72**(12): p. 6969-77.

210. **Shime, N., T. Sawa, J. Fujimoto, K. Faure, L.R. Allmond, T. Karaca, B.L. Swanson, E.G. Spack, and J.P. Wiener-Kronish**. 2001. Therapeutic administration of anti-PcrV F(ab')(2) in sepsis associated with *Pseudomonas aeruginosa*. J Immunol **167**(10): p. 5880-6.

211. **Simpson, J.A., S.E. Smith, and R.T. Dean**. 1988. Alginate inhibition of the uptake of *Pseudomonas aeruginosa* by macrophages. J Gen Microbiol **134**(1): p. 29-36.

212. **Simpson, J.A., S.E. Smith, and R.T. Dean**. 1989. Scavenging by alginate of free radicals released by macrophages. Free Radic Biol Med **6**(4): p. 347-53.

213. **Singh, P.K., A.L. Schaefer, M.R. Parsek, T.O. Moninger, M.J. Welsh, and E.P. Greenberg**. 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature **407**(6805): p. 762-4.

214. **Smith, A.J., J. Jefferies, S.C. Clarke, C. Dowson, G.F. Edwards, and T.J. Mitchell**. 2006. Distribution of epidemic antibiotic-resistant pneumococcal clones in Scottish pneumococcal isolates analysed by multilocus sequence typing. Microbiology **152**(Pt 2): p. 361-5.

215. **Smith, C., E. Andreakos, J.B. Crawley, F.M. Brennan, M. Feldmann, and B.M. Foxwell**. 2001. NF-kappaB-inducing kinase is dispensable for activation of NF-kappaB in inflammatory settings but essential for lymphotoxin beta receptor activation of NF-kappaB in primary human fibroblasts. J Immunol **167**(10): p. 5895-903.

216. **Smith, E.E., *et al.*** 2006. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A **103**(22): p. 8487-92.

217. **Smith, R.S., S.G. Harris, R. Phipps, and B. Iglewski**. 2002. The *Pseudomonas aeruginosa* quorum-sensing molecule N-(3-oxododecanoyl)homoserine lactone contributes to virulence and induces inflammation *in vivo*. J Bacteriol **184**(4): p. 1132-9.

218. **Smith, R.S., R. Kelly, B.H. Iglewski, and R.P. Phipps**. 2002. The Pseudomonas autoinducer N-(3-oxododecanoyl) homoserine lactone induces cyclooxygenase-2 and prostaglandin E2 production in human lung fibroblasts: implications for inflammation. J Immunol **169**(5): p. 2636-42.

219. **Smith, R.S., M.C. Wolfgang, and S. Lory**. 2004. An adenylate cyclase-controlled signaling network regulates *Pseudomonas aeruginosa* virulence in a mouse model of acute pneumonia. Infect Immun **72**(3): p. 1677-84.

220. **Spoering, A.L. and K. Lewis**. 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. J Bacteriol **183**(23): p. 6746-51.

221. **Stapper, A.P., G. Narasimhan, D.E. Ohman, J. Barakat, M. Hentzer, S. Molin, A. Kharazmi, N. Hoiby, and K. Mathee**. 2004. Alginate production affects *Pseudomonas aeruginosa* biofilm development and architecture, but is not essential for biofilm formation. J Med Microbiol **53**(Pt 7): p. 679-90.

222. **Stewart, P.S. and J.W. Costerton**. 2001. Antibiotic resistance of bacteria in biofilms. Lancet **358**(9276): p. 135-8.

223. **Storey, D.G., E.E. Ujack, H.R. Rabin, and I. Mitchell**. 1998. *Pseudomonas aeruginosa lasR* transcription correlates with the transcription of *lasA*, l*asB*, and *toxA* in chronic lung infections associated with cystic fibrosis. Infect Immun **66**(6): p. 2521-8.

224. **Stover, C.K.*, et al.*** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406**(6799): p. 959-64.

225. **Strom, M.S. and S. Lory**. 1993. Structure-function and biogenesis of the type IV pili. Annu Rev Microbiol **47**: p. 565-96.

226. **Strom, M.S., D.N. Nunn, and S. Lory**. 1993. A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. Proc Natl Acad Sci U S A **90**(6): p. 2404-8.

227. **Sundin, C., M.C. Wolfgang, S. Lory, A. Forsberg, and E. Frithz-Lindsten**. 2002. Type IV pili are not specifically required for contact dependent translocation of exoenzymes by *Pseudomonas aeruginosa*. Microb Pathog **33**(6): p. 265-77.

228. **Sutterwala, F.S., L.A. Mijares, L. Li, Y. Ogura, B.I. Kazmierczak, and R.A. Flavell**. 2007. Immune recognition of *Pseudomonas aeruginosa* mediated by the IPAF/NLRC4 inflammasome. J Exp Med **204**(13): p. 3235-45.

229. **Tager, A.M., J. Wu, and M.W. Vermeulen**. 1998. The effect of chloride concentration on human neutrophil functions: potential relevance to cystic fibrosis. Am J Respir Cell Mol Biol **19**(4): p. 643-52.

230. **Tateda, K., Y. Ishii, M. Horikawa, T. Matsumoto, S. Miyairi, J.C. Pechere, T.J. Standiford, M. Ishiguro, and K. Yamaguchi**. 2003. The *Pseudomonas aeruginosa* autoinducer N-3-oxododecanoyl homoserine lactone accelerates apoptosis in macrophages and neutrophils. Infect Immun **71**(10): p. 5785-93.

231. **Telford, G., D. Wheeler, P. Williams, P.T. Tomkins, P. Appleby, H. Sewell, G.S. Stewart, B.W. Bycroft, and D.I. Pritchard**. 1998. The *Pseudomonas aeruginosa* quorum-sensing signal molecule N-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity. Infect Immun **66**(1): p. 36-42.

232. **Theander, T.G., A. Kharazmi, B.K. Pedersen, L.D. Christensen, N. Tvede, L.K. Poulsen, N. Odum, M. Svenson, and K. Bendtzen**. 1988. Inhibition of human lymphocyte proliferation and cleavage of interleukin-2 by *Pseudomonas aeruginosa* proteases. Infect Immun **56**(7): p. 1673-7.

233. **Toder, D.S., M.J. Gambello, and B.H. Iglewski**. 1991. *Pseudomonas aeruginosa* LasA: a second elastase under the transcriptional control of *lasR*. Mol Microbiol **5**(8): p. 2003-10.

234. **Totten, P.A., J.C. Lara, and S. Lory**. 1990. The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J Bacteriol **172**(1): p. 389-96.

235. **Tunney, M.M., T.R. Field, T.F. Moriarty, S. Patrick, G. Doering, M.S. Muhlebach, M.C. Wolfgang, R. Boucher, D.F. Gilpin, A. McDowell, and J.S. Elborn**. 2008. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. Am J Respir Crit Care Med **177**(9): p. 995-1001.

236. **Tuomanen, E., R. Cozens, W. Tosch, O. Zak, and A. Tomasz**. 1986. The rate of killing of *Escherichia coli* by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth. J Gen Microbiol **132**(5): p. 1297-304.

237. **Turner, L.R., J.C. Lara, D.N. Nunn, and S. Lory**. 1993. Mutations in the consensus ATP-binding sites of XcpR and PilB eliminate extracellular protein secretion and pilus biogenesis in *Pseudomonas aeruginosa.* J Bacteriol **175**(16): p. 4962-9.

238. **Vallis, A.J., V. Finck-Barbancon, T.L. Yahr, and D.W. Frank**. 1999. Biological effects of *Pseudomonas aeruginosa* type III-secreted proteins on CHO cells. Infect Immun **67**(4): p. 2040-4.

239. **Vasil, M.L.** 1986. *Pseudomonas aeruginosa*: biology, mechanisms of virulence, epidemiology. J Pediatr **108**(5 Pt 2): p. 800-5.

240. **Vasseur, P., I. Vallet-Gely, C. Soscia, S. Genin, and A. Filloux**. 2005. The *pel* genes of the *Pseudomonas aeruginosa* PAK strain are involved at early and late stages of biofilm formation. Microbiology **151**(Pt 3): p. 985-97.

241. **Ventre, I., F. Ledgham, V. Prima, A. Lazdunski, M. Foglino, and J.N. Sturgis**. 2003. Dimerization of the quorum sensing regulator RhlR: development of a method using EGFP fluorescence anisotropy. Mol Microbiol **48**(1): p. 187-98.

242. **Wagner, V.E., D. Bushnell, L. Passador, A.I. Brooks, and B.H. Iglewski**. 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. J Bacteriol **185**(7): p. 2080-95.

243. **Webb, J.S., M. Lau, and S. Kjelleberg**. 2004. Bacteriophage and phenotypic variation in *Pseudomonas aeruginosa* biofilm development. J Bacteriol **186**(23): p. 8066-73.

244. **West, S.E., A.K. Sample, and L.J. Runyen-Janecky**. 1994. The *vfr* gene product, required for *Pseudomonas aeruginosa* exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. J Bacteriol **176**(24): p. 7532-42.

245. **Whitchurch, C.B., M. Hobbs, S.P. Livingston, V. Krishnapillai, and J.S. Mattick**. 1991. Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. Gene **101**(1): p. 33-44.

246. **Whitchurch, C.B. and J.S. Mattick**. 1994. Characterization of a gene, *pilU*, required for twitching motility but not phage sensitivity in *Pseudomonas aeruginosa.* Mol Microbiol **13**(6): p. 1079-91.

247. **Whitchurch, C.B., T. Tolker-Nielsen, P.C. Ragas, and J.S. Mattick**. 2002. Extracellular DNA required for bacterial biofilm formation. Science **295**(5559): p. 1487.

248. **Wilderman, P.J., A.I. Vasil, Z. Johnson, M.J. Wilson, H.E. Cunliffe, I.L. Lamont, and M.L. Vasil**. 2001. Characterization of an endoprotease (PrpL) encoded by a PvdS-regulated gene in *Pseudomonas aeruginosa.* Infect Immun **69**(9): p. 5385-94.

249. **Wilson, R., D.A. Sykes, D. Watson, A. Rutman, G.W. Taylor, and P.J. Cole**. 1988. Measurement of *Pseudomonas aeruginosa* phenazine pigments in sputum and assessment of their contribution to sputum sol toxicity for respiratory epithelium. Infect Immun **56**(9): p. 2515-7.

250. **Winstanley, C.*, et al.*** 2009. Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool Epidemic Strain of *Pseudomonas aeruginosa.* Genome Res **19**(1): p. 12-23.

251. **Winzer, K., C. Falconer, N.C. Garber, S.P. Diggle, M. Camara, and P. Williams**. 2000. The *Pseudomonas aeruginosa* lectins PA-IL and PA-IIL are controlled by quorum sensing and by RpoS. J Bacteriol **182**(22): p. 6401-11.

252. **Wolfgang, M., J.P. van Putten, S.F. Hayes, D. Dorward, and M. Koomey**. 2000. Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. Embo J **19**(23): p. 6408-18.

253. **Wolfgang, M.C., J. Jyot, A.L. Goodman, R. Ramphal, and S. Lory**. 2004. *Pseudomonas aeruginosa* regulates flagellin expression as part of a global response to airway fluid from cystic fibrosis patients. Proc Natl Acad Sci U S A **101**(17): p. 6664-8.

254. **Wolfgang, M.C., B.R. Kulasekara, X. Liang, D. Boyd, K. Wu, Q. Yang, C.G. Miyada, and S. Lory**. 2003. Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A **100**(14): p. 8484-9.

255. **Worlitzsch, D.*, et al.*** 2002. Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. J Clin Invest **109**(3): p. 317-25.

256. **Wozniak, D.J., T.J. Wyckoff, M. Starkey, R. Keyser, P. Azadi, G.A. O'Toole, and M.R. Parsek**. 2003. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. Proc Natl Acad Sci U S A **100**(13): p. 7907-12.

257. **Wu, H., Z. Song, M. Givskov, G. Doring, D. Worlitzsch, K. Mathee, J. Rygaard, and N. Hoiby**. 2001. *Pseudomonas aeruginosa* mutations in *lasI* and *rhlI* quorum sensing systems result in milder chronic lung infection. Microbiology **147**(Pt 5): p. 1105-13.

258. **Yanagihara, K., K. Tomono, Y. Kaneko, Y. Miyazaki, K. Tsukamoto, Y. Hirakata, H. Mukae, J. Kadota, I. Murata, and S. Kohno**. 2003. Role of elastase in a mouse model of chronic respiratory *Pseudomonas aeruginosa* infection that mimics diffuse panbronchiolitis. J Med Microbiol **52**(Pt 6): p. 531-5.

259. **Yahr, T.L., A.J. Vallis, M.K. Hancock, J.T. Barbieri, and D.W. Frank**. 1998. ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system. Proc Natl Acad Sci U S A **95**(23): p. 13899-904.

260. **Yates, S.P. and A.R. Merrill**. 2001. A catalytic loop within *Pseudomonas aeruginosa* exotoxin A modulates its transferase activity. J Biol Chem **276**(37): p. 35029-36.

261. **Yonezawa, M., M. Takahata, N. Matsubara, Y. Watanabe, and H. Narita**. 1995. DNA gyrase *gyrA* mutations in quinolone-resistant clinical isolates of *Pseudomonas aeruginosa.* Antimicrob Agents Chemother **39**(9): p. 1970-2.

262. **Yoon, S.S., *et al.*** 2002. *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev Cell **3**(4): p. 593-603.

263. **Zolfaghar, I., D.J. Evans, and S.M. Fleiszig**. 2003. Twitching motility contributes to the role of pili in corneal infection caused by *Pseudomonas aeruginosa.* Infect Immun **71**(9): p. 5389-93.

CHAPTER 2

The *Pseudomonas aeruginosa* Type IV Pilus-Associated Protein PilY1 is Required for Stable Fiber Formation and Host Attachment

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Pseudomonas aeruginosa is an environmental bacterium capable of causing a wide variety of infections. In addition, *P. aeruginosa* is a leading cause of hospital-acquired pneumonia and the primary pathogen associated with chronic respiratory infection in individuals with cystic fibrosis. *P. aeruginosa* pathogenesis involves bacterial attachment to host cells and tissue. Adherence is mediated by Type IV pili (TFP), which are polymeric surface fibers composed primarily of pilin subunits. In addition to their role in adherence, TFP facilitate twitching motility by undergoing rounds of extension and retraction. An exposed epitope on the terminal pilin subunit of TFP is believed to mediate *P. aeruginosa* binding to host glycosphingolipids. However, recent work has shed doubt on the direct involvement of pilin and the importance of glycolipids in *P. aeruginosa* adherence. We hypothesized that PilY1, a putative pilus-associated protein, may play a direct role in TFP-dependent bacterial adherence. To test this hypothesis, we constructed a non-polar *pilY1* deletion mutant and assessed pilus-associated phenotypes. We confirmed a previous report that *pilY1* is required for pilus

biogenesis and demonstrated that surface fiber production can be restored in the *pilY1* mutant by disruption of the pilus retraction gene *pilT*. Restoration of TFP in the non-retractile double mutant (*pilY1*, *pilT*) indicates that PilY1 is conditionally required for TFP biogenesis. We further demonstrated that association of PilY1 with surface TFP fibers involves a set of pilin-like proteins. To assess the role of PilY1 in *P. aeruginosa* adherence, we developed a biologically relevant infection model using primary well-differentiated human airway epithelial cell cultures. In this system, wild type and non-retractile (*pilT*) strains showed robust adherence. In contrast, non-piliated strains and piliated non-retractile mutants lacking PilY1 were non-adherent. Overall, our results indicate that adherence of *P. aeruginosa* to primary well-differentiated host cell requires both TFP and PilY1 and that PilY1 is a bi-functional protein with distinct roles in TFP biogenesis and adherence.

Introduction

Pseudomonas aeruginosa, an environmental bacterium, is a major source of fatal nosocomial infections and the primary cause of morbidity and mortality in patients with cystic fibrosis (CF) (20, 25). The ability of *P. aeruginosa* to cause infection requires the expression of a variety of virulence factors including Type IV pili (TFP) (49). TFP are filamentous surface appendages expressed by a wide variety of gram-negative human, animal and plant pathogens including *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Vibrio vulnificus*, enterohemorrhagic *Escherichia coli*, *Francisella tularensis*, non-typeable *Haemophilus influenzae*, *Burkholderia pseudomallei*, *Dichelobacter nodosus* and *Kingella kingae*. TFP play a crucial role in host colonization and promote numerous phenotypes including surface attachment, DNA uptake, auto-aggregation, biofilm formation and motility. In *P. aeruginosa*, TFP are required for adherence to host tissue, colonization, virulence and for the formation of robust biofilms (10, 14, 34, 42, 59). In addition, *P. aeruginosa* TFP mediate a form of

surface movement known as twitching motility through extension and retraction of pilus fibers (38, 54).

The assembly and function of TFP in *P. aeruginosa* involves more than 40 gene products (37). TFP are polymers composed primarily of a single repeating subunit termed pilin, which is encoded by the *pilA* gene in *P. aeruginosa* (56). Pilin is synthesized as a pre-protein with a short leader sequence that is removed by the integral inner membrane protease PilD prior to assembly of the mature protein into the pilus fiber (57). The mature form of pilin contains an extended, hydrophobic N-terminal α-helical region followed by a globular C-terminal domain terminating in a disulfide-bonded loop (15, 27, 43). Pilin subunits are predicted to assemble such that the N-terminal α-helical regions form the core of the pilus structure, and the globular C-terminal domains are exposed on the exterior surface of the fiber (15). The pilus is assembled on the periplasmic surface of the inner membrane and extruded across the outer membrane via a pore complex composed of the secretin protein, PilQ (5, 12, 65). Although pilin is the major structural component of the pilus, other proteins are associated with the fiber and may play structural or functional roles. In *N. gonorrhoeae*, six proteins (PilH, PilI, PilJ, PilK, PilL, and PilV) are known to be associated with the TFP fraction (62). These proteins, have homology to the highly conserved N-terminus of pilin and are thus termed "pilin-like" proteins (62). In *N. meningitidis*, PilX, a homolog of *N. gonorrhoeae* PilL, was shown to integrate into pilus fibers as a minor subunit (28). A homologous set of pilin-like proteins (FimU, PilV, PilW, PilX, and PilE) is required for TFP biogenesis in *P. aeruginosa* (1, 2, 3, 48); however, their association with the pilus fiber and specific function remains to be determined.

Type IV pili are dynamic structures that undergo cycles of extension and retraction (54), a process that involves two cytoplasmic membrane associated ATPases, PilB and PilT. PilB is required for the assembly of pilin monomers into a mature fiber (11, 41, 60), while PilT is involved in disassembly of pilin monomers and subsequent retraction of TFP (11, 61). Loss-of-function mutation

of *pilT* results in hyperpiliation and loss of twitching motility due to the inability of formed pilus fibers to retract (61). Additionally, inactivation of *pilT* results in complete loss of cytotoxicity *in vitro*, presumably due to the inability of the TFP to retract and allow for more intimate contact between the bacteria and host cell (13). However, *pilT* mutants are only slightly reduced for virulence *in vivo* (13). This reduction in virulence is likely due to the defect in twitching motility, as *pilT* mutants are able to efficiently infect mouse lungs, but are unable to spread to peripheral organs (69).

In addition to their function in twitching motility, TFP also mediate adherence of *P. aeruginosa* to eukaryotic cells (10, 19, 26, 50). While the molecular basis for this interaction has been the subject of many studies, the actual mechanism for TFP-mediated *P. aeruginosa* adhesion remains controversial. Previous studies suggest that the major structural subunit (pilin) of TFP may be directly involved in adherence of *P. aeruginosa* to host cells. Specifically, purified *P. aeruginosa* pili were shown to bind to the GalNAcβ1-4Gal moiety of the non-sialylated glycosphingolipids asialo-G_{M1} and asialo-G_{M2}, which are abundant on the apical surface of mammalian epithelial cells (36, 53). Fab fragments, generated from monoclonal antibodies, specific for the exposed C-terminal disulfide-bonded loop (DSL) region of pilin, inhibited adhesion of purified *P. aeruginosa* TFP to human buccal epithelial cells (18). Subsequent studies demonstrated that adherence of purified TFP to asialo-G_{M1} can be inhibited by a synthetic peptide corresponding to the C-terminal DSL of pilin and by the disaccharide GalNAcβ1-4Gal (52, 53, 67). Further experimentation showed that the adhesive moiety of TFP, responsible for asialylated glycosphingolipid binding, is only exposed at the distal tip of purified TFP fibers (36). Based on these studies, it is widely reported that the exposed C-terminal DSL of pilin serves as the *P. aeruginosa* TFP-associated adhesin (7, 51).

More recent experiment results have raised questions as to the role of pilin in TFP-mediated adherence as well the identity of the pilus receptor. Specifically, Emam et al. showed that intact

piliated *P. aeruginosa* are unable to bind to either asialo-G_{M1} or asialo-G_{M2} in contrast to purified pili from the same strains (21). Second, it has been established that *P. aeruginosa* preferentially binds basolateral surfaces of epithelial cell monolayers, or damaged areas of the monolayer where basolateral surfaces are exposed (22, 35). Given that asialo-G_{M1} is predominantly presented on the apical surface of airway epithelial cells (55), pilin may not be the only TFP-associated protein involved in adherence of *P. aeruginosa* to host epithelial cells.

Although the molecular interactions involved in *P. aeruginosa* adherence remain to be more clearly defined, the mechanism of TFP-mediated adhesion has been characterized in other gram-negative bacteria. In *N. gonorrhoeae*, a minor TFP-associated protein PilC has been identified as the adhesin due to its ability to bind cells and competitively block the adherence of piliated gonococci (46). Additionally, PilC is required for TFP biogenesis in both *N. gonorrhoeae* and *N. meningitidis* (31, 39, 46, 47). Association of PilC with the gonococcal TFP fraction requires expression of the pilin-like proteins PilH, PilI, PilJ, PilK, PilL, and PilV (62). PilC was initially reported to be essential for TFP biogenesis, but work by Wolfgang et al. demonstrated that deletion of the pilus retraction gene *pilT* could suppress the TFP biogenesis defect of a *pilC* mutant (64). Additionally, the pilin-like proteins in both *N. gonorrhoeae* and *N. meningitidis* are required for TFP biogenesis, but this requirement can also be suppressed by a corresponding deletion of *pilT* (9, 62). Therefore, neither PilC, nor the pilin-like proteins, are essential for TFP biogenesis but are believed to play a role in stabilizing the TFP structure against retraction by PilT (39, 65).

P. aeruginosa expresses a TFP-associated protein named PilY1 that has homology to *N. gonorrhoeae* PilC, but this protein has not been fully characterized with respect to its role in TFP biogenesis and TFP-mediated adherence (1). The *pilY1* gene was originally identified in a transposon mutant screen for genes that affect TFP biogenesis (1). Interestingly, *pilY1* is located in an operon (*fimUpilVWXY1Y2E*) with five genes that encode pilin-like proteins (described above). PilY1 is

approximately 117-kD in size and has 27% amino acid identity to *N. gonorrhoeae* PilC within a 257-amino acid region in the C-terminus. A *P. aeruginosa* mutant carrying a polar insertion in *pilY1* failed to produce TFP fibers but accumulated intracellular processed pilin subunits, suggesting *pilY1* is required for fiber assembly. Full restoration of TFP biogenesis in this mutant required complementation with *pilY1* and additional downstream genes (1). Finally, the PilY1 protein was shown to localize to TFP fractions recovered from the surface of *P. aeruginosa* (1). Given the homology between PilY1 and PilC, as well as the apparent similarities between the TFP systems of *P. aeruginosa* and *N. gonorrhoeae*, we hypothesize PilY1 plays a role in adherence of *P. aeruginosa* to host epithelial cells. Here, we demonstrate that PilY1 is conditionally required for TFP expression, such that TFP biogenesis is *pilY1*-dependent but the defect can be suppressed by inactivation of *pilT*. Additionally, we demonstrate the requirement for PilY1 in TFP-associated adherence of *P. aeruginosa* to well-differentiated human airway epithelial cell cultures following injury.

Results

PilY1 is required for TFP biogenesis. It was previously reported that polar transposon insertions in *P. aeruginosa pilY1* result in a defect in TFP production (1). To determine the role of PilY1 in TFP biogenesis, we constructed a non-polar *pilY1* deletion in wild type piliated *P. aeruginosa* strain PAK. The ability of the wild type parent and *pilY1* mutant to produce TFP was determined by transmission electron microscopy (TEM). Thin polar fibers, extending from the surface of the wild type strain, were readily detected (**Fig. 2.1**). In contrast, we were unable to detect similar fibers on the surface of the *pilY1* mutant, which was indistinguishable from a non-piliated *pilA* mutant (32) (**Fig. 2.1**). To confirm the TEM results wild type and mutant strains were grown on glass slides, and surface TFP were stained with pilin-specific antibody and examined by immunofluorescence microscopy (IF) (**Fig. 2.2**). The IF technique allowed us to specifically label TFP, and to visualize a larger population of bacteria. The wild type strain produced abundant TFP, while no TFP could be detected in association with either the *pilY1* or *pilA* mutant (**Fig 2.2**).

In addition to our qualitative assessment of TFP, we performed semi-quantitative analysis of TFP production by comparing the relative amount of pilin in pilus fractions recovered from the surface of the wild type and mutant strains (**Fig. 2.3A**). Since pilin is the major structural subunit of TFP, the amount of pilin recovered in pilus fractions correlates with TFP abundance. While pilin could be recovered from the wild type strain, we were unable to detect pilin in the TFP fraction from the *pilY1* mutant (**Fig. 2.3A**). Comparison of the pilus preparation from the *pilY1* mutant to a serial dilution of the pilus preparation from the wild type strain indicated at least a 100-fold reduction in TFP produced by the *pilY1* mutant (data not shown). Despite the lack of surface TFP, wild type levels of the pilin subunit were detected by Western blot in whole cells lysates from the *pilY1* mutant (**Fig. 2.3C**). These results indicate that that the *pilY1* mutant is defective for TFP biogenesis at a step prior to extrusion of the fiber across the outer membrane.

To rule out the possibility that the TFP biogenesis defect in the *pilY1* mutant was due to a polar effect of the deletion on neighboring genes, we assessed the ability of cloned *pilY1* to restore TFP production when expressed in *trans*. The complete *pilY1* open reading frame (GeneBank #EU234515) was cloned into plasmid pMMBGWV2 (30), such that *pilY1* expression was under the control of an IPTG-inducible *tac* promoter. The complementation plasmid (pPa-*pilY1*) was transferred to the *pilY1* mutant and expression conditions were optimized such that wild type levels of PilY1 could be detected in whole cell lysates by Western blot using PilY1-specific polyclonal antiserum (**Fig. 2.3D**). Under these conditions, expression of plasmid-borne *pilY1* was sufficient to restore TFP production (**Fig. 2.1, 2.2, and 2.3A**). Over-expression of *pilY1* at had no measurable effect on TFP abundance, indicating that wild type levels of PilY1 are not limiting for TFP biogenesis (data not shown). Overall, these results provide definitive evidence that PilY1 is required for the biogenesis of *P. aeruginosa* TFP.

Inactivation of *pilT* suppresses the TFP biogenesis defect in a *pilY1* mutant. PilT is an ATPase required for retraction of TFP in *P. aeruginosa*, *Neisseria* sp., and other gram-negative bacteria (37). Inactivation of *pilT* results in a non-retractile, non-twitching, and hyperpiliated phenotype in *P. aeruginosa* (61) (**Fig. 2.3A, and 2.4**). In *N. gonorrhoeae*, inactivation of *pilT* suppresses the TFP biogenesis defect of a mutant lacking PilC, a pilus-associated protein required for TFP biogenesis (31, 47, 64). PilC was subsequently shown to be an inhibitor of PilT-driven fiber retraction rather than an essential factor in fiber formation (39). Given these findings, as well as the limited homology between *P. aeruginosa* PilY1 and gonococcal PilC, we examined the effect of inactivation of *pilT* on TFP biogenesis in the absence of *pilY1*.

To determine if *pilT* inactivation could suppress the TFP defect of a *pilY1* mutant, we introduced the non-polar *pilY1* deletion in a *pilT* mutant background (58). The resulting *pilY1*, *pilT* double mutant produced detectable TFP (**Fig. 2.1, 2.2, and 2.3A**) in the absence of *pilY1* expression (**Fig. 2.3D**), demonstrating that the TFP biogenesis defect of a *pilY1* mutant is suppressible by inactivation of *pilT*. To confirm that the restoration of TFP production in the absence of PilY1 was due to *pilT* inactivation, we complemented the *pilY1*, *pilT* mutant with plasmid-expressed *pilT* (p*Pa-pilT*). When wild type expression of PilT was restored (**Fig. 2.3E**), TFP biogenesis was abolished, resulting in a TFP phenotype indistinguishable from a *pilY1* mutant (**Fig. 2.1, 2.2, 2.3A**). Conversely, complementation of the *pilY1, pilT* mutant with plasmid-borne *pilY1* (**Fig. 2.3D**) resulted in an increase the level of TFP similar to that seen in a *pilT* mutant (**Fig. 2.1, 2.2, and 2.3A**). Regardless of PilY1, TFP are formed in the absence of *pilT*. Given that *pilT* is involved in retraction of assembled fibers, our data suggests the TFP defect seen in a *pilY1* mutant is not associated with TFP fiber assembly. Instead, analogous to PilC in Neisseria, PilY1 may play a role in stabilizing the pilus structure by counteracting the retraction force from PilT.

Localization of PilY1 to sheared TFP fractions requires additional genes in the *pilY1* operon. PilY1 was previously shown to localize to the sheared pilus fraction of a piliated wild type strain (1). To confirm this result and to assess the contribution of other TFP-associated proteins to PilY1 localization, we used PilY1-specific antiserum to probe TFP fractions recovered from the surface of the wild type and mutant strains (**Fig. 2.3B**). As expected, PilY1 was present in the wild type TFP fraction, but not detected in fractions collected from the non-piliated *pilY1* mutant or the piliated *pilY1, pilT* double mutant. Complementation of the *pilY1* and *pilY1, pilT* mutants with plasmid-encoded *pilY1* resulted in restoration of PilY1 to the TFP fractions (**Fig. 2.3B**). The TFP fraction from the non-piliated *pilA* mutant was devoid of PilY1 (**Fig. 2.3B**), despite the fact that PilY1 could be detected in whole cell lysate from the same strain (**Fig. 2.3D**). The later result demonstrates that PilY1 localization to the TFP fraction requires the presence of surface pili. While not definitive, this result suggests that PilY1 may specifically localize to the assembled pilus fiber.

It is currently unclear if localization of PilY1 to the sheared TFP fraction requires additional proteins. However, *pilY1* is located in an operon with six additional genes (*fimU-pilVWXY1Y2E*) (1, 4). Five of the genes in this operon, encode pilin-like proteins (FimU, PilV, PilW, PilX, and PilE), which are required for pilus biogenesis (1, 2, 3, 48) and share homology with pilin-like proteins in *Neisseria* that are required for the localization of PilC to the pilus fraction (62). Given the apparent similarity between *P. aeruginosa* PilY1 and gonococcal PilC, we examined the requirement of genes in the *pilY1* operon for localization of PilY1 to the sheared pilus fraction. To this end, we created an unmarked deletion of the entire *pilY1* operon. The resulting mutant strain (*fimU-pilE*) was defective for TFP biogenesis (**Fig. 2.5A**). As was seen with the *pilY1* mutant, wild type levels of the pilin subunit were detected by Western blot in whole cells lysates from the *fimU-pilE* mutant (**Fig. 2.5C**), indicating that the defect in TFP biogenesis was not due to reduced subunit availability. Complementation of the *fimU-pilE* mutant with a plasmid-borne copy of the operon (p*Pa-fimU-pilE*) was sufficient to restore TFP biogenesis (**Fig. 2.5A**) and localization of PilY1 to the TFP fraction

(**Fig. 2.5B**). In contrast, complementation of the *fimU-pilE* mutant with plasmid-expressed *pilY1* alone (**Fig. 2.5D**) was not sufficient to restore TFP production (**Fig. 2.5A**) or localization of PilY1 to the sheared surface fraction (**Fig. 2.5B**). These results are consistent with previous studies showing that other genes (*fimU*, *pilV*, *pilW*, *pilX* and *pilE*) within the *pilY1* operon are required for TFP biogenesis and not pilin expression. Further, these results demonstrate that PilY1 cannot be detected in sheared surface fractions collected from non-piliated strains, regardless of its presence in whole cell lysates. While this result suggests that localization of PilY1 to surface fractions may require additional TFP biogenesis components, we cannot rule out the possibility that our fractionation technique is insufficient for isolation of surface localized PilY1 in the absence of TFP fibers.

In the previous section, we demonstrated the ability of a *pilT* deletion to suppress the TFP biogenesis defect of a *pilY1* mutant (**Fig. 2.3**). To determine whether other genes in the *pilY1* operon are conditionally required for TFP biogenesis, we introduced the *fimU-pilE* deletion into the *pilT* mutant background. The resulting strain (*fimU-pilE*, *pilT*) produced TFP (**Fig. 2.5A**), indicating that the entire *pilY1* operon is conditionally required for TFP biogenesis and not essential for fiber assembly. The ability to restore TFP production in the absence of the pilin-like genes (*fimU-pilE*, *pilT*) provided us with the opportunity to determine their role in PilY1 localization in an otherwise piliated background. Complementation of the *fimU-pilE*, *pilT* mutant with plasmid-expressed *pilY1* resulted in wild type levels of PilY1 in whole cell lysates (**Fig. 2.5D**); however, PilY1 was not detectable in the sheared TFP fraction (**Fig. 2.5B**). The results presented in this section indicate that localization of PilY1 to sheared, surface TFP fractions requires both the production of TFP fibers and pilin-like proteins encoded by the *pilY1* operon.

PilY1 is required for *P. aeruginosa* interaction with human lung pneumocyte cells. Previous studies have shown that TFP are required for *P. aeruginosa* adherence to mammalian cells (10, 14, 18, 19). Host cell adherence is required for *in vivo* activation of the *P. aeruginosa* contact-dependent

type III secretion system (T3SS), which causes host cell cytotoxicity (13, 14, 58, 66). Although our *pilY1* mutant lacks measurable TFP *in vitro*, the defect appears to be at the level of fiber stabilization and not TFP assembly. We hypothesized that transient fiber expression in the presence of the appropriate host cell receptor may be sufficient to promote host cell interaction. To assess the role of *pilY1* in host cell interactions, we measured the ability of the *pilY1* mutant to adhere to and cause cytotoxicity in cultured human type II pneumocyte-like carcinoma (A549) cells following infection.

Consistent with previous reports (10, 13), wild type *P. aeruginosa* adhered to A549 cells to a greater extent than the non-piliated *pilA* mutant (**Fig. 2.6**). In addition, the wild type strain was more cytotoxic than the *pilA* mutant and a *pscC* mutant, as measured by the release of the host cytoplasmic enzyme lactate dehydrogenase (LDH) (**Fig. 2.6**). The *pscC* mutant is non-cytotoxic due to a defect in the T3SS (66). The *pilY1* mutant showed a significant reduction in both adherence and cytotoxicity compared to the wild type strain, and was indistinguishable from the *pilA* mutant (**Fig. 2.6**). Complementation of the *pilY1* mutant with plasmid-encoded *pilY1* was sufficient to restore both adherence and cytotoxicity to a wild type level. Thus, the defect in adherence and cytotoxicity is specific to the *pilY1* deletion.

Our findings indicate that *pilY1* is required for *P. aeruginosa* to establish pathogenic interactions with host cells, but it is not clear whether the requirement is solely at the level of TFP production or whether PilY1 directly mediates TFP-dependent adherence. To distinguish between these possibilities, we assessed adherence and cytotoxicity of piliated *pilT* mutants in the presence or absence of *pilY1* (*pilT* versus *pilY1*, *pilT*) (**Fig. 2.6**). Both mutants were defective for adherence and cytotoxicity and were indistinguishable from the non-piliated strains (*pilA* and *pilY1*). The inability of *pilT* mutants to adhere despite being piliated suggests that pilus retraction is a prerequisite for interaction with A549 cells, and prevented us from drawing any conclusions about the role of PilY1 in this system.

TFP are required for infection and invasion of primary human airway epithelial cell cultures. Mutants lacking the pilus retraction protein PilT, were previously reported to display lower levels of adherence to non-polarized or transformed epithelial cell lines (13, 58). The lack of binding to A549 cells by the piliated *pilT* and *pilY1*, *pilT* mutants (**Fig. 2.6**) may be due to their inability to undergo pilus retraction and twitching motility (**Fig. 2.4**), a process that is likely to facilitate tight association of *P. aeruginosa* with host cells. Experiments by Fleiszig et al. and Lee et al. demonstrated preferential binding of *P. aeruginosa* to the basolateral surfaces of well-differentiated mammalian epithelial cells (22, 35). Furthermore, *pilT* mutants are more virulent in animal infection models than non-piliated *pilA* mutants, suggesting that *pilT* mutants may retain the capacity to adhere to host cells *in vivo*, despite their apparent defect *in vitro* (13). Based on these studies, we hypothesized that polarized or well-differentiated cell cultures may provide a more robust model for determining the role of PilY1 in TFP-mediated adherence. To more accurately model the host environment, we evaluated *P. aeruginosa* infection of well-differentiated human airway epithelial (HAE) cell cultures (23). HAE cell cultures are derived from primary human lung epithelial cells, which are allowed to propagate and differentiate into multi-layered cell culture that mimics the organization and structure of the airway epithelium *in vivo* (**Fig. 2.7**).

To determine the feasibility of using the HAE cell culture system as a basis for testing *P. aeruginosa* interactions with host cells, we performed infection experiments with wild type piliated *P. aeruginosa*, the isogenic non-piliated *pilA* mutant and a non-cytotoxic T3SS mutant (*pscC*). Wild type *P. aeruginosa* rarely adhered to the ciliated apical cell surface but caused local infection foci within 3 hours post-deposition onto the apical surface of HAE cell cultures **(Fig. 2.7)**. The wild type strain appeared to interact efficiently with exposed basolateral surfaces of ciliated cells and the entire surface of undifferentiated basal cells (**Fig. 2.7**). Bacterial adherence appeared to be associated with host cell rounding and detachment. By 12 hours, the wild type infection spread intercellularly to encompass the entire HAE culture. In contrast, mutants lacking *pilA* or *pscC* did not adhere to or

interact with the epithelial cultures and could be completely removed from the surface of the epithelium by gentle washing 12 hours post-deposition (**Fig. 2.7**). These results indicate that *P. aeruginosa* can efficiently interact with exposed basolateral surfaces of well-differentiated epithelial cells but that TFP and T3SS are required for initial infection and subsequent invasion of HAE cell cultures. Our observations are consistent with previous studies showing that *P. aeruginosa* preferentially binds to exposed basolateral surfaces of well-differentiated host cells and injured or remodeling epithelial tissues (16, 35, 44, 45).

PilY1 is required for adherence of *P. aeruginosa* to injured HAE cell cultures. To assess the role of PilY1 in TFP-mediated adherence, we modified the HAE infection system to artificially expose underlying basal cells. Specifically, HAE cultures were mechanically abraded to expose the basal cells prior to *P. aeruginosa* infection. To assess bacterial adherence, infections were carried out using strains harboring a plasmid that constitutively expresses GFP (pSMC21) (6). The injured HAE cultures were infected with GFP-expressing strains for 45 minutes, washed, and adherent bacteria were visualized by fluorescence microscopy. Wild type *P. aeruginosa* adhered to the injured region of the HAE culture, but not to the adjacent intact ciliated epithelium (**Fig. 2.8**). In contrast, the non-piliated *pilY1* and *pilA* mutants did not bind to the injured cell cultures (**Fig. 2.8**) indicting a requirement for TFP. Adherence of the *pilY1* mutant could be restored by complementation with plasmid-encoded *pilY1*.

To directly examine the role of PilY1 in TFP-mediated adherence, we compared binding of the piliated, non-retractile *pilT* mutant to the piliated *pilY1*, *pilT* double mutant. The *pilT* mutant, which displayed poor binding to A549 cells (**Fig. 2.6**), adhered efficiently to the damaged region of the HAE culture (**Fig. 2.8**). Conversely, the piliated *pilY1* mutant (*pilY1*, *pilT*) mutant showed little or no binding to the injured HAE cultures (**Fig. 2.8**). Complementation of the *pilY1*, *pilT* mutant, with plasmid-expressed *pilY1*, restored adherence to the injured HAE cultures (**Fig. 2.8**). To confirm the

results of our injured HAE binding experiments, we directly visualized bacterial-host cell interactions in fixed histological cross-sections of the infected cultures using light microscopy. In all cases, the bacterial strains that adhered to the HAE injury site demonstrated specific association with exposed basal cells and basolateral surfaces (**Fig. 2.9**). In contrast, the non-adherent mutants were rarely detected in association with exposed basal cells present in the injury site (**Fig. 2.9**). Our results indicate that binding of *P. aeruginosa* to exposed basolateral surfaces of well-differentiated epithelial cells is TFP-dependent, and does not require pilus retraction. Further, the adherence of *P. aeruginosa* to injured HAE cell cultures appears to require PilY1 localization to the surface TFP fraction, suggesting that PilY1 may function as a TFP-associated adhesin.

Materials and Methods

Bacterial strains and growth conditions

All mutant *P. aeruginosa* strains used in this study were derived from the prototypic laboratory strain PAK and are referenced in **Table 2.1**. The PAK*pilY1* and PAK*pilY1*, *pilT* strains were constructed by introducing a deletion allele for *pilY1* (encoded by the pEXGmΔ*pilY1* plasmid) onto the chromosome of PAK and PAK*pilT* using a previously described method (29). The pEXGmΔ*pilY1* plasmid was constructed by amplifying and splicing chromosomal fragments flanking the *pilY1* ORF by SOE PCR as previously described (66) using pilY1 5'/pilY1 SOE 3' and pilY1 3'/pilY1 SOE 5' oligonucleotide pairs (**Table 2.2**). The resulting deletion allele carries an in-frame stop codon in place of amino acid residues 51 through 801 of the *pilY1* coding sequence. Oligonucleotide pair pilY1 5'/pilY1 3' (**Table 2.2**) were used to confirm *pilY1* deletion as previously described (29). The PAK*fimU-pilE* and PAK*fimU-pilE*, *pilT* strains were constructed by introducing a deletion allele for the *pilY1* operon (*fimUpilVWXY1Y2E*, encoded by pEXGmΔ*fimU-pilE*) onto the chromosome of PAK and PAK*pilT* using a previously described method (29). The pEXGmΔ*fimU-*

pilE plasmid was constructed by amplifying and splicing chromosomal fragments that flank the *pilY1* operon by SOE PCR as previously described (66) using fimU-pilE 5'/fimU-pilE SOE 3' and fimU-pilE 3'/fimU-pilE SOE 5' oligonucleotide pairs. The resulting deletion plasmid lacks the coding sequence from the *fimU* start codon to the *pilE* stop codon. Oligonucleotide pairs fimU-pilE 5'/fimU-pilE 3' and fimU-pilE 3'/pilY1 3' (**Table 2.2**) were used to confirm deletion of the *pilY1* operon as previously described (29). For routine passage, *E. coli* and *P. aeruginosa* were grown at 37°C in Luria Bertani (LB) medium. The pMMB-based expression plasmids were maintained in *P. aeruginosa* with 150 μg/ml carbenicillin (Cb), except where noted. Bacterial growth in broth culture was assessed by optical density at 600 nm (OD_{600}).

Expression plasmid construction

The open reading frame (ORF) of *P. aeruginosa pilY1* and the entire *pilY1* operon (*fimUpilVWXY1Y2E*, referred to as "*fimU-pilE*") were amplified from chromosomal DNA from strain PAK and cloned into pMMBV2GW (p*Pa-pilY1* and p*Pa-fimU-pilE*) using Gateway Technology (Invitrogen) as described previously (66). Briefly, PCR fragments were tailed with *att*B1 and *att*B2 sequences, cloned into the pDONR201 entry vector and then transferred to the destination vector (pMMBV2GW). The pMMBV2GW plasmid is a version of the *P. aeruginosa* expression plasmid pMMBGW (66) in which the sequence of the -35 region of the *tac* promoter was changed from TTGACA to TTTACA, and the -10 region of the *tac* promoter was changed from TATAAT to CATTAT (M. Wolfgang and S. Lory, unpublished). These promoter region modifications were predicted to reduce the efficiency of transcriptional initiation at the *tac* promoter, allowing for reduced levels of expression. The *pilY1* ORF was PCR amplified using the 5' pilY1-2 and 3' pilY1-2 oligonucleotide pair. The entire *pilY1* operon was PCR amplified using 5' fimU and 3' pilE oligonucleotides. All expression plasmids were transferred to the appropriate PAK strain by conjugation (24) followed by selection on LB agar plates containing 150 μg/ml Cb and 25 μg/ml

irgasan. Unless otherwise noted, strains containing expression plasmids were grown in the presence of 20 μM (p*Pa-pilT)* or 75 μM (p*Pa-pilY1 and* p*Pa-fimU-pilE*) isopropyl-β-D-thiogalactopyranoside (IPTG).

Microscopy

Transmission electron microscopy (TEM) was performed as described previously (63), with the exception that grids were placed on a drop of bacterial suspension at 22°C for 10 minutes and the samples were stained with an aqueous 0.5% ammonium molybdate solution for 10 minutes. Immunofluorescence (IF) microscopy was performed as described previously (62), except that the *P. aeruginosa* strains were grown until OD_{600} = 0.2 prior to incubating the bacteria on poly-L-lysine-coated glass cover slips. *P. aeruginosa* pilin-specific antiserum (gift of E. C. Gotschlich, Rockefeller University) was used as a primary antibody for TFP labeling, followed by an Alexa Red-conjugated goat anti-rabbit IgG (Molecular Probes).

Pilus Preparation

To isolate TFP sheared from the bacterial surface, *P. aeruginosa* strains were inoculated for confluent growth on LB agar plates. For assays with plasmid-harboring strains, bacteria were grown on LB agar containing 30 μg/ml Cb and the indicated amount of IPTG. After incubation at 37°C for 18 hours, bacteria were collected and suspended in 10 ml of 0.15 M NaCl and 0.2% (w/v) formaldehyde. The suspensions were vortexed for 1 minute and cells were removed by centrifugation at 12,000 × *g* for 5 minutes. The supernatant was transferred to a 15 ml glass Corex tube, adjusted to 0.1 M $MgCl_2$, and incubated at 4°C for 3 hours. Following centrifugation 12,000 × *g* for 5 minutes, the resulting pellet was washed and suspended in SDS-PAGE sample buffer, resolved on an 18% (w/v) polyacrylamide gel and visualized by GelCode Blue Stain (Pierce). The relative amount of pilin

monomer demonstrated by SDS-PAGE was used as an indicator of the abundance of TFP on the bacterial surface.

Twitching motility assay

P. aeruginosa strains were grown overnight at 37°C on LB agar plates and bacteria were stab-inoculated to the bottom of 100 mm tissue culture-treated dishes (Corning) containing 5 ml LB plus 1% (w/v) agar. For assays with plasmid-harboring strains, bacteria were grown in the presence of 30 μg/ml Cb and the indicated amount of IPTG. Plates were incubated for 48 hours at 37°C in a humidified chamber. The zone of subsurface bacterial growth radiating from the point of inoculation was measured at 24 and 48 hours post-inoculation and reported as a rate (mm/hr) of twitching zone expansion.

Western Blots

Whole cell lysates for detection of pilin, PilY1, and PilT were prepared from bacteria grown in LB broth until OD_{600} = 1. Bacteria were collected by centrifugation and resuspended in 50 μl of SDS-PAGE sample buffer. Samples were run on 18% (pilin and PilT) or 7.5% (PilY1) SDS-polyacrylamide gels and transferred to nitrocellulose. Membranes were probed with the following primary antibodies: PKL1 anti-pilin monoclonal antibody (68) (1:30,000 dilution, gift of Randall Irvin, University of Alberta), anti-PilT rabbit serum (1:30,000 dilution, gift of Katrina Forest, University of Wisconsin), or anti-PilY1 rabbit serum (1:4000 dilution). PilY1-specific serum was generated in collaboration with Dr. Matt Redinbo (University of North Carolina-Chapel Hill) and Cocalico Biologicals. A horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig (Rockland) was used as a secondary antibody at a dilution of 1:80,000 (PilY1 and PilT). An HRP-conjugated goat anti-mouse Ig antibody (Promega) was used at a 1:40,000 for detection of the PKL1 anti-pilin

antibody. Blots were developed with enhanced chemiluminescence reagents (Millipore) and visualized by autoradiography.

Binding and Cytotoxicity assays

Binding of *P. aeruginosa* strains to A549 cells, as well as the associated cytotoxicity in these infections, was assessed as described previously (13). Human airway epithelial (HAE) cell cultures were collected and grown on transwell membranes as described (23), with the exception that cells were grown in the described ALI media until 1 day prior to infection, when they were transferred to antibiotic-free ALI. A plasmid that constitutively expresses GFP (pSMC21) (6) was transferred into PAK, and the indicated mutant strains, as previously described (24). Successful GFP expression was determined by visual observation. The subsequent GFP-expressing strains were grown in LB broth with 50 μg/ml Kanamycin until OD_{600} = 1.0, washed twice with sterile PBS, and resuspended in 1 ml of antibiotic-free ALI cell culture media to a concentration of $4x10^8$ cfu/ml. HAE cultures were injured by lightly dragging a sterile 200 μl pipette tip across the apical cell surface, leaving a thin layer of exposed basal cells. A total of 250 μl of each strain was added to the appropriate HAE culture at an approximate MOI of 1000. After incubation at 37°C and 5% CO_2 for 45 minutes, all HAE cultures were washed six times with pre-warmed antibiotic-free ALI media. After the final wash, all media was removed from the surface of the HAE cultures. Binding of GFP *P. aeruginosa* to the injury site was assessed visually and recorded using a Leica DMIRB Inverted Fluorescence/DIC microscope with black/white and color digital camera. Phase contrast images of the infected cultures were taken in parallel in order to locate the site of injury. After imaging, infected HAE cultures were fixed in a solution of 2% (w/v) Formaldehyde + 2% (w/v) Paraformaldehyde overnight, embedded in epon resin, and sectioned perpendicular to the injured section of the HAE culture. Semi-thin sections from the center of the cell culture preparation were stained with Richardson's stain to allow for

successful distinction of single bacteria and HAE cells. Representative sections were visualized by light microscopy and imaged.

Discussion

For many gram-negative pathogens, TFP play an important role in host infection and colonization. While these organelles are associated with a variety of species-specific phenotypes, their primary function appears to involve the presentation of adhesive molecules or epitopes that interact with receptors on host cells and tissue. The best example of a TFP associated adhesin is the PilC protein of *Neisseria gonorrhoeae* and *N. meningitidis*. Purified PilC is capable of binding to host cells and can competitively blocks epithelial cell adherence of both piliated gonococci and meningococci (33, 46). In addition to their role in adherence, TFP expressed by a number of organisms are retractile (37). When coupled with adherence to a host receptor, retraction can facilitate intimate or tight adherence (39). In addition, rounds of extension and retraction can drive surface motility (twitching motility), which can facilitate bacterial spread across abiotic surfaces as well as dissemination within host cells and tissue (39, 54). Interestingly, PilC has been shown to antagonize pilus retraction, suggesting a possible mechanistic link between the adhesin-receptor interaction, intimate attachment and pilus-dependent motility (39, 64). While the pilus-associated adhesin in *Neisseria* species has been well characterized, little is currently known about TFP-associated adhesins in other pathogenic bacteria. Here we describe the dual role of *Pseudomonas aeruginosa* PilY1 in TFP biogenesis and adherence.

With the exception of pathogenic *Neisseria*, the adhesive component of TFP produced by other species is unknown or poorly characterized. In *P. aeruginosa*, an exposed disulphide loop in the C-terminus of the pilin monomer is believed to mediate TFP adhesion to exposed asialo-G_{M1} and asialo-G_{M2} receptors on the apical surface of target host cells. Recently, studies have questioned the direct role of pilin in TFP-mediated adherence. Emam et al. demonstrated a lack of binding between

piliated *P. aeruginosa* to either asialo-G_{M1} or asialo-G_{M2} (21). This data, along with the prior PilC work in *Neisseria*, led us to examine the role of PilY1, a protein with limited homology to *Neisseria* PilC, in *P. aeruginosa* TFP-dependent adherence to human epithelial cells.

We demonstrate the requirement of PilY1 for TFP-mediated adherence to exposed basolateral surfaces of primary human epithelial cells (**Fig. 2.8 and 2.9**). *P. aeruginosa* is only able to bind to the airway epithelium when PilY1 is present in the TFP fraction. Binding is abolished in the absence of pilus fibers or when PilY1-lacking TFP are produced (*pilY1*, *pilT*). While this does not rule out the contribution of pilin to adherence, it does indicate the importance of PilY1 in the process and points to a high affinity host cell receptor, which is only present on the basolateral surface of well-differentiated cells.

It is currently unclear why our wild type strain displays relatively poor adherence to A549 cells (**Fig. 2.6**). However, *P. aeruginosa* is known to have poor binding efficiency to mammalian cells when they are grown as undifferentiated monolayers (13, 35, 66). Despite the low level of adherence of *P. aeruginosa* to undifferentiated cell lines, a clear requirement for TFP has been shown (**Fig. 2.6**). Surprisingly, adherence in these systems also requires pilus retraction, as the binding efficiency of a hyperpiliated *pilT* mutant is indistinguishable from that of non-piliated strains (**Fig. 2.6**) (13). This poor binding efficiency could be due to the absence of a critical epithelial cell receptor, or in the case of the *pilT* mutant, the lack of pilus retraction. It has been proposed that TFP are likely to have multiple mechanisms for adhering to surfaces, including reversible adherence and high-affinity adherence (8). Reversible adherence is likely to be non-specific and necessary for twitching motility. Such that, following extension, the pilus can tether to a variety of surfaces and provide an anchor for retraction. This interaction needs be relatively non-specific, so that the bacteria can move across a variety of surfaces, and of low affinity so that adherence is reversible. It is conceivable that low affinity adherence to undifferentiated cell lines involves the DSL region of pilin

and that subsequent pilus retraction may facilitate additional interactions between the bacteria and host cell surface. This may account for the discrepancy between our results with A549 monolayers and injured HAE cell cultures.

Previously, Alm et al. defined a general localization of PilY1 to the pilus fraction of a wild type strain, with a low level of protein in the membrane fraction (1). While our findings are in agreement with published data, we have extended the observation to show that additional genes in the *pilY1* operon (FimU, PilV, PilW, PilX, and PilE), which show a high degree of homology with N-terminal domain of pilin, are required for localization of PilY1 to TFP fraction (**Fig. 2.5**). Similar results were seen for *Neisseria* PilC, which also requires the expression of pilin-like proteins in order to associate with the pilus fraction (62). While our data does not prove that PilY1 is part of the pilus fiber, it does indicate the localization of PilY1 is specific to the pilus fraction. The *P. aeruginosa* pilin-like proteins may be required to trafficking PilY1 to the pilus fraction or may be part of a PilY1 containing complex that is associated with the pilus fiber. In either case, localization of PilY1 to the pilus fraction is consistent with its role as a TFP-associated adhesin.

In addition to its role in TFP-mediated adherence, PilY1 also appears to play a role in formation of the pilus fiber. Initial experiments indicated PilY1 might be essential for TFP biogenesis. When *pilY1* is inactivated in a wild type background, we are unable to detect TFP fibers (**Fig. 2.1, 2.2 and 2.3**). However, when pilus retraction is abolished by inactivating *pilT* (**Fig. 2.4**), we are able to restore detectable TFP in a *pilY1* mutant (**Fig. 2.1, 2.2 and 2.3**). Therefore, PilY1 appears to be conditionally required for TFP biogenesis.

The mechanism used by PilY1 to affect TFP biogenesis is currently unknown. However, we can define a potential mechanism using what is known about TFP in *P. aeruginosa*, as well as data from PilC in *Neisseria*. PilT is an ATPase that is required for retraction of TFP in *P. aeruginosa*, *Neisseria*, and other gram-negative bacteria (37). When *pilT* is inactivated, pili are unable to retract

and the resulting mutants are unable to twitch (**Fig. 2.4**) (38, 61). Given that TFP biogenesis is restored in a *pilY1* mutant when *pilT* is inactivated, the role of PilY1 in the biogenesis process appears to center around counteracting or antagonizing PilT-driven pilus retraction. This hypothesis is supported by data from PilC in *Neisseria*. Like PilY1, PilC is required for TFP biogenesis in a wild type background but not in a *pilT* mutant background (31, 47, 64). Further analysis in *Neisseria* described PilC as an inhibitor of PilT-driven fiber retraction rather than an essential factor in fiber formation (39). Specifically, efficient TFP assembly occurs, regardless of the amount of PilC present, in strains lacking the pilus retraction protein. In an adherence model, where pilus extension and retraction are sequentially observed, down regulation of *pilC* correlates with pilus retraction. Thus, pilus retraction accounts for the apparent defect in TFP biogenesis seen in *pilC* mutants. Given our data describing the effect of a *pilT* inactivation on the TFP biogenesis defect of a *pilY1* mutant, as well as the homology between PilC and PilY1, it is reasonable to predict that PilY1 acts as an inhibitor of PilT-driven fiber retraction rather than an as essential factor in fiber assembly.

Interestingly, the homology between PilY1 and *Neisseria* PilC it is limited to the C-terminal half of these proteins. We also find similar C-terminal homology between PilY1 and proteins in other bacteria, which are required for TFP biogenesis (data not shown). The size of PilY1 (approximately 117 kD) and other related proteins indicates the potential for two different functional domains. A similar architecture exists for hemagglutinins, which are large proteins comprised of both structural and adhesive domains (17). Given the apparent dual roles of both PilY1 and PilC in TFP biogenesis and adherence, it is plausible that the C- and N-terminal portions of these proteins play different functional roles. For instance, the C-terminal half of PilY1 may be involved in TFP biogenesis, while the N-terminal half would be responsible for adherence. If the N-terminus is involved in adherence, it would make sense that it would diverge in sequence to match the host and tissue tropism of the individual pathogen This hypothesis is supported by data from Morand et al., who demonstrated that the adhesion-promoting region of PilC is located in the N-terminal part of the molecule (40).

Therefore it appears PilY1 and PilC are members of a family of bi-functional proteins that are defined by their C-terminal homology, divergent N-terminal domains, and function in biogenesis and adherence of Type IV pili.

In summary, we have demonstrated that PilY1 plays an important and potentially central role in the function of *P. aeruginosa* TFP. Localization of PilY1 to the pilus fraction may provide a means for both antagonizing fiber retraction and providing adhesive properties to the organelle. Further, we have described a model of infection by which PilY1 is required for robust adherence to exposed basal cells and basolateral surfaces of injured HAE cell cultures. These results demonstrate the utility of HAE cell cultures in studying the early steps of *P. aeruginosa* infections. Additionally, we believe that PilY1 is a member of a larger family of bi-functional proteins that many confer similar properties to TFP expressed by a wide range of gram-negative pathogens. Though more work is required to elucidate the specific mechanisms controlling pilus retraction and adherence, we believe that understanding the function of PilY1 will provide new insight into these complex and dynamic processes.

ATTRIBUTIONS

The experiments and results described in Chapter 2 are the sole work of Ryan Heiniger, with the following exceptions:

- TEM and IF images were provided by Dr. Hanne Winther-Larsen (Laboratory of Dr. Michael Koomey).
- Dr. Raymond Pickles assisted in developing the HAE binding assay, provided HAE cells for use in infection and binding assays, and performed the actual "injury" of the HAE cultures.
- Initial HAE infection experiments with PAK, *pilA*, and *pscC* strains were performed by Dr. Matthew Wolfgang and Dr. Raymond Pickles.
- Dr. Matthew Redinbo, in conjunction with Dr. Jill Orans, provided purified C-terminal PilY1 for use in developing PilY1 anti-serum
- Cross-sections were constructed by the Histology Core of the Cystic Fibrosis Center (UNC)

Table 2.1. List of Strains and Plasmids

Strain/plasmid	Description/relevant characteristics*	Source or reference
PAK	wild type	D. Bradley
PAK*pilA*	Non-polar *pilA* deletion in PAK	Kagami et al. (1998)
PAK*pilT*	Non-polar *pilT* deletion in PAK	Sundin et al. (2002)
PAK*pscC*	Non-polar *pscC* deletion in PAK	Wolfgang et al. (2003)
PAK*pilY1*	Non-polar *pilY1* deletion in PAK	This study
PAK*pilY1*, *pilT*	Non-polar *pilY1* and *pilT* deletion in PAK	This study
PAK*fimU-pilE*	Non-polar deletion of *fimUpilVWXY1Y2E* in PAK	This study
PAK*fimU-pilE*, *pilT*	Non-polar deletion of *fimUpilVWXY1Y2E* and pilT in PAK	This study
Plasmid		
pDONR201	Gateway cloning vector; Km^r	Invitrogen
pEXGmΔ*pilY1*	Suicide vector containing *pilY1* deletion allele; Gm^r	This study
pEXGmΔ*fimU-pilE*	Suicide vector containing *fimUpilVWXY1Y2E* deletion allele; Gm^r	This study
pMMB67EH	Empty *P. aeruginosa* expression vector; Ap^r	Furste et al. (1986)
pMMBGW	Gateway-adapted version of pMMB67EH; Ap^r	Wolfgang et al. (2003)
pSMC21	Constitutive expresser of GFP; Km^r	Bloemberg et al. (1997)
pMMBV1GW	pMMBGW with altered -35 sequence; Ap^r	This study
pMMBV2GW	pMMBV1GW with altered -10 sequence; Ap^r	This study
p*Pa-pilT*	*P. aeruginosa pilT* encoded by pMMBV1GW; Ap^r	This study
p*Pa-pilY1*	*P. aeruginosa pilY1* encoded by pMMBV2GW; Ap^r	This study
p*Pa-fimU-pilE*	*P. aeruginosa fimUpilVWXY1Y2E* encoded by pMMBV2GW; Ap^r	This study

*Ap^r, ampicillin resistance marker; Km^r, kanamycin resistance marker; Gm^r, gentamicin resistance marker

Table 2.2. List of Primer Sequences

Primer	Sequence
pilY1 5’	5’-TACAAAAAAGCAGGCTGGACGCTTCGCCATGATGTTCCTG-3’
pilY1 3’	5’-TACAAGAAAGCTGGGTAACATTACCTTCTCGATGCCCAAG-3’
pilY1 SOE 5’	5’-CCTCGCATGAAATCGGTACTCCACGAATTCGGGGTCGTGGGACTCGAAATCCGA-3’
pilY1 SOE 3’	5’-TCGGATTTCGAGTCCCACGACCCCGAATTCGTGGAGTACCGATTTCATGCGAGG-3’
5’ pilY1-2	5’-TCGAGGAGGATATTCATGAAATCGGCACTCCACCAG-3’
3’ pilY1-2	5’-CAAGAAAGCTGGGTTTCAGTTCTTTCCTTCGATGGG-3’
fimU-pilE 5’	5’-TACAAAAAAGCAGGCTGTCAACAGGCCGATGACGCCAGCG-3’
fimU-pilE 3’	5’-TACAAGAAAGCTGGGTGAACTCGATCAGGTGCCGAACAACG-3’
fimU-pilE SOE 5’	5’-GACCCCCTGGAGCAACCGCATGTCTGAAACGAAAGAGCCCCTCTACGAG-3’
fimU-pilE SOE 3’	5’-CTCGTAGAGGGGCTCTTTCGTTTCAGACATGCGGTTGCTCCAGGGGGTC-3’
5’ fimU	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGAGGAGGATATTCATGTCATATCG-3’
3’ pilE	5’-GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAGCGCCAGCAGTCGTTGACGGT-3’

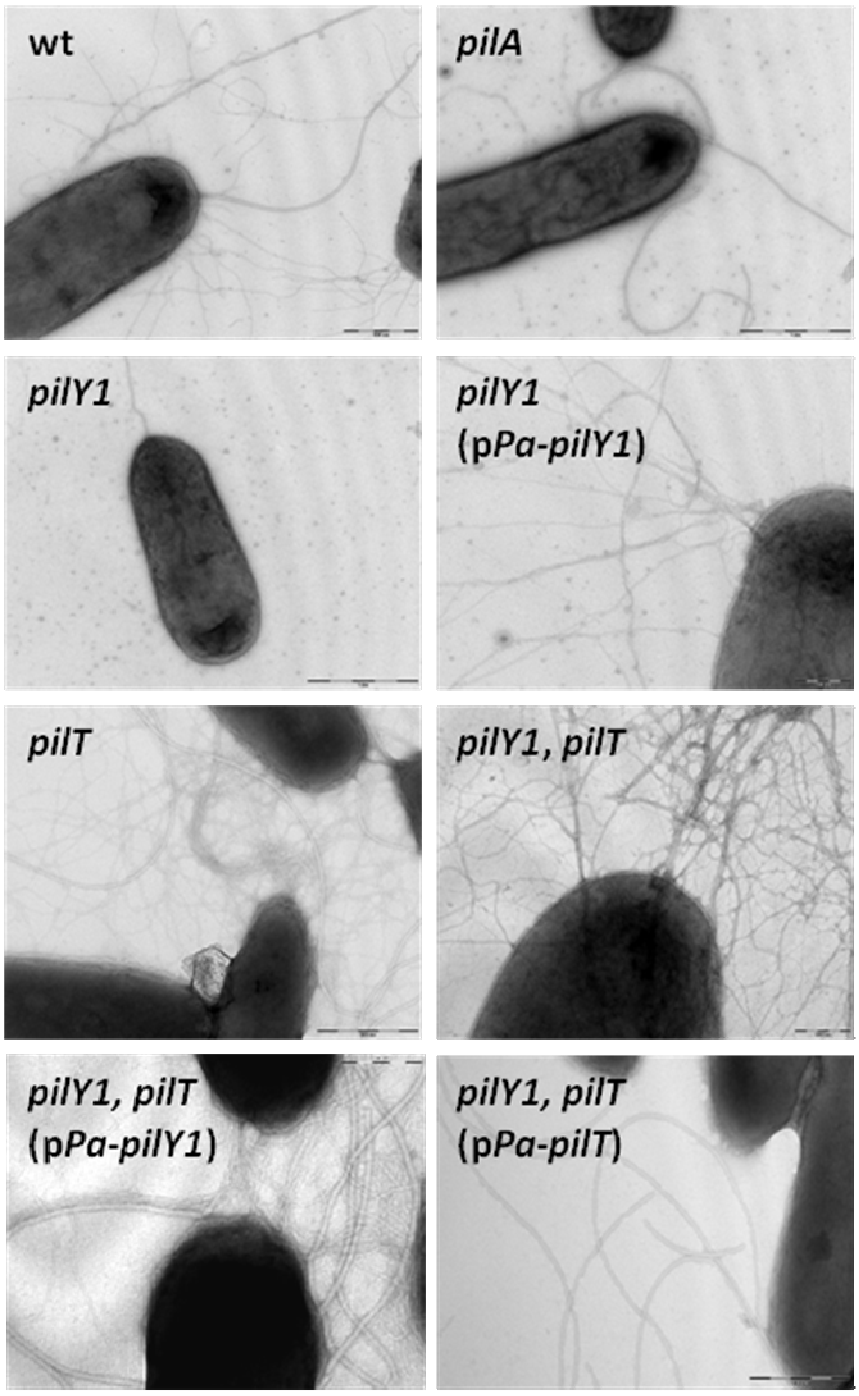

Figure 2.1. Inactivation of *pilT* suppresses the TFP biogenesis defect of a *pilY1* mutant (TEM). Pili of the indicated strains were visualized by Transmission Electron Microscopy (TEM). Visible pilus fibers are absent in a *pilY1* mutant, similar to what is seen with the negative control (*pilA*). Wild type levels of pilus fibers are restored when *pilY1* is complemented with plasmid-expressed *pilY1* (*pilY1* (p*Pa-pilY1*)), indicating the TFP biogenesis defect is specific to the *pilY1* mutation. Visible pilus fibers are also restored in a *pilY1* mutant when *pilT* is inactivated in the same strain (*pilY1*, *pilT*), indicating PilY1 is not required for essential biogenesis of the pilus fiber.

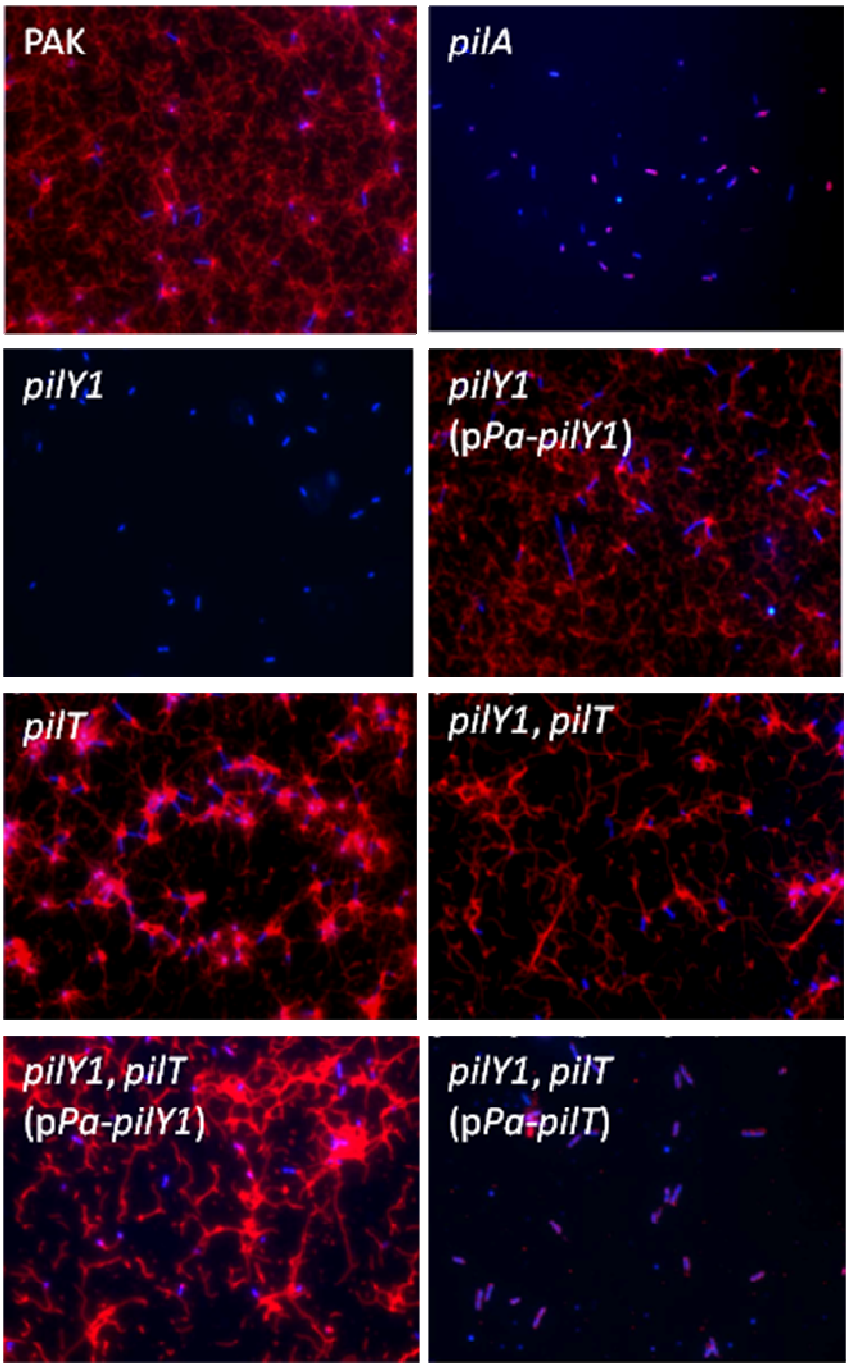

Figure 2.2. Inactivation of *pilT* suppresses the TFP biogenesis defect of a *pilY1* mutant (IF). Pili of the indicated strains were visualized by immunofluorescence microscopy. The *pilY1* mutant has no detectable pilus fibers, and appears similar to the negative control (*pilA*). Detectable fibers are restored to near wild type levels when *pilY1* is complemented *in trans* with plasmid-expressed *pilY1* (*pilY1* (p*Pa-pilY1*)), indicating the TFP biogenesis defect is specific to *pilY1*. Most importantly, detectable pilus fibers are restored in a *pilY1* mutant when *pilT* is inactivated in the same strain (*pilY1*,*pilT*), indicating PilY1 is not required for essential biogenesis of the pilus fiber.

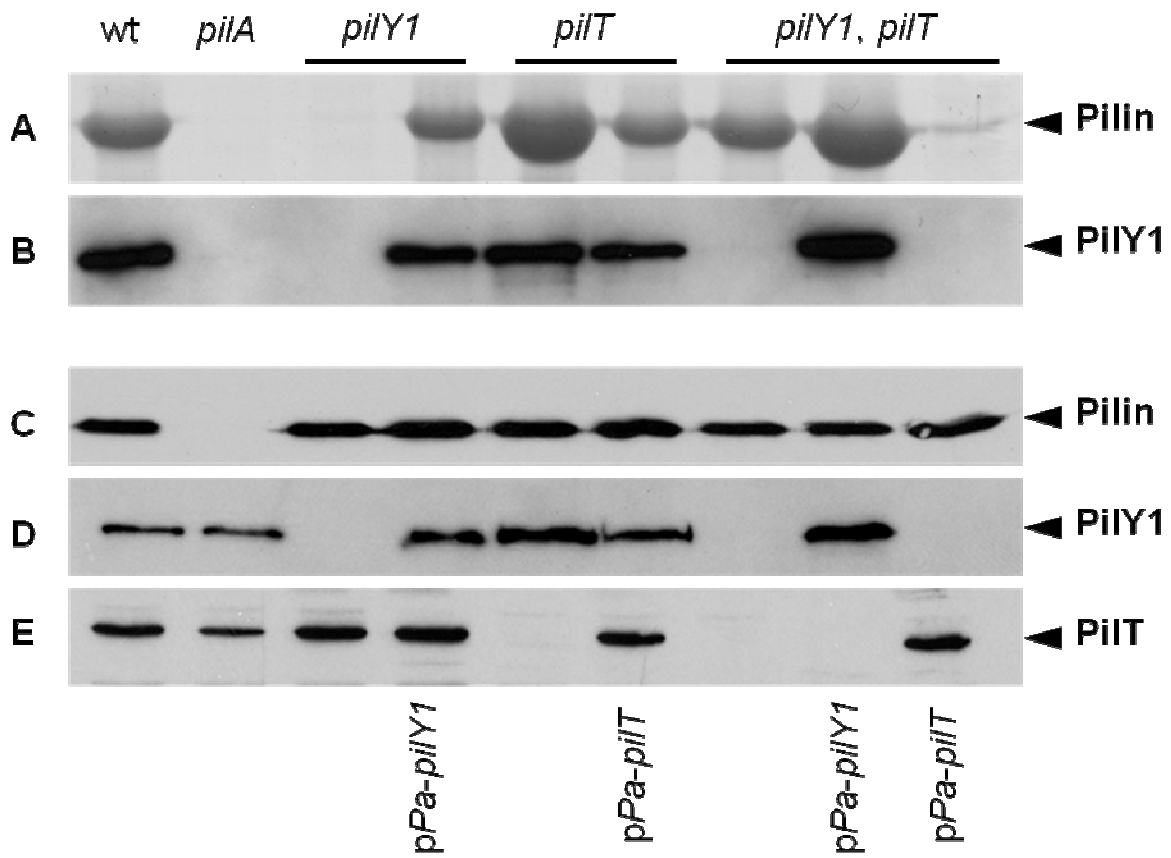

Figure 2.3. PilY1 is necessary for TFP biogenesis, but not required for pilin expression or assembly. Coomassie Blue-stained gel showing the relative abundance of recovered pilin in the pilus fractions (**A**). Western blot of separated pilus fractions probed with PilY1-specific antiserum (**B**). Whole cell lysates separated by SDS-PAGE and probed with pilin-specific (**C**), PilY1-specific (**D**) or PilT-specific (**E**) antiserum by Western blotting. Western blot of *pilT* expression in whole cell lysates is included as a control (**E**). Pilin is absent in a sheared pilus fraction of a *pilY1* mutant, but is restored when *pilT* is deleted in the same strain (*pilY1*,*pilT*) (**A**). The TFP biogenesis defect of a *pilY1* mutant is NOT associated with changes in *pilA* expression within the cell (**C**). In strains where PilY1 is present in whole cell lysates (**D**), it is also found in the sheared pilus fraction (**B**) if pilus fibers are present.

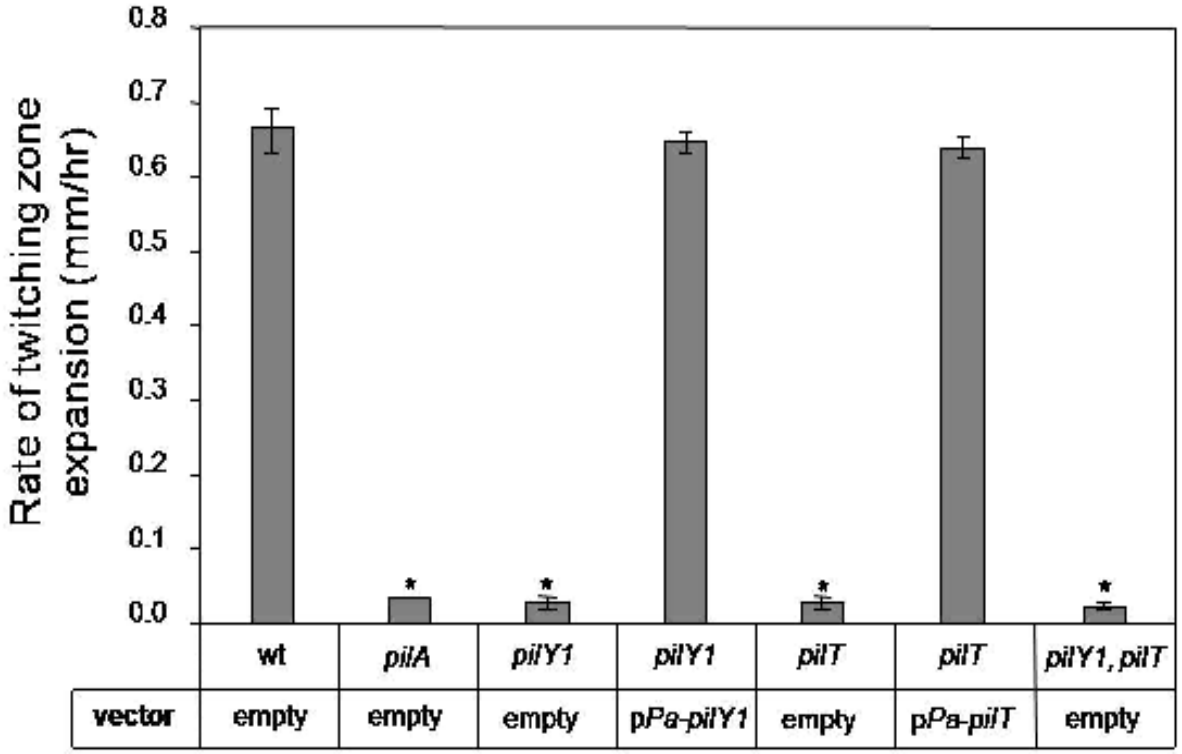

Figure 2.4. Lack of TFP biogenesis results in a twitching motility defect in a *pilY1* mutant. Recorded rates of twitching zone expansion over a period of 24 hours are described above. The *pilY1* mutant is as defective for twitching motility, similar to the negative control (*pilA*) and a *pilT* mutant. The lack of twitching motility in a *pilT* mutant is due to the absence of retractile pili. However the twitching defect of a *pilY1* mutant is most likely due to the absence of pilus fibers on the surface of the cell. Twitching in a *pilY1* mutant is restored to wild type levels when *pilY1* is complemented by plasmid expressed PilY1 (p*Pa-pilY1*), indicating the defect is specific to the *pilY1* mutation. The grey bars describe the mean of three replicates (n=3) per strain and condition, with standard deviation per mean represented by the associated error bars. The values for the indicated strains (*) are significantly different ($p<0.001$) when compared pair wise to the wild type strain.

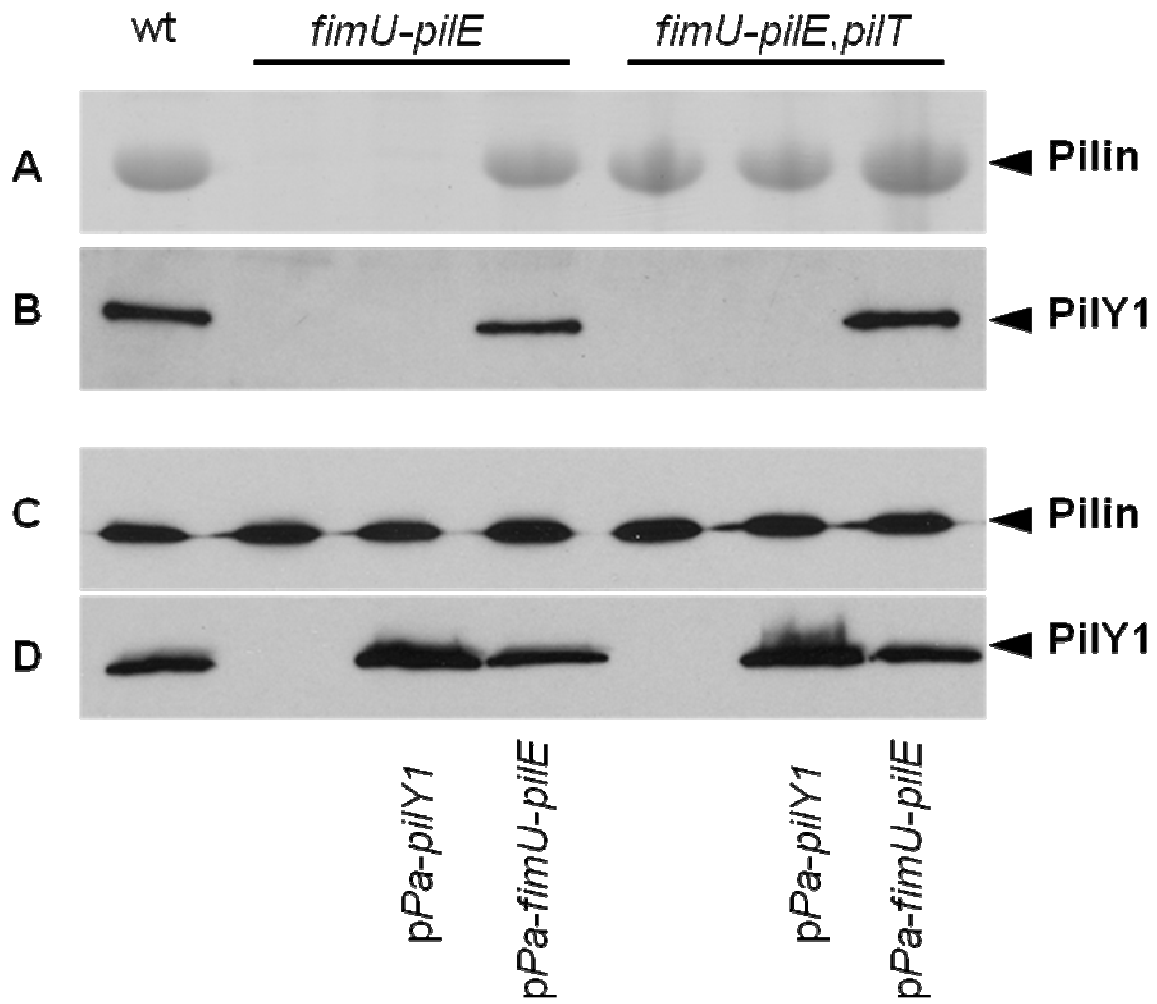

Figure 2.5. Localization of PilY1 to the sheared pilus fraction requires additional genes on the *pilY1* operon. Coomassie Blue-stained gel showing the relative abundance of recovered pilin in the pilus fractions (**A**). Western blot of separated pilus fractions probed with PilY1-specific antiserum (**B**). Whole cell lysates separated by SDS-PAGE and probed with either pilin-specific (**C**) or PilY1-specific (**D**) antiserum by Western blotting. Deletion of the *pilY1* operon (*fimU-pilE*) abolishes TFP biogenesis (**A**), but does not affect pilin expression (**C**). The defect in TFP biogenesis can be suppressed by inactivation of *pilT* (*fimU-pilE*, *pilT*) (**A**). Complementation of the piliated *fimU-pilE*, *pilT* mutant with plasmid-encoded PilY1 (p*Pa-pilY1*)(**D**) does not restore localization of PilY1 to the pilus fraction (**B**). Only complementation the entire *pilY1* operon (p*Pa-fimU-pilE*) restores localization of PilY1 to the pilus fraction (**B**), indicating one or more of the genes located on the *pilY1* operon (*fimUpilVWXY1Y2E*) are required for successful localization of PilY1 to the pilus fraction.

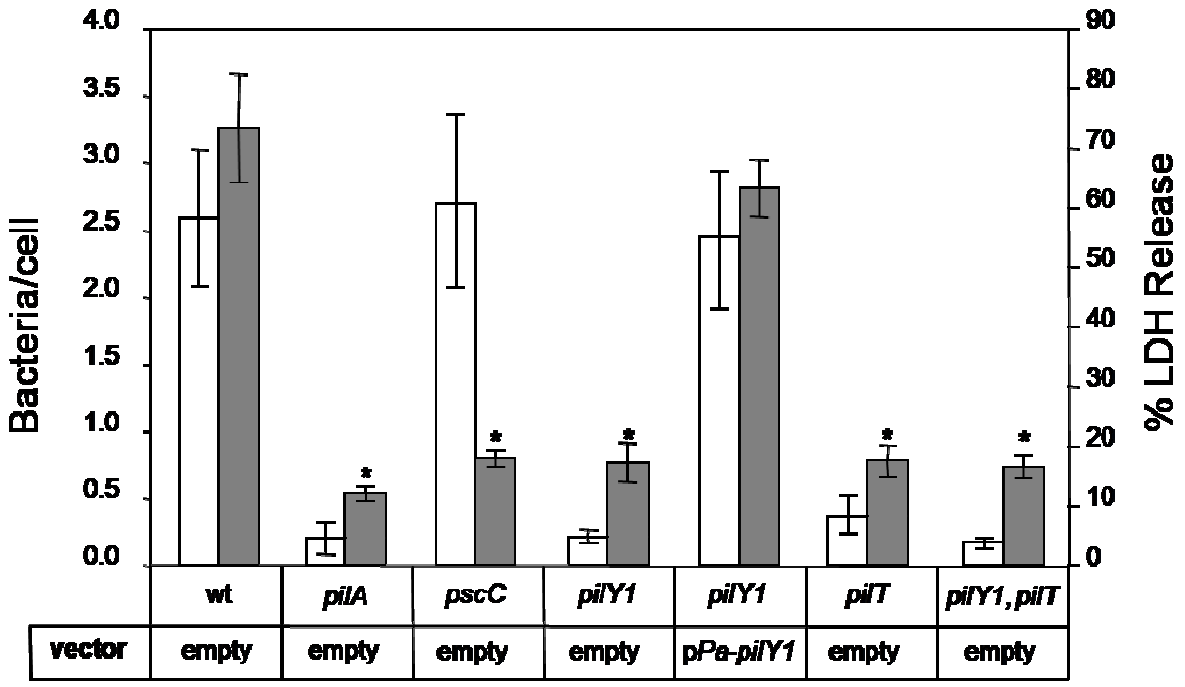

Figure 2.6. PilY1 is necessary for adherence and cytotoxicity of A549 cells *in vitro*. Levels of wild type and mutant binding to (white bars) and cytotoxicity of (grey bars) A549 cells are shown. The *pilY1* mutant is defective for both binding and cytotoxicity. This defect is reversible upon complementation with plasmid-expressed PilY1, which restores A549 binding and cytotoxicity to near wild type levels. However, the binding and cytotoxicity defect remains in strains that express non-retractile pilus fibers in the presence (*pilT*) and absence (*pilY1*,*pilT*) of PilY1. The type III secretion mutant (*pscC*) is included as a control for cytotoxicity. The bars describe the mean of three replicates (n=3) per strain and condition, with standard deviation per mean represented by the associated error bars. The values for the indicated strains (*) are significantly different ($p<0.001$) when compared pair wise to the values for the wild type strain.

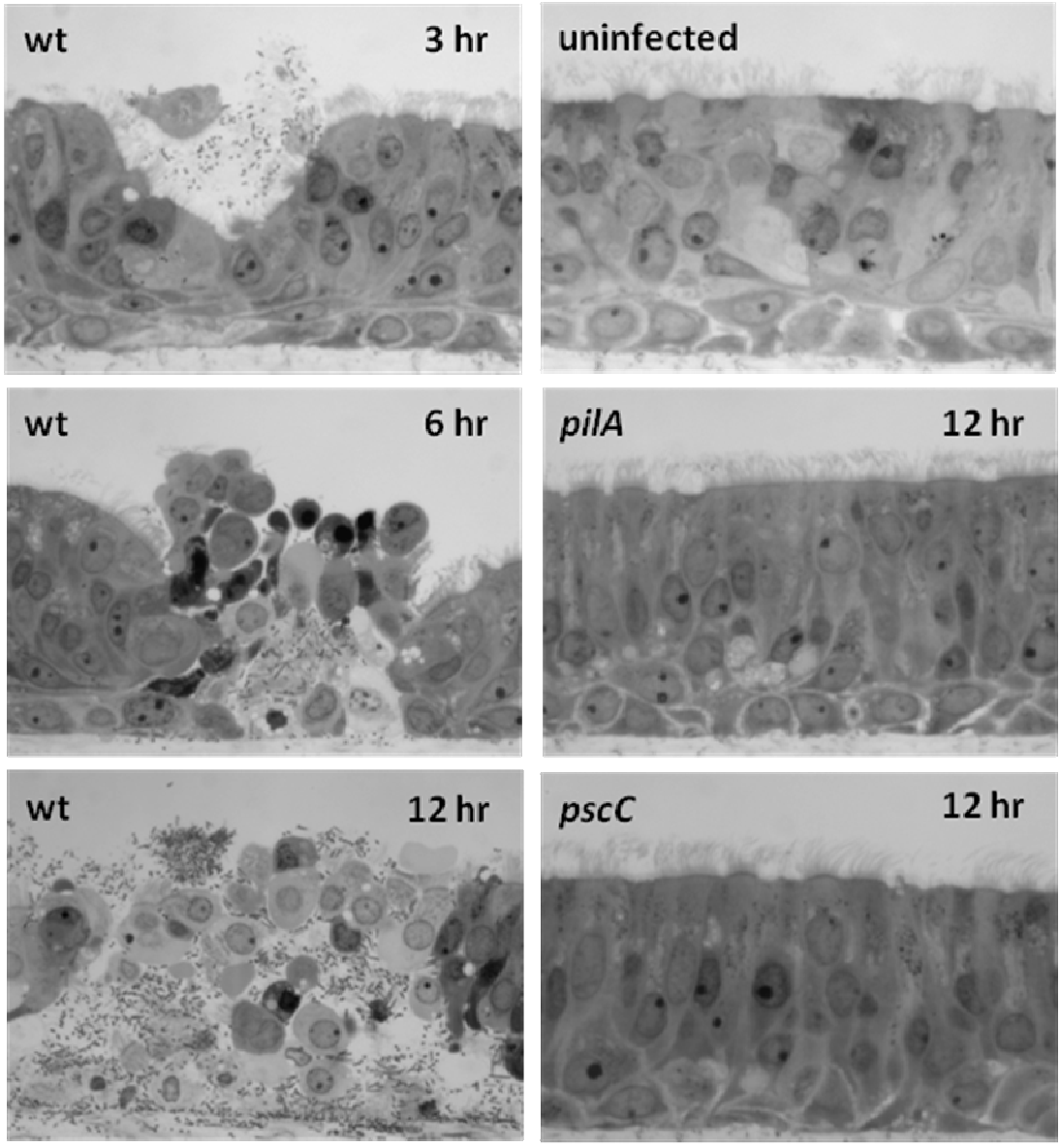

Figure 2.7. *Pseudomonas aeruginosa* requires TFP to infect well-differentiated human airway epithelial (HAE) cultures. Cross-sections of HAE cultures infected with wild type, *pilA*, and *pscC* strains. Representative images of each infection were taken at 3, 6, and 12 hours post infection. Wild type *P. aeruginosa* rarely adhered to the ciliated apical cell surface but local infection foci could be detected after 3 hours. The wild type strain interacted efficiently with the basolateral surfaces of ciliated cells and the entire surface of undifferentiated basal cells. Bacterial adherence was associated with host cell rounding and detachment. By 12 hours, the wild type infection appeared to spread intercellularly to encompass the entire HAE culture. In contrast, neither the non-piliated *pilA* mutant nor the type III secretion mutant (*pscC*) ever spread beyond the airway surface liquid.

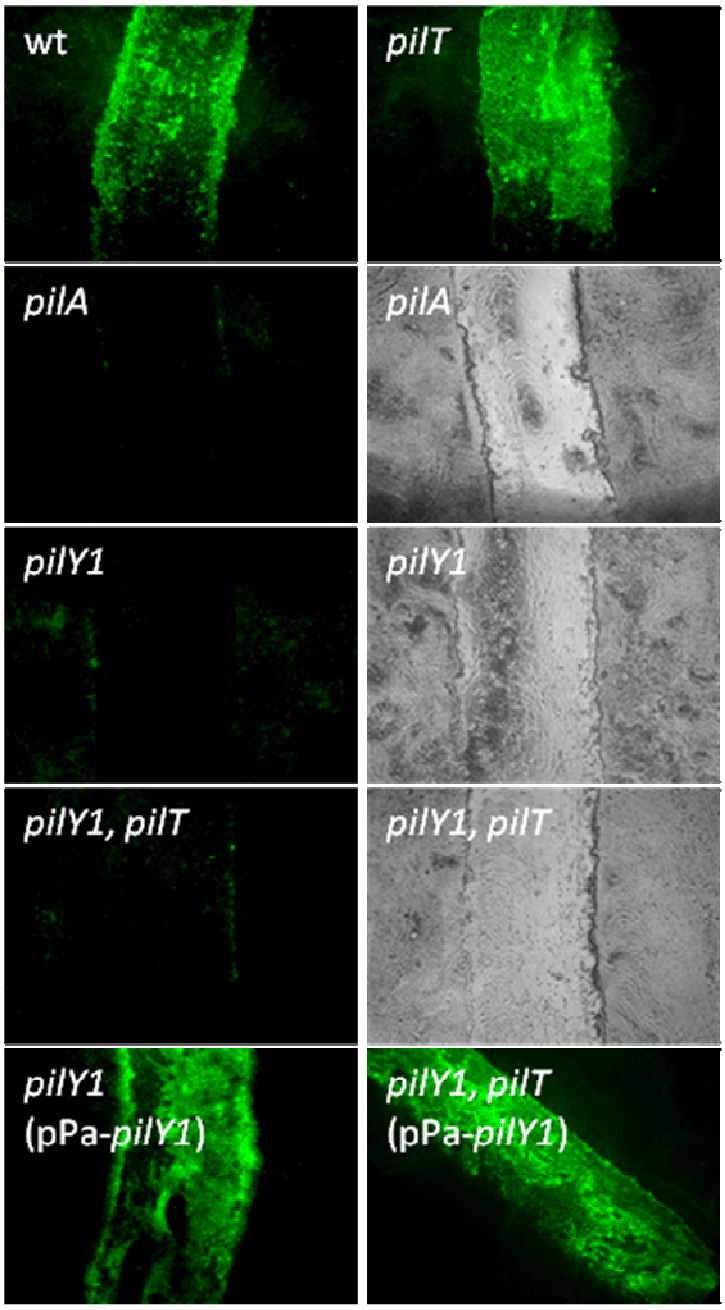

Figure 2.8. PilY1 is required for adherence of *Pseudomonas aeruginosa* to injured HAE cells. HAE cultures were imaged (top-down) by fluorescence microscopy after 45 minutes of infection with GFP-expressing strains. Wild type *P. aeruginosa* and the piliated *pilT* mutant attached efficiently to the damaged tissue and showed little interaction with the surrounding intact epithelium. The non-piliated (*pilY1*) and piliated (*pilY1*, *pilT*) mutants did not adhere to the damaged tissue and were indistinguishable from the non-piliated control mutant (*pilA*). For non-adherent strains, tissue damage was confirmed by examining the HAE cultures by both fluorescence and light microscopy. Adherence was restored for both the non-piliated and piliated *pilY1* mutants following complementation with plasmid-expressed PilY1 (p*Pa-pilY1*).

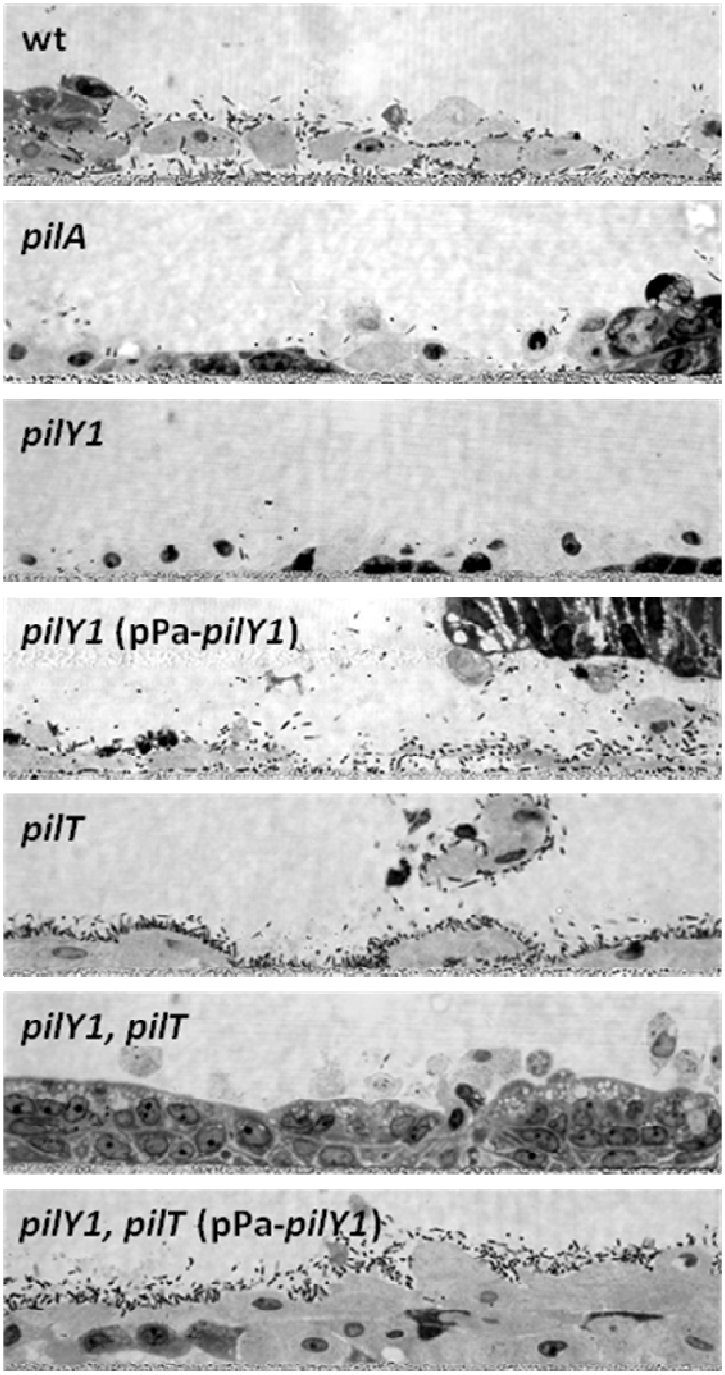

Figure 2.9. PilY1 is required for adherence of *Pseudomonas aeruginosa* to exposed basal cells of injured HAE cell cultures. Infected HAE cultures from Figure 2.8 were fixed and sectioned, with exposed basal cells from the injury site are shown. Piliated (*pilY1*) and non-piliated (*pilY1*, *pilT*) strains were defective for binding to exposed basal cells at the injury site. Adherence was restored for both the non-piliated and piliated *pilY1* mutants following complementation with plasmid-expressed PilY1 (p*Pa-pilY1*), or in the strain that expresses pilus containing PilY1 (*pilT*).

References

1. **Alm, R.A., J.P. Hallinan, A.A. Watson, and J.S. Mattick**. 1996. Fimbrial biogenesis genes of *Pseudomonas aeruginosa*: *pilW* and *pilX* increase the similarity of type 4 fimbriae to the GSP protein-secretion systems and *pilY1* encodes a gonococcal PilC homologue. Mol Microbiol **22**(1): p. 161-73.

2. **Alm, R.A. and J.S. Mattick**. 1995. Identification of a gene, *pilV*, required for type 4 fimbrial biogenesis in *Pseudomonas aeruginosa*, whose product possesses a pre-pilin-like leader sequence. Mol Microbiol **16**(3): p. 485-96.

3. **Alm, R.A. and J.S. Mattick**. 1996. Identification of two genes with prepilin-like leader sequences involved in type 4 fimbrial biogenesis in *Pseudomonas aeruginosa*. J Bacteriol **178**(13): p. 3809-17.

4. **Belete, B., H. Lu, and D.J. Wozniak**. 2008. *Pseudomonas aeruginosa* AlgR regulates type IV pilus biosynthesis by activating transcription of the *fimU-pilVWXY1Y2E* operon. J Bacteriol **190**(6): p. 2023-30.

5. **Bitter, W., M. Koster, M. Latijnhouwers, H. de Cock, and J. Tommassen**. 1998. Formation of oligomeric rings by XcpQ and PilQ, which are involved in protein transport across the outer membrane of *Pseudomonas aeruginosa*. Mol Microbiol **27**(1): p. 209-19.

6. **Bloemberg, G.V., G.A. O'Toole, B.J. Lugtenberg, and R. Kolter**. 1997. Green fluorescent protein as a marker for *Pseudomonas* spp. Appl Environ Microbiol **63**(11): p. 4543-51.

7. **Bryan, R., D. Kube, A. Perez, P. Davis, and A. Prince**. 1998. Overproduction of the CFTR R domain leads to increased levels of asialoGM1 and increased *Pseudomonas aeruginosa* binding by epithelial cells. Am J Respir Cell Mol Biol **19**(2): p. 269-77.

8. **Burrows, L.L.** 2005. Weapons of mass retraction. Mol Microbiol **57**(4): p. 878-88.

9. **Carbonnelle, E., S. Helaine, X. Nassif, and V. Pelicic**. 2006. A systematic genetic analysis in *Neisseria meningitidis* defines the Pil proteins required for assembly, functionality, stabilization and export of type IV pili. Mol Microbiol **61**(6): p. 1510-22.

10. **Chi, E., T. Mehl, D. Nunn, and S. Lory**. 1991. Interaction of *Pseudomonas aeruginosa* with A549 pneumocyte cells. Infect Immun **59**(3): p. 822-8.

11. **Chiang, P., L.M. Sampaleanu, M. Ayers, M. Pahuta, P.L. Howell, and L.L. Burrows**. 2008. Functional role of conserved residues in the characteristic secretion NTPase motifs of the *Pseudomonas aeruginosa* type IV pilus motor proteins PilB, PilT and PilU. Microbiology **154**(Pt 1): p. 114-26.

12. **Collins, R.F., L. Davidsen, J.P. Derrick, R.C. Ford, and T. Tonjum**. 2001. Analysis of the PilQ secretin from *Neisseria meningitidis* by transmission electron microscopy reveals a dodecameric quaternary structure. J Bacteriol **183**(13): p. 3825-32.

13. **Comolli, J.C., A.R. Hauser, L. Waite, C.B. Whitchurch, J.S. Mattick, and J.N. Engel**. 1999. *Pseudomonas aeruginosa* gene products PilT and PilU are required for cytotoxicity *in vitro* and virulence in a mouse model of acute pneumonia. Infect Immun **67**(7): p. 3625-30.

14. **Comolli, J.C., L.L. Waite, K.E. Mostov, and J.N. Engel**. 1999. Pili binding to asialo-GM1 on epithelial cells can mediate cytotoxicity or bacterial internalization by *Pseudomonas aeruginosa.* Infect Immun **67**(7): p. 3207-14.

15. **Craig, L.*, et al.*** 2003. Type IV pilin structure and assembly: X-ray and EM analyses of Vibrio cholerae toxin-coregulated pilus and *Pseudomonas aeruginosa* PAK pilin. Mol Cell **11**(5): p. 1139-50.

16. **de Bentzmann, S., P. Roger, and E. Puchelle**. 1996. *Pseudomonas aeruginosa* adherence to remodelling respiratory epithelium. Eur Respir J **9**(10): p. 2145-50.

17. **Delogu, G. and M.J. Brennan**. 1999. Functional domains present in the mycobacterial hemagglutinin, HBHA. J Bacteriol **181**(24): p. 7464-9.

18. **Doig, P., P.A. Sastry, R.S. Hodges, K.K. Lee, W. Paranchych, and R.T. Irvin**. 1990. Inhibition of pilus-mediated adhesion of *Pseudomonas aeruginosa* to human buccal epithelial cells by monoclonal antibodies directed against pili. Infect Immun **58**(1): p. 124-30.

19. **Doig, P., T. Todd, P.A. Sastry, K.K. Lee, R.S. Hodges, W. Paranchych, and R.T. Irvin**. 1988. Role of pili in adhesion of *Pseudomonas aeruginosa* to human respiratory epithelial cells. Infect Immun **56**(6): p. 1641-6.

20. **Driscoll, J.A., S.L. Brody, and M.H. Kollef**. 2007. The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. Drugs **67**(3): p. 351-68.

21. **Emam, A., A.R. Yu, H.J. Park, R. Mahfoud, J. Kus, L.L. Burrows, and C.A. Lingwood**. 2006. Laboratory and clinical *Pseudomonas aeruginosa* strains do not bind glycosphingolipids *in vitro* or during type IV pili-mediated initial host cell attachment. Microbiology **152**(Pt 9): p. 2789-99.

22. **Fleiszig, S.M., D.J. Evans, N. Do, V. Vallas, S. Shin, and K.E. Mostov**. 1997. Epithelial cell polarity affects susceptibility to *Pseudomonas aeruginosa* invasion and cytotoxicity. Infect Immun **65**(7): p. 2861-7.

23. **Fulcher, M.L., S. Gabriel, K.A. Burns, J.R. Yankaskas, and S.H. Randell**. 2005. Well-differentiated human airway epithelial cell cultures. Methods Mol Med **107**: p. 183-206.

24. **Furste, J.P., W. Pansegrau, R. Frank, H. Blocker, P. Scholz, M. Bagdasarian, and E. Lanka**. 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene **48**(1): p. 119-31.

25. **Garau, J. and L. Gomez**. 2003. *Pseudomonas aeruginosa* pneumonia. Curr Opin Infect Dis **16**(2): p. 135-43.

26. **Hahn, H.P.** 1997. The type-4 pilus is the major virulence-associated adhesin of *Pseudomonas aeruginosa*--a review. Gene **192**(1): p. 99-108.

27. **Hazes, B., P.A. Sastry, K. Hayakawa, R.J. Read, and R.T. Irvin**. 2000. Crystal structure of *Pseudomonas aeruginosa* PAK pilin suggests a main-chain-dominated mode of receptor binding. J Mol Biol **299**(4): p. 1005-17.

28. **Helaine, S., D.H. Dyer, X. Nassif, V. Pelicic, and K.T. Forest**. 2007. 3D structure/function analysis of PilX reveals how minor pilins can modulate the virulence properties of type IV pili. Proc Natl Acad Sci U S A **104**(40): p. 15888-93.

29. **Hoang, T.T., R.R. Karkhoff-Schweizer, A.J. Kutchma, and H.P. Schweizer**. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212**(1): p. 77-86.

30. **Jones, A.K., R.W. Heiniger, H.C. Winther-Larsen, P.M. Holiday, N.B. Fulcher, N. Roos, M.J. Cann, and M.C. Wolfgang**. 2009. Intracellular cyclic AMP controls Vfr autoregulation and virulence factor expression in *Pseudomonas aeruginosa*. Submitted for Publication.

31. **Jonsson, A.B., G. Nyberg, and S. Normark**. 1991. Phase variation of gonococcal pili by frameshift mutation in *pilC*, a novel gene for pilus assembly. Embo J **10**(2): p. 477-88.

32. **Kagami, Y., M. Ratliff, M. Surber, A. Martinez, and D.N. Nunn**. 1998. Type II protein secretion by *Pseudomonas aeruginosa*: genetic suppression of a conditional mutation in the pilin-like component XcpT by the cytoplasmic component XcpR. Mol Microbiol **27**(1): p. 221-33.

33. **Kirchner, M. and T.F. Meyer**. 2005. The PilC adhesin of the *Neisseria* type IV pilus-binding specificities and new insights into the nature of the host cell receptor. Mol Microbiol **56**(4): p. 945-57.

34. **Klausen, M., A. Heydorn, P. Ragas, L. Lambertsen, A. Aaes-Jorgensen, S. Molin, and T. Tolker-Nielsen**. 2003. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Mol Microbiol **48**(6): p. 1511-24.

35. **Lee, A., D. Chow, B. Haus, W. Tseng, D. Evans, S. Fleiszig, G. Chandy, and T. Machen**. 1999. Airway epithelial tight junctions and binding and cytotoxicity of *Pseudomonas aeruginosa.* Am J Physiol **277**(1 Pt 1): p. L204-17.

36. **Lee, K.K., H.B. Sheth, W.Y. Wong, R. Sherburne, W. Paranchych, R.S. Hodges, C.A. Lingwood, H. Krivan, and R.T. Irvin**. 1994. The binding of *Pseudomonas aeruginosa* pili to glycosphingolipids is a tip-associated event involving the C-terminal region of the structural pilin subunit. Mol Microbiol **11**(4): p. 705-13.

37. **Mattick, J.S.** 2002. Type IV pili and twitching motility. Annu Rev Microbiol **56**: p. 289-314.

38. **Merz, A.J., M. So, and M.P. Sheetz**. 2000. Pilus retraction powers bacterial twitching motility. Nature **407**(6800): p. 98-102.

39. **Morand, P.C., E. Bille, S. Morelle, E. Eugene, J.L. Beretti, M. Wolfgang, T.F. Meyer, M. Koomey, and X. Nassif**. 2004. Type IV pilus retraction in pathogenic *Neisseria* is regulated by the PilC proteins. Embo J **23**(9): p. 2009-17.

40. **Morand, P.C., P. Tattevin, E. Eugene, J.L. Beretti, and X. Nassif**. 2001. The adhesive property of the type IV pilus-associated component PilC1 of pathogenic *Neisseria* is supported by the conformational structure of the N-terminal part of the molecule. Mol Microbiol **40**(4): p. 846-56.

41. **Nunn, D., S. Bergman, and S. Lory**. 1990. Products of three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. J Bacteriol **172**(6): p. 2911-9.

42. **O'Toole, G.A. and R. Kolter**. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol **30**(2): p. 295-304.

43. **Parge, H.E., K.T. Forest, M.J. Hickey, D.A. Christensen, E.D. Getzoff, and J.A. Tainer**. 1995. Structure of the fibre-forming protein pilin at 2.6 A resolution. Nature **378**(6552): p. 32-8.

44. **Ramphal, R., P.M. Small, J.W. Shands, Jr., W. Fischlschweiger, and P.A. Small, Jr.** 1980. Adherence of *Pseudomonas aeruginosa* to tracheal cells injured by influenza infection or by endotracheal intubation. Infect Immun **27**(2): p. 614-9.

45. **Roger, P., E. Puchelle, O. Bajolet-Laudinat, J.M. Tournier, C. Debordeaux, M.C. Plotkowski, J.H. Cohen, D. Sheppard, and S. de Bentzmann**. 1999. Fibronectin and alpha5beta1 integrin mediate binding of *Pseudomonas aeruginosa* to repairing airway epithelium. Eur Respir J **13**(6): p. 1301-9.

46. **Rudel, T., I. Scheurerpflug, and T.F. Meyer**. 1995. *Neisseria* PilC protein identified as type-4 pilus tip-located adhesin. Nature **373**(6512): p. 357-9.

47. **Rudel, T., J.P. van Putten, C.P. Gibbs, R. Haas, and T.F. Meyer**. 1992. Interaction of two variable proteins (PilE and PilC) required for pilus-mediated adherence of *Neisseria gonorrhoeae* to human epithelial cells. Mol Microbiol **6**(22): p. 3439-50.

48. **Russell, M.A. and A. Darzins**. 1994. The pilE gene product of *Pseudomonas aeruginosa*, required for pilus biogenesis, shares amino acid sequence identity with the N-termini of type 4 prepilin proteins. Mol Microbiol **13**(6): p. 973-85.

49. **Sadikot, R.T., T.S. Blackwell, J.W. Christman, and A.S. Prince**. 2005. Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. Am J Respir Crit Care Med **171**(11): p. 1209-23.

50. **Saiman, L., K. Ishimoto, S. Lory, and A. Prince**. 1990. The effect of piliation and exoproduct expression on the adherence of *Pseudomonas aeruginosa* to respiratory epithelial monolayers. J Infect Dis **161**(3): p. 541-8.

51. **Saiman, L. and A. Prince**. 1993. *Pseudomonas aeruginosa* pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. J Clin Invest **92**(4): p. 1875-80.

52. **Schweizer, F., H. Jiao, O. Hindsgaul, W.Y. Wong, and R.T. Irvin**. 1998. Interaction between the pili of *Pseudomonas aeruginosa* PAK and its carbohydrate receptor beta-D-GalNAc(1-->4)beta-D-Gal analogs. Can J Microbiol **44**(3): p. 307-11.

53. **Sheth, H.B., K.K. Lee, W.Y. Wong, G. Srivastava, O. Hindsgaul, R.S. Hodges, W. Paranchych, and R.T. Irvin**. 1994. The pili of *Pseudomonas aeruginosa* strains PAK and PAO bind specifically to the carbohydrate sequence beta GalNAc(1-4)beta Gal found in glycosphingolipids asialo-GM1 and asialo-GM2. Mol Microbiol **11**(4): p. 715-23.

54. **Skerker, J.M. and H.C. Berg**. 2001. Direct observation of extension and retraction of type IV pili. Proc Natl Acad Sci U S A **98**(12): p. 6901-4.

55. **Soong, G., B. Reddy, S. Sokol, R. Adamo, and A. Prince**. 2004. TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells. J Clin Invest **113**(10): p. 1482-9.

56. **Strom, M.S. and S. Lory**. 1986. Cloning and expression of the pilin gene of *Pseudomonas aeruginosa* PAK in *Escherichia coli*. J Bacteriol **165**(2): p. 367-72.

57. **Strom, M.S., D.N. Nunn, and S. Lory**. 1993. A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. Proc Natl Acad Sci U S A **90**(6): p. 2404-8.

58. **Sundin, C., M.C. Wolfgang, S. Lory, A. Forsberg, and E. Frithz-Lindsten**. 2002. Type IV pili are not specifically required for contact dependent translocation of exoenzymes by *Pseudomonas aeruginosa*. Microb Pathog **33**(6): p. 265-77.

59. **Tang, H., M. Kays, and A. Prince**. 1995. Role of *Pseudomonas aeruginosa* pili in acute pulmonary infection. Infect Immun **63**(4): p. 1278-85.

60. **Turner, L.R., J.C. Lara, D.N. Nunn, and S. Lory**. 1993. Mutations in the consensus ATP-binding sites of XcpR and PilB eliminate extracellular protein secretion and pilus biogenesis in *Pseudomonas aeruginosa*. J Bacteriol **175**(16): p. 4962-9.

61. **Whitchurch, C.B., M. Hobbs, S.P. Livingston, V. Krishnapillai, and J.S. Mattick**. 1991. Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. Gene **101**(1): p. 33-44.

62. **Winther-Larsen, H.C., M. Wolfgang, S. Dunham, J.P. van Putten, D. Dorward, C. Lovold, F.E. Aas, and M. Koomey**. 2005. A conserved set of pilin-like molecules controls type IV pilus dynamics and organelle-associated functions in *Neisseria gonorrhoeae*. Mol Microbiol **56**(4): p. 903-17.

63. **Winther-Larsen, H.C., M.C. Wolfgang, J.P. van Putten, N. Roos, F.E. Aas, W.M. Egge-Jacobsen, B. Maier, and M. Koomey**. 2007. *Pseudomonas aeruginosa* Type IV pilus expression in *Neisseria gonorrhoeae*: effects of pilin subunit composition on function and organelle dynamics. J Bacteriol **189**(18): p. 6676-85.

64. **Wolfgang, M., H.S. Park, S.F. Hayes, J.P. van Putten, and M. Koomey**. 1998. Suppression of an absolute defect in type IV pilus biogenesis by loss-of-function mutations in *pilT*, a twitching motility gene in *Neisseria gonorrhoeae.* Proc Natl Acad Sci U S A **95**(25): p. 14973-8.

65. **Wolfgang, M., J.P. van Putten, S.F. Hayes, D. Dorward, and M. Koomey**. 2000. Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. Embo J **19**(23): p. 6408-18.

66. **Wolfgang, M.C., V.T. Lee, M.E. Gilmore, and S. Lory**. 2003. Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. Dev Cell **4**(2): p. 253-63.

67. **Wong, W.Y., A.P. Campbell, C. McInnes, B.D. Sykes, W. Paranchych, R.T. Irvin, and R.S. Hodges**. 1995. Structure-function analysis of the adherence-binding domain on the pilin of *Pseudomonas aeruginosa* strains PAK and KB7. Biochemistry **34**(40): p. 12963-72.

68. **Yu, L., K.K. Lee, R.S. Hodges, W. Paranchych, and R.T. Irvin**. 1994. Adherence of *Pseudomonas aeruginosa* and *Candida albicans* to glycosphingolipid (Asialo-GM1) receptors is achieved by a conserved receptor-binding domain present on their adhesins. Infect Immun **62**(12): p. 5213-9.

69. **Zolfaghar, I., D.J. Evans, and S.M. Fleiszig**. 2003. Twitching motility contributes to the role of pili in corneal infection caused by *Pseudomonas aeruginosa.* Infect Immun **71**(9): p. 5389-93.

CHAPTER 3

CONCLUSION

Pseudomonas aeruginosa is a versatile, opportunistic pathogen that has emerged as a major source of hospital-acquired infections and the main cause of morbidity and mortality in persons afflicted by CF (23, 36). The ability of this organism to cause life threatening infections is due to its intrinsic high resistance to most antibiotics and its capacity to express a wide range of virulence factors. These factors include extracellular toxins secreted by type II and type III secretion systems, quorum sensing, biofilm formation, and mutational adaptation leading to alginate production. While acute and chronic *P. aeruginosa* infections involve fundamentally different bacterial lifestyles and distinct sets of virulence factors, both types of infection require initial colonization of the host, a process that is mediated by TFP-dependent attachment of bacteria to host cells and tissue. Despite the important role of TFP in pathogenesis, the molecular detail of host attachment and the nature of the pilus adhesin are currently unclear. The purpose of this thesis was to characterize the role of PilY1, a TFP associated protein, in mediating bacterial-host interactions.

TFP are polymeric surface fibers used by a variety of bacteria to mediate attachment to host cells (25). Pilus fibers mediate intimate interactions between *P. aeruginosa* and host cells, and facilitate contact-dependent type III secretion of toxins (6, 7, 39, 45). In addition to facilitating host attachment, TFP in *P. aeruginosa* contribute to biofilm formation, and drive a specialized form of movement known as twitching motility, which contributes to bacterial dissemination (4, 6, 20, 26, 29, 35, 42). Previous experiments indicate that the disulfide loop (DSL) region of pilin, exclusively

exposed at the tip of the pilus filament, serves as the adhesive epitope for binding of the pilus fiber to the GalNAcβ1-4Gal moiety present on asialylated glycosphingolipids (GSLs) (3, 9, 10, 12, 14, 16, 22). Evidence for direct binding of pilin to asialylated GSLs is primarily derived from studies using purified pilus fibers. More recent experiments demonstrate that TFP producing strains of *P. aeruginosa* do not bind to asialylated GSLs (11). In fact, most studies report relatively poor binding of piliated *P. aeruginosa* strains (1-5 bacteria/cell) to the apical surface of cultured cells (21), despite the fact that asialylated GSLs are predominantly presented on the apical surface (38). In contrast, several studies have reported the preferential binding of *P. aeruginosa* to the basolateral surfaces of cultured epithelial cells, or damaged areas of cell culture monolayers where basolateral surfaces are exposed (13, 21). These results strongly suggest that asialylated GSLs are not the primary receptor for TFP-dependent binding and indicate that another component of TFP may serve as the adhesin. Using a heterologous expression system, we recently showed that *Neisseria gonorrhoeae* could assemble surface TFP composed of *P. aeruginosa* pilin (43). Surprisingly, the bacteria were unable to bind to primary human epithelial cells in the absence of the gonococcal TFP-associated adhesin PilC2. However, gonococcal binding could be restored when *P. aeruginosa* pilin was co-expressed with gonococcal PilC. In this system, PilC was able to associate with the heterologous fibers containing *P. aeruginosa* pilin. These results strongly suggest that *P. aeruginosa* pilin does not have intrinsic adherence properties and that other *P. aeruginosa* TFP-associated proteins are necessary for adherence.

The lack of clear data supporting a direct involvement of pilin in *P. aeruginosa* adherence prompted our search for a TFP-associated adhesin. In Chapter 2, we describe the characterization of *P. aeruginosa* PilY1, a previously identified TFP-associated protein, with limited homology to the C-terminal region of gonococcal PilC2. Specifically, we show that PilY1 is conditionally required for TFP biogenesis and that it may antagonize pilus retraction. Second, we show that PilY1 specifically associates with the TFP fraction of *P. aeruginosa*, and that localization requires a set of genes with

homology to pilin ("pilin-like"). Lastly, we demonstrate that association of PilY1 with the extracellular pilus fraction is necessary for adherence of *P. aeruginosa* to injured human airway epithelial (HAE) cells, indicating a potential role for PilY1 as a TFP-associated adhesin.

These findings represent a paradigm shift in our view of *P. aeruginosa* pathogenesis and have broader implication for the mechanism of TFP-mediated adherence by other gram-negative pathogens. Despite their limited homology, *P. aeruginosa* PilY1 and *Neisseria* PilC proteins appear to be members of a large family of proteins found in a number of TFP-expressing bacteria. We hypothesize that other members of this family are likely to be required for TFP-mediated adherence to host cells. This hypothesis is supported by recent data from the gram-negative pathogen *Kingella kingae*. *K. kingae* expresses TFP that are essential for adherence to both synovial and respiratory epithelial cells (19). The *K. kingae* genome encodes two PilY1/PilC homologs. Mutants lacking either gene show reduced adherence and a double mutant lacking both *pilY1*/*pilC* alleles is unable to adhere to host cells, suggesting the these proteins may function as adhesins (19). However, the direct involvement of *K. kingae* PilY1/PilC proteins in adherence remains to be determined, given that the double mutant fails to produce pili. The defect of pilus production seen in mutants lacking PilY1/PilC homologs represents a substantial hurdle in assigning a definitive and direct role in adherence. The fact that biogenesis can be restored in these mutants by eliminating the easily identifiable pilus retraction protein (PilT) suggests a general strategy for bypassing the biogenesis defect. The application of a similar strategy is likely to be useful in other systems.

While our findings have identified a potentially key protein (PilY1) in the function of TFP in *P. aeruginosa*, many questions remain unanswered. First, it is well established that many components of the TFP biogenesis machinery are highly conserved among phylogenetically distant gram-negative organisms. If PilY1 and PilC represent a general family of TFP-associated adhesins, why are their primary sequences, and the sequences of other family members, so divergent? As

mentioned above, homology between *P. aeruginosa* PilY1 and *N. gonorrhoeae* PilC (as well as other members of this family) is limited to the C-terminal half of the protein. Is it possible that the N- and C-terminus of these proteins represent different functional domains? We hypothesize that the conserved C-terminal half of PilY1 is responsible for TFP biogenesis or "anti-retraction". Consequently, we hypothesize that the N-terminal half of PilY1/PilC proteins is involved in adherence and has diverged in sequence according to the specific tissue and host tropism of the individual pathogen. This hypothesis is supported by data from *Neisseria meningitidis*. In this gram-negative pathogen, there are two *pilC* alleles; either is sufficient to support pilus assembly. Yet, only PilC1 is able to support TFP-mediated adherence (28). When studies were performed to identify the regions of PilC1 required for adherence, using PilC1-PilC2 hybrids, it was demonstrated that the adhesion-promoting domain of PilC1 is located within the N-terminal region of the protein (28). Further, regardless of the combination of PilC1- and PilC2-specific regions, all strains were piliated, suggesting that PilC-mediated adherence and PilC-dependant TFP biogenesis involve distinct regions of the protein (28). However, even with this supporting evidence, our hypothesis is speculative and requires further work in *P. aeruginosa* to determine whether there is a functional difference in the C- and N-terminal domains of PilY1.

The mechanism used by PilY1, and related proteins, to control TFP biogenesis is currently unknown, but it deserves substantial consideration. Without PilY1 in the pilus fraction, we are unable to detect pilus fibers on the surface of the bacterium. It is possible that the level of *pilY1* expression, and subsequent levels of PilY1 in the cell, may function to enhance extension (high PilY1 levels) or retraction (low PilY1 levels) of the pilus fiber in response to environmental cues. Support for this model comes from the regulation of the *pilY1* operon by both cAMP/Vfr and the 2-component regulatory system AlgZ/R (2, 18). In particular, phosphorylation of AlgR is required for successful twitching motility in *P. aeruginosa* (2). Twitching motility only occurs due to the successful extension and retraction of TFP from the surface of the bacterium (37). Therefore, levels of *pilY1*

expression may control extension and retraction of the pilus fiber. This phenomenon appears to be specific to the *pilY1* operon, as the level of *pilA* (pilin) expression is unchanged in a Vfr mutant (18), and RT-PCR analysis of surface grown *P. aeruginosa* suggests the *pilY1* operon fully accounts for the effect of AlgR on TFP expression and biogenesis (2). This would suggest that there are conditions where it is favorable for the bacterium to modulate the level of PilY1, and associated proteins, independent of the rest of the TFP biogenesis machinery. Supporting this theory is data demonstrating positive regulation of the TFP-associated adhesin in *N. meningitidis* (PilC1) by contact of the bacteria to viable host cells (40). Additionally, downregulation of PilC in *N. meningitidis* is associated with pilus retraction (27). Taken together, this data suggests *P. aeruginosa* controls pilus extension (biogenesis) and retraction by modulating the amount of PilY1 present in the cell.

While the control of PilY1 levels may be a general mechanism for altering pilus dynamics under certain conditions, our results suggest that the expression of PilY1 is required for host adherence. The fact that PilY1 appears to function as both an adhesin and a biogenesis factors implies that these two functions may be mechanistically linked. We propose that during colonization, pilus-associated PilY1 initially binds to its host receptor, tethering the bacteria to the surface of host cells. However, given the shear forces encountered by the bacteria during infection, and the fact that TFP are brittle structures, tethered bacteria are likely to detach. Subsequent retraction would allow the bacteria to intimately interact with the host cells. Contact-dependent TFP retraction has been documented to occur in *N. meningitis* following attachment to host cells (27). Hypothetically, the binding of PilY1 to its cognate host receptor could alter the flexibility of the filament. While currently undocumented, it is possible that the TFP filament rotates during polymerization, a phenomenon that has been recently described for conjugative pili (5). Attachment of the pilus to a fixed surface or receptor could restrict rotation and cause a switch in polymerization dynamics resulting in retraction. Similarly, attachment of PilY1 to its receptor may restrict pilus extension, generating a change in force, which then favors retraction. Experiments with lazer tweezers have

shown that the application of external force on gonococcal pilus fibers can drive the switch between extension and retraction (24). Alternatively, the interaction between PilY1 and its receptor could alter PilY1 interaction with the pilus resulting in a subtle change in the conformation of pilin. This conformational change could be propagated through the entire pilus fiber, resulting in retraction and a closer interaction between *P. aeruginosa* and the host cell. Similar long range interactions have been documented for actin, where the binding of gelsolin to the growing or barbed end of the actin filament results in a conformational change in actin protomers that is propagated through-out the filament (31).

The data presented in Chapter 2 indicates the localization of PilY1 to the pilus structure requires the expression of pilin-like proteins from the *pilY1* operon. However, it is unclear if all or only certain combinations of these proteins are required for successful PilY1 localization. Regardless, it appears that pilin-like proteins can integrate into the pilus at low levels and may be distributed at regular intervals along the length of the fiber (15). This may provide a mechanism for the association of PilY1-like proteins at similar discrete intervals. As such PilY1 could antagonize retraction by stabilizing the elongating pilus incrementally. It has been postulated that extension and retraction may occur by a ratchet like mechanisms (44), where the fiber is built and retracted in blocks or segments. The length of these segments may be determined by the PilY1 interval. Such a model is consistent with the stepwise extension and retraction of TFP that has been directly visualized and measured using laser tweezers (24, 37). A more thorough understanding of TFP fiber structure and the distribution of minor proteins within and along the pilus fiber shaft will provide important clues as to the mechanisms controlling pilus dynamics.

Lastly, if PilY1 is the TFP-associated adhesin in *P. aeruginosa*, what is the corresponding receptor on the surface of host cells? Given the data presented, the PilY1 receptor is likely to be located on the basolateral surface of well-differentiated epithelial cells or may be upregulated for expression due to tissue injury or stress. Currently there is no candidate, other than asialo-G_{M1}/G_{M2},

for a *P. aeruginosa* TFP receptor. Interestingly, exposure of a basolateral receptor may require prior tissue damage. Such a model is consistent with the propensity of *P. aeruginosa* to infect hosts with injured or damaged tissue (8, 30, 33, 34, 46). Damage could occur from a prior medical condition, mechanical abrasion due to the use of catheters and ventilators (33), serious burns or other wounds, inflammation or from prior exposure to a variety of inhaled and infectious agents such as *Haemophilus influenza* (17) or *P. aeruginosa* itself (1, 41). Consistent with this hypothesis, it is well documented that *P. aeruginosa* can only cause eye infections following abrasion of the corneal epithelium (32). Identification of PilY1 as the TFP-associated adhesin is likely to expedite the search for the *P. aeruginosa* host receptor. Specifically, purified PilY1 protein would provide a useful reagent for cross-linking and pull-down experiments designed to identify potential interacting host proteins.

The work represented in this thesis represents only the tip of the iceberg in understanding the early events in the pathogenesis of *P. aeruginosa* infection. Future studies are necessary to elucidate the mechanism of PilY1-dependant pilus retraction, to identify components of TFP that are required for PilY1-pilus associations, and to confirm the direct involvement of PilY1 in host adherence. Our findings, combined with studies in pathogenic *Neisseria*, suggest that PilY1 and PilC are part of a larger family of proteins that have a bi-functional role in TFP biogenesis and host adherence. This family of proteins is likely to play a vital role in the pathogenesis of other gram-negative infections and may represent a conserved and novel target for anti-infective therapies.

References

1. **Azghani, A.O.** 1996. *Pseudomonas aeruginosa* and epithelial permeability: role of virulence factors elastase and exotoxin A. Am J Respir Cell Mol Biol **15**(1): p. 132-40.

2. **Belete, B., H. Lu, and D.J. Wozniak**. 2008. *Pseudomonas aeruginosa* AlgR regulates type IV pilus biosynthesis by activating transcription of the *fimU-pilVWXY1Y2E* operon. J Bacteriol **190**(6): p. 2023-30.

3. **Campbell, A.P., D.L. Bautista, B. Tripet, W.Y. Wong, R.T. Irvin, R.S. Hodges, and B.D. Sykes**. 1997. Solution secondary structure of a bacterially expressed peptide from the receptor binding domain of *Pseudomonas aeruginosa* pili strain PAK: A heteronuclear multidimensional NMR study. Biochemistry **36**(42): p. 12791-801.

4. **Chi, E., T. Mehl, D. Nunn, and S. Lory**. 1991. Interaction of *Pseudomonas aeruginosa* with A549 pneumocyte cells. Infect Immun **59**(3): p. 822-8.

5. **Clarke, M., L. Maddera, R.L. Harris, and P.M. Silverman**. 2008. F-pili dynamics by live-cell imaging. Proc Natl Acad Sci U S A **105**(46): p. 17978-81.

6. **Comolli, J.C., A.R. Hauser, L. Waite, C.B. Whitchurch, J.S. Mattick, and J.N. Engel**. 1999. *Pseudomonas aeruginosa* gene products PilT and PilU are required for cytotoxicity in vitro and virulence in a mouse model of acute pneumonia. Infect Immun **67**(7): p. 3625-30.

7. **Comolli, J.C., L.L. Waite, K.E. Mostov, and J.N. Engel**. 1999. Pili binding to asialo-G_{M1} on epithelial cells can mediate cytotoxicity or bacterial internalization by *Pseudomonas aeruginosa.* Infect Immun **67**(7): p. 3207-14.

8. **de Bentzmann, S., P. Roger, and E. Puchelle**. 1996. *Pseudomonas aeruginosa* adherence to remodelling respiratory epithelium. Eur Respir J **9**(10): p. 2145-50.

9. **Doig, P., P.A. Sastry, R.S. Hodges, K.K. Lee, W. Paranchych, and R.T. Irvin**. 1990. Inhibition of pilus-mediated adhesion of *Pseudomonas aeruginosa* to human buccal epithelial cells by monoclonal antibodies directed against pili. Infect Immun **58**(1): p. 124-30.

10. **Doig, P., T. Todd, P.A. Sastry, K.K. Lee, R.S. Hodges, W. Paranchych, and R.T. Irvin**. 1988. Role of pili in adhesion of *Pseudomonas aeruginosa* to human respiratory epithelial cells. Infect Immun **56**(6): p. 1641-6.

11. **Emam, A., A.R. Yu, H.J. Park, R. Mahfoud, J. Kus, L.L. Burrows, and C.A. Lingwood**. 2006. Laboratory and clinical *Pseudomonas aeruginosa* strains do not bind glycosphingolipids *in vitro* or during type IV pili-mediated initial host cell attachment. Microbiology **152**(Pt 9): p. 2789-99.

12. **Farinha, M.A., B.D. Conway, L.M. Glasier, N.W. Ellert, R.T. Irvin, R. Sherburne, and W. Paranchych**. 1994. Alteration of the pilin adhesin of *Pseudomonas aeruginosa* PAO results in normal pilus biogenesis but a loss of adherence to human pneumocyte cells and decreased virulence in mice. Infect Immun **62**(10): p. 4118-23.

13. **Fleiszig, S.M., D.J. Evans, N. Do, V. Vallas, S. Shin, and K.E. Mostov**. 1997. Epithelial cell polarity affects susceptibility to *Pseudomonas aeruginosa* invasion and cytotoxicity. Infect Immun **65**(7): p. 2861-7.

14. **Hazes, B., P.A. Sastry, K. Hayakawa, R.J. Read, and R.T. Irvin**. 2000. Crystal structure of *Pseudomonas aeruginosa* PAK pilin suggests a main-chain-dominated mode of receptor binding. J Mol Biol **299**(4): p. 1005-17.

15. **Helaine, S., D.H. Dyer, X. Nassif, V. Pelicic, and K.T. Forest**. 2007. 3D structure/function analysis of PilX reveals how minor pilins can modulate the virulence properties of type IV pili. Proc Natl Acad Sci U S A **104**(40): p. 15888-93.

16. **Irvin, R.T., P. Doig, K.K. Lee, P.A. Sastry, W. Paranchych, T. Todd, and R.S. Hodges**. 1989. Characterization of the *Pseudomonas aeruginosa* pilus adhesin: confirmation that the pilin structural protein subunit contains a human epithelial cell-binding domain. Infect Immun **57**(12): p. 3720-6.

17. **Jackson, A.D., P.J. Cole, and R. Wilson**. 1996. Comparison of *Haemophilus influenzae* type b interaction with respiratory mucosa organ cultures maintained with an air interface or immersed in medium. Infect Immun **64**(6): p. 2353-5.

18. **Jones, A.K., R.W. Heiniger, H.C. Winther-Larsen, P.M. Holiday, N.B. Fulcher, N. Roos, M.J. Cann, and M.C. Wolfgang**. 2009. Intracellular cyclic AMP controls Vfr autoregulation and virulence factor expression in *Pseudomonas aeruginosa*. Submitted for Publication.

19. **Kehl-Fie, T.E., S.E. Miller, and J.W. St Geme, 3rd**. 2008. *Kingella kingae* expresses type IV pili that mediate adherence to respiratory epithelial and synovial cells. J Bacteriol **190**(21): p. 7157-63.

20. **Klausen, M., A. Heydorn, P. Ragas, L. Lambertsen, A. Aaes-Jorgensen, S. Molin, and T. Tolker-Nielsen**. 2003. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Mol Microbiol **48**(6): p. 1511-24.

21. **Lee, A., D. Chow, B. Haus, W. Tseng, D. Evans, S. Fleiszig, G. Chandy, and T. Machen**. 1999. Airway epithelial tight junctions and binding and cytotoxicity of *Pseudomonas aeruginosa.* Am J Physiol **277**(1 Pt 1): p. L204-17.

22. **Lee, K.K., P. Doig, R.T. Irvin, W. Paranchych, and R.S. Hodges**. 1989. Mapping the surface regions of *Pseudomonas aeruginosa* PAK pilin: the importance of the C-terminal region for adherence to human buccal epithelial cells. Mol Microbiol **3**(11): p. 1493-9.

23. **Lyczak, J.B., C.L. Cannon, and G.B. Pier**. 2002. Lung infections associated with cystic fibrosis. Clin Microbiol Rev **15**(2): p. 194-222.

24. **Maier, B., M. Koomey, and M.P. Sheetz**. 2004. A force-dependent switch reverses type IV pilus retraction. Proc Natl Acad Sci U S A **101**(30): p. 10961-6.

25. **Mattick, J.S.** 2002. Type IV pili and twitching motility. Annu Rev Microbiol **56**: p. 289-314.

26. **Merz, A.J., M. So, and M.P. Sheetz**. 2000. Pilus retraction powers bacterial twitching motility. Nature **407**(6800): p. 98-102.

27. **Morand, P.C., E. Bille, S. Morelle, E. Eugene, J.L. Beretti, M. Wolfgang, T.F. Meyer, M. Koomey, and X. Nassif**. 2004. Type IV pilus retraction in pathogenic *Neisseria* is regulated by the PilC proteins. Embo J **23**(9): p. 2009-17.

28. **Morand, P.C., P. Tattevin, E. Eugene, J.L. Beretti, and X. Nassif**. 2001. The adhesive property of the type IV pilus-associated component PilC1 of pathogenic *Neisseria* is supported by the conformational structure of the N-terminal part of the molecule. Mol Microbiol **40**(4): p. 846-56.

29. **O'Toole, G.A. and R. Kolter**. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol **30**(2): p. 295-304.

30. **Pereira, S.H., M.P. Cervante, S. Bentzmann, and M.C. Plotkowski**. 1997. *Pseudomonas aeruginosa* entry into Caco-2 cells is enhanced in repairing wounded monolayers. Microb Pathog **23**(4): p. 249-55.

31. **Prochniewicz, E., Q. Zhang, P.A. Janmey, and D.D. Thomas**. 1996. Cooperativity in F-actin: binding of gelsolin at the barbed end affects structure and dynamics of the whole filament. J Mol Biol **260**(5): p. 756-66.

32. **Ramphal, R., M.T. McNiece, and F.M. Polack**. 1981. Adherence of *Pseudomonas aeruginosa* to the injured cornea: a step in the pathogenesis of corneal infections. Ann Ophthalmol **13**(4): p. 421-5.

33. **Ramphal, R., P.M. Small, J.W. Shands, Jr., W. Fischlschweiger, and P.A. Small, Jr.** 1980. Adherence of *Pseudomonas aeruginosa* to tracheal cells injured by influenza infection or by endotracheal intubation. Infect Immun **27**(2): p. 614-9.

34. **Roger, P., E. Puchelle, O. Bajolet-Laudinat, J.M. Tournier, C. Debordeaux, M.C. Plotkowski, J.H. Cohen, D. Sheppard, and S. de Bentzmann**. 1999. Fibronectin and alpha5beta1 integrin mediate binding of *Pseudomonas aeruginosa* to repairing airway epithelium. Eur Respir J **13**(6): p. 1301-9.

35. **Saiman, L., K. Ishimoto, S. Lory, and A. Prince**. 1990. The effect of piliation and exoproduct expression on the adherence of *Pseudomonas aeruginosa* to respiratory epithelial monolayers. J Infect Dis **161**(3): p. 541-8.

36. **Santucci, S.G., S. Gobara, C.R. Santos, C. Fontana, and A.S. Levin**. 2003. Infections in a burn intensive care unit: experience of seven years. J Hosp Infect **53**(1): p. 6-13.

37. **Skerker, J.M. and H.C. Berg**. 2001. Direct observation of extension and retraction of type IV pili. Proc Natl Acad Sci U S A **98**(12): p. 6901-4.

38. **Soong, G., B. Reddy, S. Sokol, R. Adamo, and A. Prince**. 2004. TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells. J Clin Invest **113**(10): p. 1482-9.

39. **Sundin, C., M.C. Wolfgang, S. Lory, A. Forsberg, and E. Frithz-Lindsten**. 2002. Type IV pili are not specifically required for contact dependent translocation of exoenzymes by *Pseudomonas aeruginosa.* Microb Pathog **33**(6): p. 265-77.

40. **Taha, M.K., P.C. Morand, Y. Pereira, E. Eugene, D. Giorgini, M. Larribe, and X. Nassif**. 1998. Pilus-mediated adhesion of *Neisseria meningitidis*: the essential role of cell contact-dependent transcriptional upregulation of the PilC1 protein. Mol Microbiol **28**(6): p. 1153-63.

41. **Tsang, K.W., A. Rutman, E. Tanaka, V. Lund, A. Dewar, P.J. Cole, and R. Wilson**. 1994. Interaction of *Pseudomonas aeruginosa* with human respiratory mucosa in vitro. Eur Respir J **7**(10): p. 1746-53.

42. **Whitchurch, C.B. and J.S. Mattick**. 1994. Characterization of a gene, *pilU*, required for twitching motility but not phage sensitivity in *Pseudomonas aeruginosa.* Mol Microbiol **13**(6): p. 1079-91.

43. **Winther-Larsen, H.C., M.C. Wolfgang, J.P. van Putten, N. Roos, F.E. Aas, W.M. Egge-Jacobsen, B. Maier, and M. Koomey**. 2007. *Pseudomonas aeruginosa* Type IV pilus expression in *Neisseria gonorrhoeae*: effects of pilin subunit composition on function and organelle dynamics. J Bacteriol **189**(18): p. 6676-85.

44. **Wolfgang, M., H.S. Park, S.F. Hayes, J.P. van Putten, and M. Koomey**. 1998. Suppression of an absolute defect in type IV pilus biogenesis by loss-of-function mutations in *pilT*, a twitching motility gene in *Neisseria gonorrhoeae.* Proc Natl Acad Sci U S A **95**(25): p. 14973-8.

45. **Wolfgang, M.C., B.R. Kulasekara, X. Liang, D. Boyd, K. Wu, Q. Yang, C.G. Miyada, and S. Lory**. 2003. Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A **100**(14): p. 8484-9.

46. **Zoutman, D.E., W.C. Hulbert, B.L. Pasloske, A.M. Joffe, K. Volpel, M.K. Trebilcock, and W. Paranchych**. 1991. The role of polar pili in the adherence of *Pseudomonas aeruginosa* to injured canine tracheal cells: a semiquantitative morphologic study. Scanning Microsc **5**(1): p. 109-24; discussion 124-6.